STANDARDIZATION OF SOME METHODS FOR EXPERIMENTS WITH BACTERIOPHAGE P22 AND SALMONELLA TYPHIMURIUM

by

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TABLE OF CONTENTS

INTRODUCTION

Among collections of protocols for scientific experiments, the most widely accepted has been Professor Mark Adams' "Methods of Study of Bacterial Viruses" (1950; in revised form, 1959). It is a tribute to his craftsmanship that the most sophisticated experiments of today rigidly adhere to many of his protocols.

Adams' methods dealt with the virulent T phages of Escherichia coli. In working with other phages, it has been desirable to modify some of these methods, to attempt variations, and to select from these variations the procedure that yields the most desired result. The purpose of the work reported in this thesis was to standardize some of the methods used with the temperate phage P22 and its host, Salmonella typhimurium.

In general, the techniques used in the microbial genetics laboratory at Kansas State University were those adopted from protocols in the scientific literature, or from observations by Dr. A. Eisenstark in other laboratories. However, there were indications that modifications of the standard techniques might yield better results in some situations. Accordingly, certain modifications of the accepted methodology were compared as to yield, sensitivity, convenience, and reproducibility. The results of these studies are presented as a contribution to the methodology of studying the transduction and conjugation systems in phage P22 and its host bacterium S. typhimurium.

REVIEW OF THE LITERATURE

The experiments discussed in this thesis involved two phenomena, transduction and conjugation. On each of these phenomena, volumes of literature have been written. The author has chosen to restrict the review to those contributions in the literature that directly pertain to the methodology involved.

Bacterial recombination

The discovery of bacterial recombination by Lederberg and Tatum (1946) marked the opening of a new era in biology. This process involves the transfer of chromosomal material from one bacterial cell to another by cell-to-cell contact. The chromosome transfer requires the presence in the donor cell of an autonomous, transmissible genetic element which is called the F, for fertility (Lederberg et al., 1952; and Hayes, 1953). The discovery of the F factor made possible the recognition of two mating types in E. coli. The first, F+, harbors F and behaves as a genetic donor or male, while the second, F-, lacks F and behaves as genetic recipient or female.

When a population of F+ cells is mixed with an excess of F- cells, only about 1 in 10^4 donor cells transfer chromosomal deoxyribonucleic acid (DNA) to recipients. This transfer is due to the presence in F populations of rare mutant types called Hfr for high frequency of recombination. It has been shown that isolation of such mutants produce populations in which 1% or more of the cells are active donors (Clark, 1963; and Hayes, 1953).

According to status of the F factor there are two kinds of males; namely F males which possess the factor in the infectious state and Hfr males which possess F attached to the chromosome (Richter, 1957). Males

with their F factor in the infectious state are characterized by two properties. First, the F factor is readily transmissible to females upon cell contact and as a result the female is converted to a male (Lederberg, Cavilli, and Lederberg, 1952). Second, the factor is susceptible to disinfecting action of the dye acridine orange (Hirota and Iijima, 1957; Hirota, 1958). In contrast, the F factor of Hfr males is neither contagious (Cavilli, Lederberg, and Lederberg, 1953; Hayes, 1953) nor disinfectible (Hirota, 1958). Richter (1957) described a Hfr 3 strain which alternated back and forth between two states of fertility (F+ and Hfr). In general, the Hfr property is not inherited in fertile crosses (Cavalli, Lederberg and Lederberg, 1953; Hayes, 1953; Richter, 1957).

F+ strains may give rise to F- mutants. Females may be selectively enriched by passage through soft agar (Skaar, Richter and Lederberg, 1957). The F- obtained in both cases are reconvertible to F+ by growth in mixed culture with an F+ strain. Proof that the F factor is located on the chromosome was provided by showing that F in one of the two parents in a cross must exist before a cross can occur; therefore F is carried in the latent state (Cavilli, Lederberg and Lederberg, 1953). Hfr strains may revert to F+ (Hayes, 1953). If strain Hfr 2 is transferred to fresh broth several times, F+ cultures will arise by mutation (Richter, 1959). Hfr 2 is not infectious (Hayes, 1953).

Stability of Salmonella F factor on storage

In 1966 Sanderson made several interesting observations concerning the stability of Salmonella donor cells. He noted that the Hfr is unstable, reverting to F+ and on to F- during storage. He found that after storage there are so few donors in the population that re-isolation is required each

time it is to be used. It has been shown that Hfr A reverts to F+ in 25% to 75% of the cell after a few weeks in nutrient broth. Hfr A reverted to F+ in 100% of the cells after 2 years in soft agar stabs. Thus it may be seen that the preservation of male strains is a special problem that is in need of attention.

The preservation of bacteria

The preservation of bacterial cultures has always presented problems. These problems are of two kinds, the insurance of culture viability, and the retention of heritable characteristics. There is no universal method of preservation suitable to all cultures. Even closely related cultures may and often do differ in their responses to the same preservation procedure. The genetic constitution of a culture, which is responsible for its physiological and metabolic behavior, is a factor that must be considered not only in respect to viability, but also in the stability of individual culture characteristics. Some culture characteristics are more stable than others and some are so essential to a cell that their alteration or loss can be lethal. The loss or alteration of other traits not resulting in lethality can still cause much concern. The retention of these characteristics is in many instances much more important than maintaining 100 per cent viability. Some of these characteristics, for example, are those concerning antibiotic production, sensitivities to various toxic agents, special nutritional responses, virulence, and the fertility factor of male cells. Possession of one or more of these traits by a culture is frequently the reason its preservation is desired. Maintenance of such heritable traits depends upon the successful control of mutation and adverse selection (Braendle, 1962).

With the above generalizations in mind, a review of the literature of

factors influencing the survival of S. typhimurium is presented. Since almost every worker introduced some new variant in technique, the techniques have been obscured by two main facts. The first is the enormous differences in criteria of survival accepted by different workers; the second is the frequent lack of precise details of the method employed (Fry, 1954).

Estimation of survival

Regardless of the method of preservation and the kind of organism the success of the method can be satisfactorily estimated only in terms of the number of surviving organisms. The variations in most work have occurred here (Fry, 1954). The minority of workers have consistently performed viable counts on their cultures before and after preservation for different periods, and these results give a true evaluation of the preservation technique. In contrast, the majority of the workers have estimated their success in terms of "the death or survival" method. In the death or survival method, no attempt is made even to estimate the approximate number of organisms present, but instead, the tube culture is filled with a suitable fluid medium, incubated until growth has taken place, and then subcultured. The only positive information given is that growth occurred and thus at least one viable organism was present (Fry, 1954). It has been shown that when the survivors are reduced to very small numbers, selection may have occurred, and the surviving culture may not represent the original before preservation (Braendle, 1962).

Nature of the organism

All workers who have used a variety of organisms have agreed that some are more sensitive to drying than others. Fry and Greaves (1951) showed

that two strains of hemolytic streptococci (groups B and E) showed very high survival rates of 100 and 72% after a year, Staphylococcus aureus, typhimurium, and Bact. coli gave much lower survival rates, between 10 and 30%, and Vibrio cholerae and Neisserea gonorrhoeae showed a survival of the order of 1% or much lower. Stamp (1947) showed that a particular strain of typhimurium showed a survival rate of 75% 2 to 3 days after drying, 34.7% after 2 years' storage, and 21.8% after 4 years' storage.

The suspending medium

A large number of different fluids have been used for making the bacterial suspension before drying, inactivated blood serum and broth being the two most common (Fry, 1954). Leifson (1936) compared times of survival of S. typhimurium, Vib. cholerae, and N. meningitidis after drying in five different media including water, 0.3% beef extract, 1.0% peptone, pork infusion, and pork infusion with blood. Suspensions of bacteria in these media were dried both on pieces of paper and on glass beads, stored in vacuo over calcium chloride, and examined at intervals up to 64 days. S. typhimurium survived to the 64th day in all media. Vib. cholerae and N. meningitidis both died off quickly in the first four but were viable after 64 days in pork infusion with blood, Vib. cholerae only on the glass beads. From the experiment just described, water or saline were inferior to broth, and broth was inferior to serum.

Conditions of growth and the age of the culture

Very little information is available about the conditions under which a culture should be grown to insure the best survival after preservation. Naylor and Smith (1946) found that cultures of Serratia marcescens grown at

30 C to 34 C for 18 to 24 hours were more resistant to drying than those grown for longer or shorter periods. They also found (confirmed by Fry and Greaves, 1951) that cultures of the same organism aerated by vigorous shaking during growth were more resistant than non-aerated cultures.

Cell concentration of the suspension

The effect of the concentration of the bacterial suspension has been investigated by three workers, with three different results. Otten (1930) dried S. typhimurium, Shigella shigae, and Vib. cholerae in concentrated suspensions and in the same suspensions diluted tenfold and found that in each case the more concentrated had the higher survival rate. Stamp (1947) found that with a diminution in cell concentration, the percentage of survival rate increases. Fry and Greaves (1951) found that with three different suspensions of the same organism, with a factor of 10,000 between the weakest and the strongest, there was no significant difference in the survival rates after storage for 3 months and for 8 months. When cultures are dried from the frozen state with an adequate protective colloid the percentage survival is independent of the initial concentration (Fry, 1954).

Atmosphere of storage

The choice lies between air, nitrogen or some other inert gas, and vacuum. Very little work has been done; therefore, there is little evidence available as a guide. Roger (1914) stored dried cultures of bacteria in vacuo, and in air, oxygen, nitrogen, hydrogen, and carbon dioxide. He found the highest survival with vacuum storage, and the lowest survivals with air and oxygen; results with nitrogen, hydrogen, and carbon dioxide were intermediate. Roger's results were later confirmed by Naylor and

Smith (1946). Stark and Herrington (1931) carried out tests which showed that exposure of extremely dry bacteria to free oxygen gas causes a pronounced killing of bacteria. Swift (1937) suggested that it might be better to pass the air over calcium chloride, but he does not appear to have done this as a routine.

Temperature of storage

This is another area in which there is very little experimental evidence. Roger (1914) reported the results which revealed that, with lactic acid bacteria dried in milk, the higher the temperature of storage, the lower was the survival rate. His cultures were held for 30 and 60 days at temperatures from -6 C to 37 C. Weiser and Hennum (1947) reported that dried cultures of Bact. coli gave higher survival rate when stored in the refrigerator than at room temperature. Proom and Hemmons (1949) confirmed the results of Weiser and Hennum (1947).

Residual moisture

Roger (1914) reported that after drying a culture of lactic acid bacteria in milk and after storage for 157 days at 28 C, the culture with the highest moisture content (5.77%) showed the lowest viability and the culture with lowest moisture content (0.90%) showed the highest viability. Fisher (1950) found that with an increase in drying time an increase in the killing effect of the process resulted. Fry and Greaves (1951) confirmed Fisher's work (1950).

Absorption of water

The chemical desiccants which have been used are sulfuric acid, phosphorus pentoxide, calcium chloride, and anhydros calcium sulfate. All of

them will work well provided they are used properly. Each of them have their advantage and disadvantages. Sulfuric acid must be agitated to prevent dilution, and it could burn if allowed to come in contact with the skin. Phosphorus pentoxide is a convenient desiccant, but expensive in comparison to calcium sulfate or calcium chloride. Calcium sulfate has the property of being regenerated on heating; therefore it can be used over and over. Larger amounts of calcium chloride and calcium sulfate are required than of phosphorus pentoxide (Fry, 1954).

Discovery of transduction

In 1952, Zinder and Lederberg (1952) reported that certain temperate bacterial viruses could act as vectors in the transfer of bacterial genes from one cell line to another. Transduction is a mechanism of genetic transfer in bacteria whereby genetic material is conveyed from one cell to another by means of a phage. (See below.) This phenomenon was first observed with phage PLT22 in Salmonella, but has also been observed in diverse genera (Holloway and Monk, 1959; Thorne, 1962; and Morse, 1959).

Transducing phage and transductants

Transducing phage is prepared by infecting a phage-sensitive donor strain with a temperate phage under conditions which favor lysis and the production of phage progeny. The lysate is freed from bacterial cells and concentrated by centrifugation. The recipient cell, also phage sensitive, but differing genetically from the donor, is infected with lysate under conditions which favored lysogeny rather than lysis. After adsorption of the phage, the bacteria are plated on a medium which selects for recombinants. Clones derived from cells possessing the newly acquired characters

are selected from the large population of recipient bacteria. These clones appear with a frequency of about 1 per 10^5 to 10^6 recipients (Goodgall et al., 1965).

Bacteriophage assay

Three principal assay methods have been used: (1) plaque counts on nutrient agar plates seeded with phage-susceptible bacteria, (2) dilution end-points using lysis of fluid bacterial cultures as an indicator for the presence of phage, and (3) measurement of the length of time required of lysis of a standard fluid culture (Adams, 1950). Only the first method is generally useful. It was originally described by d'Herelle in 1917. An appropriate dilution of phage preparation is mixed with a concentrated suspension of susceptible bacteria and an aliquot of the mixture is spread on the surface of an agar plate.

The agar layer method for plating bacteriophages was first described by Gratia (1936), and is generally used by all workers. The host bacteria and virus particles are mixed in a small volume of warm 0.7 per cent agar and the mixture is poured over the surface of an agar plate and allowed to harden to form a thin layer. The advantages of the agar layer method are that the host bacteria and virus particle may be more uniformly distributed over the surface of the plate than by the spreading technique and that a larger volume of virus may be plated. The greater porosity of the soft agar layer permits more rapid diffusion of the phage particles and development of larger plaques than are obtained by the spreading technique, and hence variation in plaque morphology may be studied. The main disadvantage is that melted agar must be maintained at 46 C until used.

Phage preparation and assay on bottomless agar layer

Mora and Rizvi (1963) described a method by which plaque counts and bacteriophage stocks could be performed on plates without the bottom agar layer. Their method was essentially the same as the agar layer method without the bottom layer. The efficiency of the method as compared with the standard agar overlay method for titering and phage preparation was as good or better than the standard.

The concentration and purification of phage

The first successful purification of phage was achieved by Schlesinger (1933) using coliphage WLL related to the T2-C16 serological group. Northrop (1938) concentrated and purified Staphylococcus phage by applying the techniques used for enzyme purification. The final phage was precipitated with ammonium sulfate and further purified by ammonium sulfate fractionation. Kalmanson and Bronfenbrenner (1939) concentrated and purified a strain of coliphage T2, using collodian membranes. Hershey, Kalmanson, and Bronfenbrenner (1943) prepared highly concentrated phage suspensions by washing the phage from the surface of agar plates on which large populations of bacteria had been lysed. From 10^{11} to 10^{12} phage particles per ml were obtained.

Concentration with organic agents

Bronfenbrenner and Korb (1925), and Wahl and Blum-Emerique (1949) obtain good results with ethanol. Putnam, Kozloff, and Niel (1949) used 30% cold ethanol to precipitate E. coli phage T6. Hotchin (1954) precipitated Staphylococcus phages with 30-35% acetone in water for 18 hrs at 0 C, and 50 to 100% of viable phages were recovered. Acetone was tested by Colwell (1937) and he found that it inactivated coliphage, but acetone did

not inactivate Staphylococcus phage K at low concentrations (Adams, 1959).

Quick concentration of phage by carbowax

Soller (1961) described a method by which phage could be concentrated by osmotically forced dialysis. He used carbowax, a high molecular weight polyethylene glycol (Union Carbide Chemical Co.) to force the dialysis. Phage λ and ØX174 showed no loss of biological activity after concentration.

The extraction of phage from agar plates

After confluent lysis occurred on E. coli - coliphage plates, about 3 ml of broth was added to each plate and allowed to stand for 15-20 minutes (Adams, 1959). Eisenstark (1966) recommended that the diluent be allowed to sit at room temperature from 3-5 hours before decanting off the diluent (after the diluent has been allowed to extract phage from the agar layer). The phages which are extracted come from many single plaque populations. It has been reported that an E. coli plaque 2 mm in diameter may contain between 10^7 and 10^9 recoverable phage particles (Adams, 1959).

Phage typing of bacteria

The technique of phage typing is simple. It was first described by Craigie and Yen in (1938). The culture to be examined is inoculated into nutrient broth and incubated until a turbidity equivalent to about 5×10^8 organisms per ml is attained. The culture is spread on the surface of a nutrient agar plate, either as a series of discrete areas or as a complete lawn, allowed to dry, and the phages are spotted serially onto these areas in standard amounts of the routine test dilution delivered either by a standardized loop or pipette. When the spots are dried the plated are

incubated at a temperature suitable for the system. When members of the Enterobacteriaceae are the bacterial hosts, the plates can be read after about 5 to 7 hours incubation (Adams, 1959).

RNA phages sensitivity to RNAse

The RNA containing phages were first discovered by Loeb and Zinder (1960). The RNA phages are male specific: f2; MS-2; fr; R-17; M-12; Fh5; B; and F. A Pseudomonas phage which contains RNA has been described (Feary, Fisher, 1963). Feary showed that Pseudomonas phage 7s lost 99% of its plaque titer when diluted 1:100 in phosphate buffer containing 20 ug ribonuclease/ml when incubated at 37 C for 30 minutes. At the same dilution a 30 minute incubation at 25 C resulted in a 90% reduction of plaque titer, while a 1:9 dilution incubated at 37 C resulted in a loss of only 13%. The presence of 0.005 M ethylenediamine-tetracetic acid (EDTA), independent of dilution, reduced the loss in plaque titer observed in the presence of 20 mg ribonuclease alone. Feary (1963) attributed the inactivation of the Pseudomonas phage 7s to the presence of host proteolytic enzyme exposing the ribonucleic acid to attack by ribonuclease.

Transduction frequency of normal lysate

In normal lysate, transduction of a particular gene is a rare event, i.e. 10^{-5} to 10^{-8} per plaque-forming particle (Zinder, 1953). Phage competent in general transduction appears capable of incorporating any portion of the bacterial genome, including both chromosomal genes and episomal factors (Arber, 1960; and Jacob, 1955). It has been shown that markers are not transduced with the same frequency, and that the frequencies may vary by a factor of 20 or more. However, the variations are usually reproducible

(Lennox, 1955; Jacob, 1955; Ozeki, 1959). The reason genetic markers transduce at different frequencies is unknown at the present time.

MATERIALS AND METHODS

Unless otherwise specified, the following shall be considered true:

1. Dehydrated nutrient broth was secured from Difco
2. All agar used was Ionagar
3. All incubations were carried out at 37 C
4. Media and solutions were sterilized with steam under pressure at 121 C for 15 minutes
5. Source of strains

   All strains of bacteria and bacteriaphages were obtained from the culture collection maintained by Dr. A. Eisenstark in the Microbial Genetics Laboratory at Kansas State University.

Bacterial Strains

Host strains	Name	Bacteriophages specific for host
Au 1 or Hfr Cu 1895	*E. coli*	MS2, F2
Au 2 or LT 2	*S. typhimurium*	P22
Au 156 or 1813	*E. coli*	M13, M12
Au 170	*S. typhimurium*/P22	ØX174
arg E 116	*S. typhimurium*	P22
leu 124	*S. typhimurium*	P22
Hfr B3	*S. typhimurium*	P22
826	*E. coli*	T3

Materials

Media

Nutrient broth	8 gm nutrient broth
	5 gm NaCl
	1000 ml distilled water
Nutrient agar	8 gm nutrient broth
	5 gm NaCl
	8.5 gm Ionagar
	1000 ml distilled water
2X agar	18 gm Ionagar
	1000 ml distilled water
2X minimal salt	9 gm KH_2PO_4
	21 gm K_2HPO_4
	2 gm $(NH_4)_2SO_4$
	0.94 gm Na Citrate
	1000 ml distilled water
	0.1 gm $MgSO_4$
Nutrient overlay agar	8 gm nutrient broth
	5 gm NaCl
	6 gm Ionagar
40% Glucose	40 gm Dextrose
	100 ml distilled water

Singly enriched minimal	400 ml 2X minimal salts
	8 ml Glucose (40%)
	400 ml 2X agar
	8 ml Nutrient broth
Doubly enriched minimal	400 ml 2X minimal salts
	8 ml Glucose
	400 ml 2X agar
	16 ml Nutrient broth
Double enriched soft agar	10 ml 2X agar
	20 ml Phosphate Buffer Solution
	0.4 ml Glucose (40%)
	1 ml Nutrient broth
Phosphate solutions	(A) 268 gm $NaHPO_4 \cdot 7H_2O$/2 liters distilled water
	(B) 138 gm $NaH_2PO_4 \cdot H_2O$/2 liters distilled water
Phosphate Buffer Solution stock	
	386 ml $Na_2HPO_4 \cdot 7H_2O$ (Solution A)
	144 ml $NaH_2PO \cdot H_2O$ (Solution B)
	500 ml distilled water
Phosphate Buffer Solution	80 ml Phosphate Buffer Solution stock
	17 gm NaCl
	1920 ml distilled water
Nuclease Solutions	DNAse, 400 ugm/ml in tris buffer (pH 7.5)
	RNAse, 400 ugm/ml in tris buffer (pH 7.5)
Preservative Solutions	Glycerol
	Normal horses serum

Preparation of cell stock

To obtain a single colony isolate, a drop of the bacterial culture of the stock to be grown was spiral streaked on double enriched minimal media and incubated overnight. A single isolated colony was picked by touching a sterile tooth pick to that isolated colony. This colony was then inoculated into a 500 ml Erlenmyer flask which contained 100 ml of warm (37 C) nutrient broth. The suspension was grown to a concentration of 2×10^9 cells/ml at 37 C, thus yielding a saturated cell culture for transduction. For phage production, the overnight cells were diluted 1/40 in warm nutrient broth (37 C) with shaking for three hours to a titer of ca. 2×10^8 cells/ml.

Assay of cell stock

The cell stock was shaken twenty-five times. A 0.1 ml aliquot was then removed and serially diluted in 9.9 ml of PBS blanks to the final dilution of 10^{-9}. An aliquot of each serial dilution of bacterial culture was added to 2 ml of soft agar of nutrient overlay (47 C) with mixing with the pipette. The mixture was then plated on nutrient agar poured two days earlier and stored at 25 C. After the top layer had hardened, another 2 ml of soft agar was added over the layer containing the cells. Once the last layer had hardened, the plates were inverted and incubated overnight. The colonies were scored with a colony counter. The titer was calculated by multiplying the number of colonies counted by the reciprocal of the dilution used and by the reciprocal of the volume used. All dilutions were plated in triplicate.

Preparation of high titer phage stocks

Phage stocks were prepared by the plate method. Depending on the plaque size made by the phage, the phage stock was diluted until near

confluent lysis was produced. For phage P22 approximately three thousand phage were needed to give overlapping plaques near confluent lysis. Host cells grown to a concentration of 2×10^9 cells/ml were put in their exponential phase by diluting them 1/40 in warm (37 C) nutrient broth and incubating them for three hours. To 2 ml of soft agar were added a 0.1 ml of phage stock (wet nutrient agar plates, 3.6×10^3 P22 phages/plate; dry plates, 23×10^3 P22 phages/plate) and 0.1 ml of exponential growing host cells (2×10^8 cells/ml) with mixing by blowing through the pipette. The top layer (phage, cells, and soft agar) was poured onto a nutrient agar plate. The plates were incubated overnight and then observed to see if near confluent lysis resulted. If so, 3 ml of PBS were added to each plate and allowed to remain for three hours at room temperature. The crude phage lysate was harvested by pipetting off the liquid. The cells contained in the liquid were removed by centrifugation for thirty minutes at 3000 rpm (4 C). The supernate was collected and 1/20 of its volume of chloroform was added. After shaking, the supernate containing the phage was removed from the heavier layer of chloroform. The phage stock was then titered, dated, and labeled.

Assay of phage stock

To a 9.9 ml PBS blanks was added a 0.1 ml of phage stock. The mixture was diluted to a final dilution of 10^{-9}, 0.1 ml of each dilution was added to 2 ml of soft agar (47 C) and a 0.1 ml of overnight host cells (ca. 10^8 cells/ml) was added with mixing by blowing gently from a pipette. The mixture of soft agar, cells, and phage was poured on a nutrient agar plate. The plate was then tilted up and down to force the top layer to cover the entire plate. Once the top layer hardened, the plate was incubated from

three to five hours. The plaques were scored with a colony counter. The number of plaque forming units per ml was calculated by multiplying the number of plaques counted by the reciprocal of the dilution used and by the reciprocal of the volume used. The nutrient agar plates used must be at least two days old or dried for four hours at 45 C with the lid removed.

Standard assay of Hfr B3 before storage

Donor cells and recipient cells were grown up overnight from a single colony isolate with shaking in nutrient broth. Next, a 1/40 dilution was made of each recipient cell culture and of each donor cell culture into warm nutrient broth. Each culture was further incubated for approximately three more hours with shaking until each had reached the approximate titer of 5×10^8 cells per ml.

Each recipient cell culture (.05 ml) and donor cell (.05 ml) culture were pipetted onto a surface of a plate containing minimal medium and the mixture spread evenly over the surface with a glass spreader. After incubation for 48 hours, the plates were scored for the number of recombinants.

Preparation and storage of Hfr B3 donor cells

A known potent donor was selected and grown to a concentration of 5×10^8/ml from a single colony isolate. The donor cell culture was divided into four parts and each portion was centrifuged at 3000 rpm at 4 C for 30 minutes to form a pellet. Each pellet was washed twice with warm PBS solution and re-pelleted. To each pellet a preservative (see Materials and Methods) was added. Into each storage vial, 0.5 ml of the donor cells and preservative suspension or three glass beads to which donor cells have been attached, were dispensed. Each vial was corked with a sterile cork and

sealed with hot paraffin. The samples were stored at the following temperatures: -18 C, 11 C, 25 C, and 37 C. At selected intervals, vials of each sample were removed from storage. The donor cells were then revived and assayed for their ability to transfer their chromosomes.

Revivification of stored donor cells

After each storage vial was removed from the selected environmental temperature, approximately 0.1 ml of the bacterial suspension was removed from each vial. In the case where the donor cells were attached to glass beads, three glass beads were removed. The donor cells and preservatives or glass beads with cells attached were inoculated into screw-capped tubes containing 1 ml of warm nutrient broth at 37 C. The suspension was incubated with shaking. Aliquots of 0.1 ml were spiral streaked on nutrient agar plates and minimal agar. After incubation overnight the nutrient agar plates were observed for small colonies which were considered to be histidine requiring Hfr B3. The minimal plates were observed for contaminants and prototrophs which could have reverted from histidine requiring. Five small colonies were chosen at random.

The quick Hfr assay

The recipient cells (leu-134 and arg E 116) were grown from a single colony isolate, after overnight incubation on the shaker, the recipient cells were diluted in warm nutrient broth and further incubated with shaking for three hours. Each recipient (0.5 ml) was spread on one-half of the minimal medium plate leaving a one-half inch strip separating each recipient. The cells were allowed to air dry 10 minutes at room temperature with the petri plate covered. Each of five small circled Hfr B3 colonies was

made into a suspension by removing them from the nutrient plate with a sterile stick after revivification. Each was placed into a drop of phosphate buffer solution. The suspension was placed on an inverted lid of the petri plate and made homogeneous by moving a sterile stick vertically in the drop. The minimal plates which were spread with a different recipient on each half of the plate were inoculated by using two sterile sticks which had been placed in the donor cell suspension. One stick was used to streak across one recipient in a straight line and the other stick was used to streak the other recipient in the same manner. Five donor cell suspensions were streaked on each of the two recipients of each minimal plate. In order to retain cells having the property exhibited by the donor cells being tested, a nutrient plate was inoculated and labeled with each of the five donor cell suspensions. After incubation for 48 hours each of the plates was scored by counting the number of recombinants which grew on each recipient from each donor cell suspension.

Plaque-count assay on plates without the bottom layers

One ml of host cell suspension in the log phase of growth and 0.1 of appropriate dilutions of bacteriophage in PBS were mixed with 5 ml of melted nutrient overlay which had been cooled to 47 C. This mixture was poured on sterile petri plates immediately; it solidified in about 20 minutes. The plates were incubated in an inverted position from 4 to 8 hours. The plaques which could readily be seen were counted. The plates were then tilled in such a manner to observe any plaques which were formed near the edge of the petri plate. A level pour area was necessary to obtain good plaque distribution.

Phage stock preparation on plates without the bottom layers

Two ml of an overnight suspension of host cell were infected with a 0.1 ml of enough bacteriophage specific for the host to cause confluent lysis. The titer of bacteriophage needed to cause confluent lysis varied among the bacterium-phage systems. The mixture of host cells and bacteriophages were incubated for five minutes and to it was added 5 ml of melted nutrient overlay (47 C) with mixing. This mixture was then poured on sterile petri plates and incubated overnight. The bacteriophages were extracted by the addition of 5 ml of PBS to each plate. Three hours later the solution was pipetted off. After the removal of the host by centrifugation at 3000 rpm and treatment with chloroform as described earlier, the bacteriophage stock was titered.

Concentration of bacteriophage P22 with Carbowax

The method used to concentrate bacteriophage P22 with Carbowax is essentially the same as that described by Soller (1961). The crude bacteriophage P22 lysate of 200 ml was placed into narrow dialysis tubes (Visking (8/32 or 18/32)) of which one end was sealed (tied into a knot twice). After the tubing was filled with P22 phage lysate and sealed with a rubber band, the dialysis tubing containing the crude P22 bacteriophage lysate was placed into a liter beaker with about 50 gm of Carbowax 4000 on and around it. After about three hours the volume of the dialysis tubing had been reduced to about 15 ml. Portions of this concentrated bacteriophage P22 lysate was dispensed into storage vials and stored at temperatures: -56 C, -18 C, 11 C, 25 C, and 37 C. At selected intervals each of the vial's contents was sampled to determine titer.

Pour plate method of transduction

To soft double enriched overlay at 46 C were added 0.05 ml of overnight host cells (ca. 2 x 10^9/ml) and 0.05 ml of P22 bacteriophage (ca. 10^{10}/ml). The phage and host cells were not allowed to incubate at all, but were plated immediately or in less than one minute after being added to the soft agar on a double enriched minimal plate. After the overlay had hardened, the plates were incubated at 37 C. The number of transductants was scored at twenty-four and forty-eight hours.

Spread plate method of transduction

To the surface of a double enriched minimal agar plate was added host cells and bacteriophage as above. The mixture was spread evenly over the surface immediately with a sterile glass spreader. Once the surface had dried, the plates were incubated. The number of transductants were scored at twenty-four and forty-eight hours.

Tube method of transduction

To each warm (37 C) agglutination tube, bacteriophage host cells as above were added with mixing. This mixture was then pipetted immediately on the surface of a double enriched plate and spread, incubated and scored as for the spread plate method.

Method of prolonged incubation

Broth cultures of each recipient were shaken overnight. To pre-warmed agglutination tubes (37 C) were added 1 ml of recipient cells (ca. 2 x 10^9/ml of bacteriophage P22. The suspension which was composed of the recipient and the phage was mixed and incubated from 0 to 90 minutes. Each suspension was then sampled at selected intervals from 0 to 90 minutes by

removing 0.1 ml aliquots. The aliquots were spread on double enriched minimal plates. The number of transductants were scored after incubation for twenty-four and forty-eight hours.

Method of concentrating bacteriophage P22

To each of three groups of ten screw capped tubes were added the following volumes of lysates of bacteriophage P22 (ca. 2 x 10^9/ml) 1,1,2,3,4,5, 6,7,8, and 9 ml. To each group of ten screw capped tubes, one and only one concentrating agent was added to make the volume in each tube 10 ml. The pH of each tube was checked and if the pH of each tube was not in the range of 6.5 to 7.5, 0.1 N HCl or 0.1 N NaOH was added until the appropriate pH range was reached. All tubes were incubated at 11 C for four hours before further treatment. The group of tubes which contained ammonium sulfate as concentrating agent was centrifuged in the cold (11 C) for 15 hours at 15,000 rpm in the Servall RC2B centrifuge. The methanol and acetone series were centrifuged as above for thirty hours. The phage pellet of each tube was then resuspended in 1 ml of PBS and stored overnight in the cold before titering.

The incidence and isolation of bacteriophage and host cells from laboratory air

Detection of Bacteriophage. To 2 ml of soft agar (NOV) at 47 C, 0.1 ml (ca. x 10^9/ml) of appropriate host cells was added. S. typhimurium was used to detect phage P22 and E. coli K-12 served as the indicator of coliphage. The mixture of cells and soft agar was plated on nutrient agar. The cover was removed and left off for thirty minutes. The plates were incubated for about five to eight hours and examined for plaques.

Detection of Host Cells. To detect the presence of host cells in

laboratory air, nutrient agar plates covered with 2 ml of nutrient overlay agar were exposed for 30 minutes. Colonies appearing after 24 hours incubation were identified as host or non-host by phage typing.

Studies on the variation of bacteriophage P22 populations

To determine the number of phages adhering to a toothpick on stabbing a plaque, a sterile toothpick was used to stab a bacteriophage plaque (4 hours old) and then placed into a tube which contained 1 ml of PBS and a drop of chloroform. The phage suspension thus obtained was titered by the plaque assay method (Adams, 1959).

To determine the number of bacteriophages per whole plaque, a sterile Pasteur pipette mouth piece was used to remove entire or whole plaques from the surface of the plate's top layer and transfer them to 1 ml of PBS and drop of chloroform. The phage suspension made in this way was titered as above.

Comparison of bacteriophage P22 extraction techniques

To determine if modifications of the standard extraction method would yield more phages per ml of lysate, two modifications were tested. The standard method was designated "A" (as described in Materials and Methods under preparation of high titer phage stock) while the others were assigned "B" and "C". Extraction method "B" consisted of adding 5 ml of PBS to the surface of the plates and immediately removing the PBS and the top layer to a beaker. The suspension was shaken for 3 hours at 25 C, before centrifugation and titration of the supernatant. Method of extraction "C" consisted of the removal of the top layer of agar to a beaker and then the addition of 5 ml of PBS. This suspension was allowed to sit for 3 hours before the supernatant was removed, centrifuged to remove host cells, and titered.

Multiple Extraction. Phage stocks were grown using the agar overlay method (Adams, 1959). To each plate, five ml of PBS was added and allowed to remain for one hour. At the end of that hour, the crude phage lysate was removed by pipette. The above process was repeated four times. Each extraction was titered by the plaque count assay method of Adams, 1959.

Optimum time of Extraction. Phage stocks were grown using the agar overlay method of Adams, 1959. To each plate was added 3 ml of PBS. The crude phage lysate was harvested from three plates for each time. The times at which plates were harvested were 0, .5, 1, 2, 3, 6, and 12 hours. The phage suspension collected at each time was titered.

Optimum Volume of Extraction. Phage stocks were grown using the agar overlay method of Adams, 1959. To four groups of five plates each, the following volumes of PBS were added 1, 3, 5, and 8 ml respectfully. The PBS was allowed to remain for 1.5 hours and harvested. The lysate from each group was titered.

Rapid bacteriophage typing of bacteria in hemagglutination trays

Into each well of the hemagglutination tray was pipetted 0.5 ml of host cells (ca. 2×10^9/ml) followed by 1 ml of soft agar or nutrient overlay (47 C). The suspension was mixed by gently moving the tray in a clockwise horizontal motion about twenty-five times before solidification which occurred in about ten minutes. Each well was inoculated with a toothpick which had been dipped into a known phage suspension of high titer. The tray was covered with an aluminum foil paper to prevent drying during the incubation period of five to eight hours. After incubation each well was observed closely for lysis of the host bacterium used.

Identification of an RNA containing bacteriophage (M12) with RNAse

To determine if the plaque forming ability of bacteriophage M12 was sensitive to RNAse, host cells (ca. 2 x 10^9/ml) (0.1 ml) and 0.1 ml of bacteriophage (ca. 1000 phage/ml) were added to 2 ml of nutrient overlay or soft agar at 47 C. After gentle mixing by blowing into the mixture with the pipette, the mixture was plated on a layer of nutrient agar. Once the top layer had hardened, 0.2 ml of a solution containing 400 ugm/ml RNAse in Tris buffer at pH 7.5 was added to the edge of the surface as a drop and opposite this drop was added another drop of a solution composed of DNAse (0.2 ml of 400 ugm/ml) in Tris buffer. Upon the drying of the two spots, the plates were incubated from 3 to 8 hours and observed for plaques. If plaques were absent, the bacteriophage nucleic acid was considered to be sensitive to the enzyme solution. The enzyme solution which prevented plaque formation indicated the type of nucleic acid the phage contained. The presence of plaques in both spots indicated that the phage's plaque forming ability was not affected by either enzyme solution.

RESULTS

Sanderson (1966) established that the S. typhimurium male strain Hfr B3 reverts from Hfr to F+ to F- on storage. His methods of maintaining donor strains, in order of increasing stability for the donor properties of Hfr A, and Hfr 2 are: broth tubes, slants, soft agar, stab cultures, and lypophile preservation. The present study was initiated to develop a method for preventing strains of S. typhimurium Hfr B3 from losing the Hfr characteristic during storage. Basically, the experiment consisted of determinations of frequency of recombination in a given batch of cells immediately after harvest and after storage in various solutions at -18 C, 11 C, 25 C, and 37 C. For this work the early marker, arg E116, was chosen because it indicated the transfer of the beginning of the chromosome. However, late marker also present indicated that in these experiments most of the chromosome was transferred.

The assay and storage of S. typhimurium Hfr B3

Assay of S. typhimurium Hfr B3 before storage revealed that 89% of the single colony isolates gave arg^{+}E116 recombinants, and 11% gave leu^{+}124 recombinants (see Table 1). The standardization assay provided a base against which recombination rates in future batches of Hfr B3 cells grown under the same conditions could be compared. The culture assayed was freshly isolated. Immediately after the cells were mixed with each of the preservatives, the population was determined by colony count, this count being designated as the titer at zero time (Table 2). After a storage period of eight months, the titer was again determined in the same manner.

Table 1

Recombinants formed by crosses of freshly prepared cells of recipients and Hfr B3

Number of bacteria per plates		Recipient strain	Recombinants per 20 plates	Per cent of total recombinants
Hfr B3	Recipient			
1×10^8	1×10^8	argE (Early)	2931	89
1×10^8	1×10^8	leu (Late)	586	11
			3517	100

On a minimal medium plate, 0.05 ml of recipient cells and 0.05 ml of donor cells were spread.

After incubation at 37 C for 24-48 hours, each plate was scored for recombinant colonies.

Table 2

Cells counts of strain Hfr B3 before and after storage in different preservatives at different temperatures

	Preservative used			
	Glycerol	Normal serum	Glass beads ($CaSO_4$)	Nutrient broth
Initial counts	204×10^5/ml (100%)	128×10^7/ml (100%)	72×10^7/ml (100%)	308×10^7/ml (100%)
Counts after eight months of storage at				
-18 C	4×10^5 (2%)	35×10^5 (0.3%)	220×10^5 (3%)	33×10^5 (0.1%)
+11 C	0	2100×10^5 (16%)	100×10^5 (1.4%)	1×10^5
+25 C	0	142×10^5 (1%)	0	1000×10^5 (3%)
+37 C	0	106×10^5 (0.8%)	0	197×10^5 (0.6%)
	2%	18.1%	4.4%	3.7%

Comparison of the viable cell count showed that at least 80% of the cells had died regardless of the preservative used. The samples showing the highest percent cell survival were those stored in normal horse serum. They retained at least 16% viability when stored at a temperature of 11 C.

Nishioka's (1966) method for quick Hfr assay was used in strains in following loss of Hfr in storage. Its primary purpose was to determine whether single colony isolates had retained this Hfr property. If one wishes to compare roughly the potency of single colony isolates, one merely streaked several of them on the same type of recipient and compared the number of recombinants which resulted. The data obtained by the quick Hfr assay are presented in Tables 3, 4, 5, and 6. The sample which showed the highest percent of donor cells still potent and capable of transferring their chromosome was that stored in normal horse serum at a temperature of 11 C. A photograph of plates used for the quick Hfr assay is presented in Plate I.

Thus, of the various procedures used to store S. typhimurium Hfr B3, normal horse serum and a temperature of 11 C was the most successful. This treatment gave highest viability of bacteria (16%) and the highest retention. This correlation of viability of the Hfr property and retention of Hfr was expected since the primary requirements for recombination in these cells are viability and presence of a functional Hfr character.

The use of the bottomless agar layer method for phage titration and stock

The conventional phage plating methods using a nutrient agar layer as base was compared with a method in which the soft agar overlay was added to the bare plate. Performance was judged on the basis of phage titers obtained and on the concentration of phage produced for stock preparations.

Table 3

Number of recombinants upon "quick Hfr assay" of Hfr B3 after storage in nutrient broth at different temperatures

Storage Temperature	Recipient	Months of Storage 2	4	6	8	10
-18	arg E 116[1]	21	390	298	76	233
	N[2]	5	3	5	5	5
	leu 124[1]	0	155	73	21	195
	N	0	5	4	5	5
+11	arg E 116	3	301	129	50	216
	N	2	5	5	5	5
	leu 124	8	196	37	15	121
	N	2	5	4	4	5
+25	arg E 116	12	187	79	45	214
	N	4	5	5	5	5
	leu 124	0	67	60	10	42
	N	0	5	3	4	3
+37	arg E 116	52	192	238	25	204
	N	5	5	5	5	5
	leu 124	0	29	75	9	98
	N	0	4	5	4	5

[1] The total number of recombinants (either arg$^+$E or leu$^+$) obtained when ca. 1 x 10^6 recipient cell were cross-streaked with ca. 2 x 10^5 Hfr B3 cells.

[2] The number of isolated colonies that yielded recombinants. Five were tested; therefore 5 in this table means that 5 out of 5 tested yielded recombinants.

Table 4

Number of recombinants upon "quick Hfr assay" of Hfr B3 after storage in serum at different temperatures

Storage Temperature	Recipient	Months of Storage 2	4	6	8	10
-18	arg E 116	44	113	73	62	503
	N*	5	5	3	5	5
	leu 124	13	102	55	14	252
	N*	3	5	3	4	5
+11	arg E 116	25	258	133	57	394
	N*	4	5	5	5	5
	leu 124	22	130	55	13	463
	N*	5	4	5	4	5
+25	arg E 116	49	123	558	67	138
	N*	5	5	5	5	2
	leu 124	2	32	40	3	0
	N*	2	5	4	3	0
+37	arg E 116	44	420	240	51	213
	N*	5	5	5	4	5
	leu 124	0	129	110	10	48
	N*	0	5	5	4	5

Table 5

Number of recombinants upon "quick Hfr assay" of Hfr B3 after storage in glycerol at different temperatures

Storage Temperature	Recipient	Months of Storage 2	4	6	8	10
-18	arg E 116	0	134	237	50	277
	N*	0	5	5	5	5
	leu 124	0	160	97	12	152
	N*	0	5	5	3	5
+11	arg E 116	2	363	217	0	358
	N*	1	5	5	0	5
	leu 124	13	144	73	0	257
	N*	1	5	4	0	5
+25	arg E 116	0	0	0	0	0
	N*	0	0	0	0	0
	leu 124	0	0	0	0	0
	N*	0	0	0	0	0
+37	arg E 116	11	0	0	0	0
	N*	3	0	0	0	0
	leu 124	2	0	0	0	0
	N*	1	0	0	0	0

Table 6

Number of recombinants "quick Hfr assay" of Hfr B3 after storage on glass beads at different temperatures

Storage Temperature	Recipient	Months of Storage 2	4	6	8	10
-18	arg E 116	26	190	113	63	178
	N*	3	4	5	5	5
	leu 124	2	209	19	9	128
	N*	3	5	4	2	5
+11	arg E 116	9	86	116	0	162
	N*	4	4	5	0	5
	leu 124	0	176	75	0	214
	N*	0	5	4	0	5
+25	arg E 116	40	249	0	0	0
	N*	5	5	0	0	0
	leu 124	1	96	0	0	0
	N*	1	4	0	0	0
+37	arg E 116	0	0	0	0	0
	N*	0	0	0	0	0
	leu 124	0	0	0	0	0
	N*	0	0	0	0	0

EXPLANATION OF PLATE I

The photograph of the petri plate shows the results of the quick Hfr assay method. The colonies on the minimal media represent recombinants of Salmonella typhimurium Hfr B3 and Salmonella typhimurium recipients arginine E 116 or leucine 124. The section of the petri plate which had arginine recipients was designated "A" and the section which had leucine recipients was designated "L".

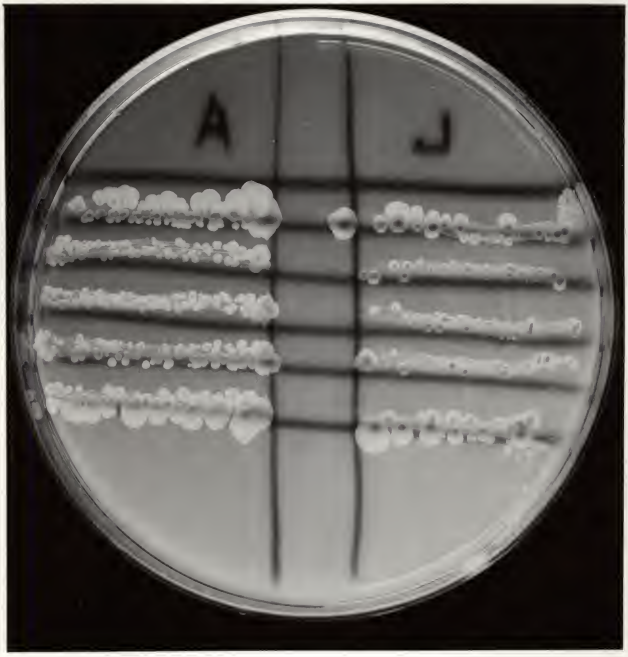

PLATE I

The result of this comparison is presented in Table 7 and 8. Five plates of each type were used and the average or mean number of plaques per plate was compared. The mean number of plaques per bottomless agar layer plate was 67, while the mean number per bottom agar layer plate was 63. When the spread of the counts was considered for the two types of plates, it was found that the conventional plates yielded the smallest spread or range. Bottomless agar plates provided an estimate of the population mean that compared favorably with that of the bottom agar layer plates.

To test the ability of the two types of plates for phage preparation, ten plates of each type were used to grow phage P22. After harvesting and pooling the phage stocks from the two types of plates, the phage stocks were titered by the plaque-count assay method. The titers of the stocks from the two types of plates is presented in Table 8. The titer of the phage stock grown on the bottomless agar plates was found to be 300 x 10^9/ml whereas the titer of the phage stock grown on the plates with the bottom layer present was 250 x 10^9/ml. This difference in stock titer was not significant. Therefore, it can be concluded that the bottomless agar layer plates can yield stocks with titers as high as those arrived at by the use of plates with the bottom agar layer present.

The effect of Carbowax on bacteriophage P22 after storage

The assay of bacteriophage P22 before and after concentrating with Carbowax is shown in Table 9. The samples of concentrated phage P22 were stored at five different temperatures (-56 C, -18 C, 11 C, 25 C, and 37 C) for 60 days. The phage samples were titered before and after concentrating. The concentrated P22 maintained a higher per cent of recovery for all temperatures as opposed to the non-concentrated phage. The best storage

Table 7

Bacteriophage P22 assay on plates without bottom agar layers

Plate no.	Type of plate: Bottom layer present Pfu / 0.1 ml	Type of plate: Bottom layer absent Pfu / 0.1 ml
1	82	85
2	71	67
3	80	92
4	73	96
5	73	66
Total	379	406
Mean	63.1	67.6
Range	11	30

Table 8

Bacteriophage P22 stock preparation on plates without bottom agar layers

Type of plates used	No. of plates	Titer of pooled lysate Pfu / ml
Bottom agar layer present	10	250×10^9
Bottom agar layer absent	10	300×10^9

Table 9

Effect of concentration by Carbowax on storage survival of phage P22

Storage Temperature	Treatment	Titer* after storage for				
		0 days	30 days		60 days	
		pfu	pfu	per cent survival	pfu	per cent survival
-56	Control	60	31	52	9	15
	Concentrated	300	241	80	247	80
-18	Control	66	49	82	55	82
	Concentrated	300	253	84	234	78
11	Control	60	47	79	36	60
	Concentrated	300	267	89	269	88
25	Control	66	55	93	39	65
	Concentrated	300	251	84	291	84
37	Control	60	11	20	---	---
	Concentrated	300	237	79	106	36

*pfu x 10^9

---lost sample

temperature for concentrated phage was found to be 11 C while the best storage temperature for non-concentrated was a -18 C.

The influence of three different methods of phage-host contact upon transduction frequencies

The results obtained by three different methods of carrying out transduction experiments can be found in Table 10. Two widely separated markers were used, one possessed a relative high frequency transducing ability (leucine) and the other a lower frequency transducing ability (arginine). From Table 10, it can be seen that no matter what method was used the leucine gene was transduced three times for every arginine gene transduced. The statistical analysis (analysis of variance) in Table 10 shows that arginine and leucine gene transducing frequency is significantly different, but the methods being tested were not significantly different from each other in terms of producing a change in the transduction frequency.

The effect of prolonged incubation of infected host cells on the frequency of transduction

The effect of prolonged incubation of infected host on the frequency of transduction is presented in Table 11. After a 10 minute period to allow for full adsorption of the transducing bacteriophage P22, the transducing frequencies for each of the genes (arg E 116 and leu 124) were relatively stable. Stability, in this instance, is defined in terms of variations of transduction frequency which remained in the statistical limits of non-significance. The application of a two sample "t" test to each gene's transduction frequency distribution yielded the same results (see Table 12). Therefore, it may be concluded that prolonged incubation of infected cells

Table 10

Comparison of the number of transductants produced by three different methods for determining the frequency of transduction

Type of Transductants	Method					
	Pour		Spread		Tube	
	arg	leu	arg	leu	arg	leu
	124	368	157	424	65	341
	123	337	154	470	63	398
	106	352	163	425	80	391
	123	359	138	559	87	479
	112	423	162	433	62	488
Sum	587	1839	774	2311	357	2097
Mean	117	368	154	462	71	419

Statistical Analysis[1]

Source	Degrees of Freedom	Mean Square	F Table	F Calculated
Blocks	1	136,806	18.51	115*[3]
Arg leu				
Treatments	2	2,798	19.00	2.36[2]
Pour Spread Tube				
Error	2 5	1,185		

*Indicates significance at .05 level.

[1]Analysis of variance.

[2]The methods used were independent of the transducing frequencies.

[3]The transducing frequency of arg E 116 differed significantly from that of leu 124.

Table 11

The effect of prolonged incubation of infected host cells

on transduction frequency

The transduction frequency versus the incubation period at 37 C

Incubation period (in minutes)	Number of Transductants: Recipients	
	leu 124	arg E 116
0	141	100
5	314	23
10	359	74
15	352	87
20	349	79
25	349	74
30	382	112
35	421	88
40	421	82
45	403	73
50	283	84
55	277	55
60	429	76
80	307	92
90	392	77
95	374	121

One ml of overnight recipient cells (2×10^9/ml) and one ml of transducing phage P22 (ca. 10^{10}/ml) were incubated at 37 C in a test tube.

At selected intervals 0.1 ml samples were removed and plated on double enriched minimal plates.

After about 24-48 hours of incubation the plates were scored for transductants.

Table 12

Statistical Analysis[1]

	Arg recipient		
	A_1	A_2	
N	7	7	
Mean	85	83	
Degrees of Freedom	6	6	
$s_{x_1-x_2}$	"t" value	P	H_0
15.8	.126	0.2	$u_1 = u_2$
	leu recipient		
	B_1	B_2	
N	7	7	
Mean	376	352	
Degrees of Freedom	6	6	
$s_{x_1-x_2}$	"t" value	P	H_0
13.1	1.83	0.1	$u_1 = u_2$

[1] Two sample "t" test.

beyond 10 minutes does not affect the frequency of transduction.

Concentration of phage P22 with various chemical agents: comparison of yields

Table 13 presents the data obtained on testing three different concentrating agents. Since phage samples treated with acetone and methanol were centrifuged for the same length of time they will be compared. Methanol and acetone seem to inactivate phage since the controls without the concentrating agent yielded a higher per cent recovery of viable phage. Of the two (methanol and acetone), the results showed methanol to be the better precipitating or concentrating agent. Saturated ammonium sulfate solution and crude P22 lysate were centrifuged for 15 hours at 11 C and gave a 48% recovery of viable phage with one part crude phage lysate and nine parts saturated ammonium sulfate solution. The ammonium sulfate precipitation process did not inactivate P22. From these results, it is concluded that one part crude phage solution to nine parts saturated ammonium sulfate solution is a better way to concentrate phage P22 than by precipitation with methanol or acetone.

The incidence and isolation of bacteriophage and host cells from the laboratory air

It was found that bacteriophage P22 and its host, Salmonella typhimurium, were present in the air of the laboratory in which about five students and technicians were performing experiments with these biological materials. The number of host cells or phage isolated seem to be influenced by the number of persons working in the laboratory at any particular time as would be expected. Tables 14 and 15 show the numbers of phage and host

Table 13

A comparison of the power of various agents to concentrate bacteriophage P22 in terms of the number of plaque-forming units recovered after low speed centrifugation

Amount of ppt. agent added (ml)	Amount of crude phage suspension added (ml)	Expected titer after conc. (Pfu x 10^9/ml)	Centrifugation time (hrs.) 30 Acetone Pfu x 10^9/ml	15 $(NH_4)_2SO_4$ Pfu x 10^9/ml	30 Methanol Pfu x 10^9/ml
9	1	163 (100%)	0	78 (48%)	0
8	2	326 (100%)	2 (0.2%)	116 (18%)	7 (1%)
7	3	489 (100%)	3 (0.2%)	275 (19%)	104 (7%)
6	4	650 (100%)	0	375 (15%)	322 (13%)
5	5	815 (100%)	90 (2.2%)	468 (12%)	318 (7%)
4	6	975 (100%)	265 (4.5%)	602 (10%)	281 (5%)
3	7	1140 (100%)	363 (4.6%)	653 (8%)	471 (6%)
2	8	1305 (100%)	649 (6.2%)	623 (6%)	637 (6%)
1	9	1470 (100%)	363 (2.8%)	910 (7%)	827 (6%)
Control					
9 ml, PBS	1	163 (100%)	107 (67%)	5 (3%)	89 (55%)

Table 14

The number of Salmonella bacteriophage and Coliphage appearing on 50 plates after 30 minute exposure period to air in laboratory

No. of Salmonella phages	No. of Coli phages	Condition of the laboratory
2	0	No people present
5	0	People present and working

An overlay of soft agar containing S. typhimurium or E. coli host cells was plated on nutrient agar plates. Plates were exposed to the laboratory air for 30 minutes, the cover replaced, and plates were incubated for about 5 to 8 hours before scoring plaques.

Table 15

The number of S. typhimurium cells and E. coli cells isolated from laboratory air after 30 minute exposure period

No. of S. typhimurium cells	No. of E. coli cells isolated	Condition of the laboratory
0	0	No people present
2	0	People present and working

An overlay of soft nutrient agar was poured over nutrient agar plates. They were exposed to the air by removing the cover. After 30 minutes of exposure, these plates were covered and incubated at 37 C for 24 to 48 hours. Each plate was observed carefully for colonies.

cells which appeared on plates exposed to the laboratory air for 30 minutes. Since the exposures were longer than normally encountered in laboratory experiments, it is concluded that contamination is a serious problem only when conventional rules of pure culture technique governing exposure of cultures are disregarded.

Studies on the variation of bacteriophage P22 populations in single plaques and in "stabs" from plaques

The visible indication of a single virus presence on a lawn of host bacterial cells is designated as a "plaque." This plaque is the equivalent of a single colony of bacteria. The colony of bacteria originated from a single cell. The plaque, too, had its origin from a single virus. Since these infective centers or plaques can be isolated, they represent a finite population. Phages originating from single infective centers have been used in many ways by Geneticists. They used them to obtain a pure stock of virus, to identify mutants and recombinants, and to purify stocks of mixed virus. Also, virologists attempting to grow virus stock used the area covered by an infective center to obtain high titered virus stock.

These studies on the variation of P22 populations were to establish the average number of bacteriophage P22 picked up on a single toothpick simply by touching a fresh (four hours old) plaque at random and to establish the average number of bacteriophage P22 recoverable from a single plaque. Since each plaque contained a different number of phages and each toothpick picked a different number of phages, a certain amount of variation was known to exist. But how much variation? This variation was expressed in terms of the range. The results are presented in Tables 16 and 17. In Table 16 are presented the results from recovering bacteriophage P22 from freshly

Table 16

The number of plaque-forming units of bacteriophage P22 per plaque recovered upon the isolation and assay of the contents of randomly selected whole plaques

Plaque number		Pfu x 10^7/plaque
1		117
2		17
3		44
4		250
5		307
6		18
7		127
8		13
9		24
10		46
11		6
12		2
13		32
14		12
15		31
16		8
17		3
18		6
19		46
	Total	1087 x 10^7
	Average	57 x 10^7
	Range	2 x 10^7 to 307 x 10^7

Table 17

Variations in the number of phage particles picked up by stabbing plaques with sterile toothpicks (Phage P22)

Toothpick number	Pfu/toothpick	Toothpick number	Pfu/toothpick
1	360×10^5	16	25×10^5
2	34×10^5	17	24×10^7
3	56×10^5	18	690×10^5
4	764×10^5	19	176×10^5
5	24×10^7	20	863×10^5
6	454×10^5	21	163×10^5
7	4×10^5	22	5×10^5
8	12×10^5	23	17×10^7
9	41×10^5	24	754×10^5
10	6×10^5	25	434×10^5
11	26×10^5	26	387×10^5
12	637×10^5	27	57×10^5
13	1×10^5	28	1×10^5
14	48×10^7	29	21×10^5
15	976×10^5	30	48×10^5
	Total number	$18{,}225 \times 10^5$	
	Average/toothpick	202×10^7	
	Range	1×10^5 to 48×10^7	

isolated plaques and titering them. From the data it can be shown that a fresh P22 plaque isolated at random will contain on the average about 57×10^7 phages (ranging from 2×10^7 to 307×10^7). In Table 17 are presented the results from stabbing thirty fresh plaques with sterile toothpicks and titering the phage population found on each toothpick. It was found that 202×10^7 bacteriophage P22 on the average (ranging from 1×10^5 to 48×10^7). How can this information be used?

In routine laboratory work, this information is extremely useful, for a knowledge of the number of recoverable phages per plaque allows one to calculate the number of viruses needed per plate to obtain high titered phage. It also aids in determining the limiting number of phages which can be grown on a plate. The upper limits of the titer of P22 obtainable by the plate method has been set at 10^{12}. Why was the upper limit set at 10^{12}? First, the average P22 plaque area is 3.2 mm^2 and yields approximately 10^9 recoverable phages. The area of the petri plate is 5645 mm^2. Therefore, about 3600 phages are needed to cause confluent lysis (calculated by dividing the area of a single plaque into the area of the petri plate). Thus, one could not expect to obtain a phage titer higher than the number of phages needed to cause confluent lysis times the number of recoverable phages per plaque (3.6×10^{12} per plate).

Since toothpicks are used a great deal by geneticists for picking plaques, it has now been established that by merely touching a toothpick to a plaque one can obtain on the end of a toothpick not less than 10^5 phage. This number represents about one per cent of the total number of recoverable phages that are in the entire plaque. Thus, when one samples a plaque by the use of a sterile toothpick, there may be a marked sampling

error if a large number of similar type plaques aren't picked.

Extraction studies of bacteriophage P22

The term extraction is defined in this paper as the separation of the bacteriophage P22 from its host cells, host cell parts, and from the media used to maintain the host. When bacteriophages are grown on nutrient agar plates in overlay, the first step of the extraction is the removal of the phages and some unwanted cells from the nutrient overlay. This is usually done in several steps beginning with application of PBS to the surface of the plate at room temperature. After about three hours, this PBS is decanted off. After a low speed centrifugation, nearly all the host cells are removed. This constitutes step number two. To the supernant is added chloroform (1/20 of the volume of the supernant) with shaking. With the separation of the phage stock from the chloroform, the stock suspension is ready for use.

The above description represents the standard procedure used in our laboratory. With the above scheme in mind, studies were initiated to determine the following:

1. whether the nutrient overlay containing bacteriophage P22 could be extracted more than once and the per cent of the total phage obtained by each extraction;

2. the volume of PBS which should be added to yield the highest number of pfu/ml;

3. whether the routine method for extracting bacteriophage P22 could be modified to extract more phages per ml of lysate;

4. whether the time for decanting PBS from plates to yield the highest pfu/ml of bacteriophage P22.

In Table 18 data are presented concerning the multiple extraction of the phage P22 from nutrient overlay (top layer) with PBS. Four separate

Table 18

Volumes and titers of bacteriophage P22 stocks obtained on separate extractions of the same nutrient agar plates with PBS

Extraction	Titer* of P22	Volume collected	Volume added
1	565×10^9	5 ml	5 ml
2	331×10^9	5 ml	5 ml
3	176×10^9	5 ml	5 ml
4	125×10^9	5 ml	5 ml

*Each titer represents an average of 20 plates.

Twenty plates of P22 bacteriophage was grown up by the agar-overlay method.

On each of the plates 5 ml of PBS was pipetted.

After three hours the PBS was decanted and titered for its pfu/ml. This represented extraction one.

On the same plate, 5 ml of fresh PBS was again pipetted, allowed to remain for 1 hour, decanted and then titered for its pfu ability. This represented extraction two.

The extraction procedure was repeated for a total of 4 times on the same plate.

extractions were made with 5 ml of PBS each time. Each extraction yielded titers higher than 125 x 10^9 pfu/ml. The first extraction yielded the highest per cent recovery while all others gave titers considerably lower. After 4 extractions it was calculated that about 32.2% of phages present on the plate had been recovered. Therefore, the conclusion of this study is that successive extractions of Phage P22 from the top layer of nutrient plate does result in a greater recovery of the total number of phages present on the plate, but, on the other hand, multiple extraction and pooling of extracted lysates results in a drop in titer. Since titer depends on the number of pfu's/ml, it is a function of the volume, not the total number of particles present.

After determining that a nutrient plate on which phage stock had been grown could be extracted many times, the problem arises as to what is the best volume of PBS to add to plates to obtain the highest pfu/ml. The results of this determination are presented in Table 19. Four different volumes of PBS were applied for 1.5 hours to the surface of nutrient plates on which phage P22 stock was present. The greatest volume of PBS decanted from the plates yielding the highest number of pfu/ml was considered the best volume to use. It was found that by applying a volume of 3 ml of PBS for 1.5 hours to the surface of nutrient plates, 1.8 ml of lysate was recovered yielding a phage titer of 10^{11}. Thus, according to the method used, the best volume of PBS to apply to the surface of a nutrient plate to extract bacteriophage P22 is 3 ml.

With the establishment of the optimal volume of PBS to use when extracting phage stock from a nutrient plate, the problem of what time to decant the PBS from the nutrient plate can be determined. The results of this

Table 19

Determination of volume of PBS added to plates to yield highest pfu/ml in preparation of phage stocks

Volume added (ml)	Volume removed (ml)	Time (hrs)	Pfu/ml
1	0.2	1.5	102×10^{10}
3	1.8	1.5	67×10^{10}
5	3.8	1.5	56×10^{10}
8	6	1.5	36×10^{10}

Bacteriophage P22 was grown by the agar overlayer method.

To groups of 10 plates each, the above indicated volume of PBS was added.

After 1.5 hours, the PBS from each group of 10 plates each was decanted and pooled.

The pooled PBS from each group was titered for its pfu/ml.

The largest volume of PBS yielding the highest pfu/ml was considered to be the best volume to use.

determination are presented in Table 20. Twelve different times were used. They ranged from 0 to 12 hours. The zero hour sample gave the lowest titer (56×10^9) while the 12 hour sample gave the highest titer (41×10^{11}). Although the 12 hour sample gave the highest titer, it is not considered the best time to decant the PBS. By definition, the best time to decant the PBS was determined to be the earliest time at which the highest number of pfu/ml was obtained. Another factor was considered in determining the best time, that is, the volume recovered. It was revealed by the experiment that after two hours of absorption of the PBS into the nutrient agar top layer, the volume taken off remains constant. Thus, according to the method employed, 2 hours is both necessary and sufficient to obtain the highest pfu/ml per 3 ml of PBS applied.

During the introduction to the results of the extraction studies (page 53), a procedure was described for obtaining phage stock. This procedure is a standard one used in our lab. This next experiment was undertaken to determine if the routine method for extracting bacteriophage P22 could be modified to yield more phage per ml of lysate (Table 21). For a detail description of how each modification was performed see Materials and Methods. Essentially the standard consist of decanting the PBS by pipette from the surface of the agar plate (Technique A). Technique B consisted of adding the PBS and then removing PBS and top agar layer before extracting the phages. Technique C consisted of removing the top agar layer and then adding to it PBS before extracting the phage. It was found that modifications in the standard method resulted in titers which were not significantly better than those obtained by the standard method itself.

Table 20

Determination of time for decanting PBS from plates to yield highest pfu/ml of bacteriophage P22

Time (hrs)	Ml of PBS added	Ml of PBS removed	Pfu/ml
0	3	2	56×10^{9}
0.5	3	1.8	35×10^{10}
1	3	1.8	74×10^{10}
2	3	1.5	150×10^{10}
3	3	1.5	174×10^{10}
6	3	1.5	182×10^{10}
12	3	1.5	41×10^{11}

Bacteriophage P22 was grown by the agar overlayer method.

To each plate 3 ml of PBS was added.

At selected times, 10 plates were harvested by decanting and pooling the PBS.

The pooled PBS was titered to determine the average pfu/ml.

The earliest time at which the highest pfu/ml was found was considered to be the best time for removing the PBS.

Table 21

A comparison of techniques for extracting phage from top layer of agar plates

Techniques	Titer of P22 recovered
A (decanted after adding 5 ml PBS)	245×10^9
B (5 ml PBS added and top agar removed)	310×10^9
C (top agar removed, then to it was added 5 ml PBS)	245×10^9

*Each titer is a mean which represents 10 plate counts.

Rapid bacteriophage typing

Plate II shows the use of a hemagglutination tray in bacteriophage typing. The entire test, from preparing the individual wells with nutrient overlay and host cells to observing the wells for lysis, was completed in less than 5 hours.

RNAse inactivation of an RNA bacteriophage M-12

Plate III and Table 22 shows the effect of RNAse on an RNA phage (M-12). RNAse at a concentration of 80 ug/ml inactivated the plaque-forming ability of bacteriophage M-12.

EXPLANATION OF PLATE II

The plate on the opposite page shows the use of a hemagglutination tray in the rapid bacteriophage typing technique. The vertical columns contain four strains of host bacteria. The horizontal rows contain four separate phages plus two controls without phage. See the protocol below.

Lysis shown by the phage

	Column no.	1	2	3	4
	Bacterial strains	AU156	AU170	AU826	LT2
Horizontal rows	Phage strains				
1	none (control)	-[2]	-	-	-
2	M12	+[1]	-	+	-
3	ØX174	-	+	+	-
4	T3	-	-	+	-
5	P22	-	-	-	+
6	none (control)	-	-	-	-

[1] + Indicates lysis.

[2] - Indicates no lysis.

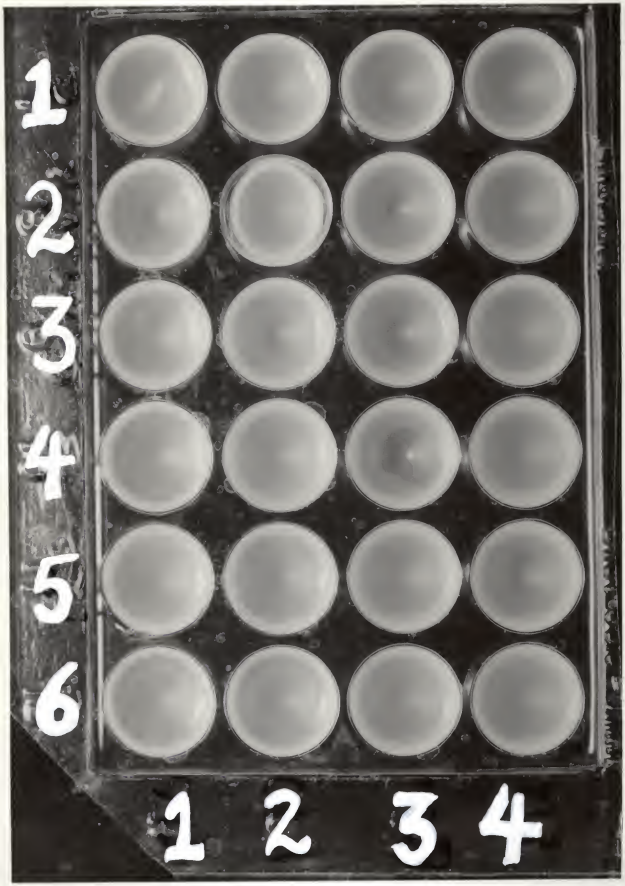

PLATE II

EXPLANATION OF PLATE III

The photograph shows two petri plates on which M12 bacteriophage was plated. On each plate in opposite spots, was placed either 0.2 ml of 400 ugm/ml of DNAse solution or RNAse solution. M12 failed to form plaques in the presence of RNAse solution, but in the presence of DNAse solution plaque formation was not inhibited.

PLATE III

Table 22

The effect of DNAse and RNAse on pfu ability of different types of bacteriophages

Bacteriophages				Effect of enzyme	
Phage	Type of DNA	Type of RNA	Host	DNAse	RNAse
P22	D.S.[3]		LT2	-[1]	-
M13	S.S.		1813	-	-
M12		S.S.[4]	1813	-	+[2]
T3	D.S.		826	-	-
ØX174	S.S.		AU170	-	-
MS2		S.S.	AU1	-	-
F2		S.S.	AU1	-	-

[1]Minus signs indicate that the enzyme did not prevent plaque formation.

[2]Plus signs indicate that the enzyme did prevent plaque formation.

[3]The term double stranded is indicated by D.S.

[4]The term single stranded is indicated by S.S.

DISCUSSION

The assay and storage of Hfr B3

The purpose of this experiment was to determine the optimal conditions for maintaining viability in the donor, S. typhimurium Hfr B3. This problem was undertaken because of the high frequency with which Hfr B3 loses its F factor on storage (Sanderson, 1966). Before using this strain for an experiment, Hfr cells frequently had to be re-isolated from a mixed population consisting of only a few fertile cells within the population. The optimal conditions were described in terms of the medium in which the greatest number of fertile donors were found to have survived storage, and the temperature at which the greatest number of fertile donors were present after storage.

In order to determine the potency of Hfr B3 cells before storage, a standardization assay was carried out immediately. The standardization assay described the ability of Hfr B3 single colony isolates to transfer an early marker and a late marker to F- cells in terms of the recombinants. The results of the standardization assay (Table 1) show that 89% of the Hfr B3 single colony isolates will transfer the arginine E gene and 11% of the single colony isolates will transfer the leucine gene when the specified conditions are maintained. Thus, this assay of the donor cells before mixing them with the preservative can be used as a means of comparing the potency of other Hfr B3 culture which will be stored in the future.

Once a fertile Hfr B3 was obtained, cells were mixed with each preservative: nutrient broth, normal horse serum, glycerol, and glass beads. The fertile cells and preservative were then transferred to storage vials.

After storage of each set of samples at -18 C, 11 C, 25 C, and 37 C,

the vials were removed every two months and the contents were placed into a tube of warm nutrient broth at 37 C with shaking. Since this sub-culturing technique was used before the "quick" Hfr assay was used, survival means only that at least one viable organism was present, and if that organism was a fertile donor the "quick" Hfr assay will indicate it. From Tables 3, 4, 5, and 6 it can be observed that at least one viable fertile donor remained since the "quick" Hfr assay indicated chromosome transfer not only of the early marker (arg E) but of the late marker (leu) also. From the same tables it can be seen that normal horse serum allowed the greatest number of donors to survive. A viable cell count was carried out at the end of eight months. This count was compared with the cell count at zero time. It was confirmed that normal horse serum contained the greatest number of viable cells (Table 2).

The findings on the storage temperature at which the greatest number of organisms survived agree in principle with those of Weiser and Hennum (1947) who found that E. coli gave a higher survival rate when stored in the refrigerator than at room temperature. Since S. typhimurium survived well on all storage media at the optimal temperature, this shows good confirmation of Leifson's (1936) work. Leifson showed that S. typhimurium survived well (even after drying) in water, 0.3% beef extract, 1.0% peptone, pork infusion and pork infusion with blood.

From this study the following was confirmed:

1. Normal horse serum (undiluted) maintained the greatest number of viable cells of the four preservatives tested.
2. Cells stored in normal horse serum showed the greatest number of viable cells at 11 C.
3. According to the quick Hfr assay, there was no significant detectable loss of the F factor by Hfr B3.

The use of the bottomless agar layer method for phage titration and stock

In Tables 7 and 8 results are presented concerning the use of bottomless agar layer plates. When bottomless agar plates were used to titer bacteriophage P22 and the results compared with that of plates with bottom agar layers, no significant difference could be detected. The comparison of the bottomless agar layer plates to the plates with bottoms in their use for bacteriophage P22 stock preparations yielded stocks which when titered showed no significant difference. The bottomless agar layer plates offered such advantages as the following: (1) economy of time, because the bottomless agar layer plates did not have to be poured in advance of their use; (2) economy of media, because the bottomless layer agar plates required less media; (3) and the same high efficiency which was observed with the plates which contained the bottom agar layer. This work confirms the work reported by Mora et al. (1963) who first reported the use of bottomless agar layer method.

It should be noted, however, that the media which was poured into the bottomless agar layer plates adhered better to plastic petri dishes than it did to glass petri dishes. Also, when using the bottomless agar layer method for titration, plaque-count assay, the critical concern here was to use just enough cells to be able to observe lysis. If too many cells were used, then the plaques (P22) were small and difficult to see. If too few cells were used, then the plaques were only observable after enough cell divisions had occurred. Even with these disadvantages, the bottomless agar method should soon come into common use.

The effect of Carbowax on bacteriophage P22 after storage

The results presented in Table 9 provide evidence which shows the

stabilizing effect of Carbowax on the plaque-forming ability of bacteriophage P22. This work confirmed the results reported by Soller (1961) that Carbowax could be used to concentrate bacteriophage without the loss of biological activity. The temperature at which the Carbowax concentrated bacteriophage P22 showed the highest per cent recovery after 60 days of storage was 11 C. In contrast, the temperature at which the unconcentrated bacteriophage P22 showed the highest per cent recovery after 60 days of storage was that of -18 C. Fujimura (1961) showed that a concentrated phage solution of ØX174 was more stable than a dilute solution. If Fujimura (1961) were correct then the stability observed in concentrated bacteriophage P22 solution could be due not to the Carbowax but to the concentration of phages themselves.

The influence of three different methods of phage-host contact upon transduction frequencies

The data presented in Table 10 provided evidence that the transducing frequencies of genes or transducing fragments found in normal lysates (P22= S. typhimurium lysate) were independent of the method used to obtain transduction. To test the hypothesis that the transducing frequency of genes was independent of the method, three methods were chosen, the pour plate, the spread plate, and the tube method. The number of transductants were scored for each of these methods. The frequency of each locus transduced was summed and to the data an analysis of variance (one-way) was performed. The results of the analysis of variance indicated that regardless of the method used the transducing frequency in normal lysate was fixed or constant, and that the transducing frequency of leucine occurred with a greater probability than that of arginine relating to their presence in normal lysate. Therefore,

bacteriophage P22 can transduce different markers but not at same frequency (Lennox, 1955; Jacob, 1955; Ozeki, 1959).

Since each method varied only in the way the transductants were handled and not in the number of transductant received the three methods yielded essentially the same results. Thus, the method used to perform complete transduction has no influence on the transducing frequency of the genes or transducing fragments located in a normal bacteriophage P22 lysate.

The effect of prolonged incubation of infected host cells on the frequency of transduction

In Table 11, the data presented provide evidence which indicates that on prolonged incubation after a 10 minute absorption period of the transducing bacteriophage P22 to its host cells, the transduction frequency remains relatively constant. It was suspected at first that if a transduction mixture (transducing bacteriophage P22 and S. typhimurium in nutrient broth at 37 C) were incubated longer than usual (10 minutes) that perhaps the conditions of the environment would favor lysis of some of the cells which would cause a great reduction in the transduction frequency. But, on applying the two sample "t" test (Table 12) to each transducing frequency distribution it was concluded that the transducing frequency of each gene was relatively constant. The inconsistencies which were observed at 0, 5, 30, and 95 minutes are explained in terms of poor adsorption of the transducing phage P22 for 0 and 5 minutes for both the leucine gene and the arginine gene; the larger than usual value for the transduction frequency of the arginine gene after incubation for 30 and 95 minutes are explained by sampling errors. This work confirms the necessity for the 15 minute absorption period for the transducing bacteriophage P22 (Zinder, 1952). From the evidence provided in

Table 12, it is as concluded that prolonged incubation (37 C) of the transduction mixture up to 95 minutes after infection produced no detectable effect on the transducing frequencies of the genes tested (arginine gene and the leucine gene). It must be noted, however, that if the transduced cells were allowed to remain in the nutrient broth until after the transducing fragment was incorporated and cell division had occurred, then the number of transductants would not be equivalent to the previous transducing frequency of the bacteriophage P22 lysate.

Concentration of phage P22 with various chemical agents: Comparison of yields

The evidence presented in Table 13 indicated that of the three concentrating agents compared, saturated ammonium sulfate was best in the ratio of 9 parts of sautrated ammonium sulfate solution to 1 part crude phage lysate (P22). The ammonium sulfate apparently showed no inactivation of bacteriophage P22 plaque-forming ability since the control's per cent recovery of viable phage was less than those which included ammonium sulfate. The use of ammonium sulfate to concentrate bacteriophage further confirms the work of Northrop (1938). The near 50 per cent recovery agrees well with results obtained with Newcastle Disease virus, which was concentrated with saturated ammonium sulfate (Minocha, 1966).

Methanol and acetone did not compare favorably with ammonium sulfate. Both seemed to be inactivating to the plaque-forming units of bacteriophage P22 since there was a greater per cent of recovery in the controls than the tubes containing the concentrating agents. These results concerning the ability of acetone to serve as a concentration agent disagree with that obtained by Hotchin (1954). According to Hotchin the conditions he used

allowed him to recover 50 to 100% viable staphylococcus phages. However, Colwell (1937) found acetone to be inactivating to coliphages. There are two possibilities. Either bacteriophage P22 is extremely sensitive to acetone or the amount of time the bacteriophage P22 and acetone remained together influenced the inactivation. Although methanol showed the same inactivating characteristics as acetone, it did so to a lesser degree. Thus, if acetone and methanol were compared as concentrating agents, methanol, under the condition used, would be chosen. Since methanol and acetone were centrifuged twice as long as ammonium sulfate, phage P22 was in contact with them longer. It can be seen from the phage counts in their (methanol and acetone) controls that centrifugation at 5000 rpm (Servall) brought down at least 50% of the phages as compared to ammonium sulfate's control which had about 3% brought down in half the time. This is further evidence of inactivation by acetone and methanol of the phage P22. The inactivation ability of methanol and that of acetone decreased the recovery per cent observed in the tubes containing these concentrating agents.

The use of ammonium sulfate as a concentrating agent enables one to obtain with a low speed centrifuge (5000 rpm, centrifuge) high titers and greater volumes of viable bacteriophage P22 in about 15 hours or less.

The incidence and isolation of bacteriophage and host cells from laboratory air

The evidence presented in Tables 14 and 15 demonstrated the presence of bacteriophage P22 and its host cell, S. typhimurium, in the air of the laboratory where several people were working with this phage and its host.

The method employed to show the presence of bacterial cells and bacteriophages in the laboratory air was essentially the same as that used by Koch

(Salle, 1961). This method was one of the simplest procedures which could be used for air examination. This method has no quantitative value since it does not indicate the number of organisms present in a known volume of air. It does, however, supply relative results.

The results in Table 14 showed fair agreement with the reference by Salle (1961) that bacterial populations increased in proportion to the activity of the atmosphere. This was tested by sampling the air while the laboratory was empty and by sampling the air when the laboratory was filled with working students and instructors. The results shown (Table 15) if tested statistically would probably not be significant but if many more sampling were done the increase in cell count and phage count during laboratory activity would result in a significant difference.

Since no microorganisms are indigenous to the air, they had to have come by some process from air environment in which they were able to multiply. Johansson et al. (1946) showed by means of high speed photography and air sampling techniques that certain accepted bacteriological laboratory operations, such as pipetting, pouring, and vigorous agitation of dilution blanks, often produce bacterial contamination of the surrounding air and environment. More than 50 per cent of the laboratory operations revealed droplet aerosols formed by blowing the last drop out of pipettes or removing the stoppers from dilution blanks that had been vigorously agitated. Since microorganism can be introduced into the laboratory air by routine operations, many suggestions have been made concerning their elimination. Reitman (1954) suggested the use of soaked cotton pledget when making dilutions in vaccine bottles, and Wedum (1953) recommended the use of protected ventilated cabinets for working with infections or contaminating material. The

author suggests the use of good techniques, the use of a special room with an exhaust fan for making dilutions and pouring of infectious material, and finally the use of ultraviolet light to keep the special room sterile.

Studies on the variation of bacteriophage P22 populations in single plaques and in "stabs" from plaques

From the results described in Table 16, the average number of bacteriophage P22 per plaque was about 5.7×10^8. Since phage geneticists and mutation experts sample plaques by inserting in the plaque or by simply touching the plaque with a sterile toothpick, it was interesting to assay the number of phage P22 collected on the end of a toothpick from stabbing at random (fresh, 4 hours old) P22 plaques. It was found that 70% of the toothpicks collected in the range from 1×10^5 to 50×10^7 bacteriophages per ml (see Table 17). The average number of bacteriophages P22 per 30 toothpicks was 6.7×10^7/ml. Anderson (1948) reported that from a single plaque 2 mm in diameter one may obtain between 10^7 and 10^9 recoverable phage particles (E. coli phage). This observation agrees well with the above finding even though the above data was obtained from P22, a S. typhimurium phage. Therefore, on the end of a toothpick a concentration of about 10^7 can be obtained on the average by touching a P22 plaque.

Recovery of bacteriophage P22 from top layer agar plates

Multiple extractions: Table 18 contains data which shows that on multiple extraction of a nutrient overlayer in which phage P22 was released, the extracting fluid (PBS) continue to yield relatively high titers. Since a large number of phages remain after primary extraction it is only logical that some portion of the remaining unabsorbed phages can be extracted. Each

P22 plaque on the average is about 2 mm in diameter and each contain about 10^9 phages/plaque. In order to get confluent lysis, about 3600 plaques are needed. The approximate total number of phages on the plate after confluent lysis is about 3600 x 10^9 phages. The first extraction removed on 14% of the phages. The second extraction removed 9.2% of the phages. The third and fourth removed 4.9% and 3.5% respectively. Thus, the approximate total per cent of phages removed equal about 32.2%; thus 67.8% of the phages remained even after four extractions.

A comparison of three different methods of extracting phage P22 from top layer agar plates

Three methods for separating bacteriophage P22 from its nutrient overlay were compared (see Table 21). Method "A" which consisted of removing the fluid (PBS) only from the surface of the nutrient overlay into which phage was released, method "B" which consisted of removing the fluid (PBS) and the top agar layer (nutrient overlayer), and method "C" which consisted of removing the top layer first and then adding to the top layer 5 ml of PBS, all yielded approximately the same titer of phage stock and about the same volume of stock. Method "A" consumed the least amount of time. Method "B" would probably be chosen over "C", but each was nearly equally time consuming. Method "B" was expected to yield the higher titer, but it didn't. Such factors as readsorption of the phage progeny to the host cell wall, phage progeny being trapped in the media, failure to synchronize culture and failure to separate phage and cells at the instance of phage release probably contributed to the failure to get a distinction between the methods and the low titers (Adams, 1959). Thus, no difference could be detected between the methods according to the plaque count.

Optimal volume of diluent for bacteriophage P22 extraction

In Table 19 the data presented indicates that the optimal volume of PBS to be used in extracting bacteriophage P22 from its top agar at room temperature should be 3 ml with a 3 hour period of extraction. This observation is consistent with references described by Hershey, Kalmanson, and Bronfenbrenner (1943). Although Hershey et al. (1943) used the same volume of diluent, the kind of diluent they used was broth, the phage was a coli phage, and the time they allowed the fluid to stand was only 15-20 minutes. Swanstrom and Adams (1951) report good yields with a 5 ml per plate diluent of broth. The amount of diluent used will depend on several factors. First, the amount of time the plates are allowed to stand before extraction. Second, the method of extraction used (scraping off of the top layer may allow the addition of more diluent than usual because of the loss in decanting and centrifugation). Third, the approximate titer wanted (the addition of 1 ml instead of 3 ml will result in a 10-20% phage/ml increase if removed in 15 minutes. Thus, the optimal volume for plates prepared by the agar layer technique is approximately 3 ml.

Optimal time for bacteriophage P22 extraction

As described (Table 20), the optimal time for allowing the diluent (PBS) to stand on the surface of the top layer for extracting bacteriophage P22 was about two hours. Some investigators recommended 30 minutes (Swanstrom and Adams, 1951). Others reported 15 to 20 minutes as the period necessary for the standing of the diluent (Hershey et al., 1943). Eisenstark (1966) reported the use of 3-5 hours as the time the diluent should be allowed to remain on the plate. The amount of time that the diluent should be left on the plate still is an open question, for the literature shows

little if any agreement. In the future phage extraction should be done in the cold at 11 C to prevent evaporation of the diluent, and instead of stopping at 12 hours, the experimenter should continue for at least 30 hours using about 10-20 plates for each time. In conclusion, at least 2 hours should be used in extracting phage P22 in order to realize a higher titer and this extraction should occur in the cold (11 C).

Rapid bacteriophage typing

From Figure 2 it can be observed that the phage typing in a hemagglutination tray offers certain distinct advantages. First, only 1 ml of soft agar is used per well where as a regular size petri plate requires about 15 ml. Second, each well serves as test area for either the bacteriophage (when unknown) or host cells. Since this test area is isolated from the others, it can be considered as a separate petri plate. Third, there is no need to pour plates before hand and wait for them to dry (to allow a certain amount of evaporation of water to occur from the media). Last, one can test more phages or cultures because hemagglutination tray occupys less space than the bulky petri plates. The disadvantages are the following:

1. One-half of an ml of overnight host cells are needed per well.
2. About 15 minutes are required before the soft agar solidifies.
3. The reading time of the well is based on the phage-host system used.

For those laboratories operating on low funds, these hemagglutination trays can be cleaned by removing the used media from each well and submerging the trays into a Lysol solution (20 ml of Lysol per liter) for about 24 hours. They should then be rinsed with tap water for four hours. After they have air dried, the wells can be tested for purity (absence of bacteriophage) by placing in each well, cells and media and incubating for 5 hours.

If no plaques result, the trays are clear for further use.

Since the advantages for using the above method for phage typing exceeds the disadvantages, this technique should be considered on the basis of its merit.

RNAse inactivation of an RNA bacteriophage M-12

The evidence presented in Plate 3, Table 22 demonstrates the sensitivity of M-12, and RNA containing phage, to RNAse. This sensitivity was expressed by failure of M-12, after exposure to RNAse (approximately 80 ug/ml in Tris-buffer), to form visible plaques on its host cells. This phenomenon does not seem to be restricted to M-12, for a small P. aeruginosa phage, (7v, RNA containing), has been reported to be sensitive to RNAse of about the same concentration (Feary, 1963). Some of the possible explanations for the above observations are as follows:

1. Accidental damage of the phage coat during assemblage which left an opening by which the RNAse molecule could penetrate.
2. A heritable property of the phage's RNA molecule which is expressed as a defect in the phage coat through which the RNAse can enter.
3. A mistake during packaging of the phage's RNA molecule which leaves a part of the molecule exposed to the external environment.
4. RNAse serving in some capacity to cause the release of the RNA molecule from its coat thus exposing it to the inactivating properties of RNAse.

Since RNAse sensitivity has been observed in a totally unrelated RNA phages (Feary, 1963), it can be concluded, although not confirmed, that RNAse sensitivity is a heritable property of many RNA containing phages. This would rule out any mistakes as a possible explanation. Since M-12

showed sensitivity to RNAse, one could propose that the RNAse did some or all of the following:

1. Inactivated the RNA molecule before it left the phage coat (evidence: no plaques).
2. Inactivated the RNA molecule as it left the phage coat if one is willing to assume the coat remains outside of the host. (Evidence: same as (1).)

Since it has been established that double stranded RNA is nuclease-resistant and that single-stranded RNA is nuclease-sensitive (Weissman, 1964), can be attributed to the RNAse inactivating the RNA molecule of the phage either before the RNA molecule entered the host cell or after the release of phage progeny from the host.

Since sensitivity to RNAse has been found in two totally unrelated phages, it is very possible that this property may be characteristic of most or all of the RNA containing bacteriophages. Thus, the use of RNAse may be considered as a rapid technique for determining when a bacteriophage contains RNA.

CONCLUSIONS

These studies indicated the following:

1. Hfr, a male Salmonella typhimurium, when stored in undiluted normal horse serum at 11 C showed a high viable cell count and a high chromosomal transfer frequency up to ten months.
2. The use of plates without the bottom agar layer produced bacteriophage P22 stock preparations and bacteriophage P22 titrations as good or better than those performed with bottom agar layer.
3. Carbowax when used to concentrate bacteriophage P22 showed no inactivating characteristics on the biological activity of bacteriophage P22 even after storage.
4. The method used to perform transduction was found to be independent of the transducing frequency of genes found in normal bacteriophage P22 lysate.
5. It was found that after 10 minutes adsorption of transducing bacteriophage P22 has occurred, prolonged incubation showed no relationship to the frequency of transductants.
6. Of the bacteriophage P22 concentrating agents compared, saturated ammonium sulfate showed the highest per cent of recovery of viable phage P22 (50%). Methanol, and acetone were both inactivating in terms of plaque forming units to bacteriophage P22 at almost all concentrations.
7. Bacteriophage P22 and its host, Salmonella typhimurium, were demonstrated to be present in KSU P22-Salmonella Laboratory's air but in extremely low numbers.
8. The range of the number of viable bacteriophage P22 found on a

toothpick after stabbing a fresh (4 hours old) plaque was found to be from 1×10^5 to 5×10^8 phages per ml. The average number of phage P22 which can be expected on the end of a toothpick per 30 stabs was 202×10^7 phages/ml.

9. The mean number of bacteriophage P22 contained in 30 randomly selected plaques was found to be approximately 57×10^7 phages per ml.

10. Nutrient agar plate on which bacteriophage P22 has been grown can be extracted as many as five times with Phosphate buffer solution at room temperature.

11. Three methods of extracting bacteriophage P22 were compared. Not any of them showed any advantage over the other.

12. The optimal volume of Phosphate buffer solution which should be used in extracting bacteriophage P22 from its nutrient agar overlay was found to be 3 ml per plate at room temperature.

13. The optimal time for removing the extracting fluid (Phosphate buffer solution) from the surface of the nutrient agar overlay was determined to be approximately 2 hours.

14. Rapid bacteriophage typing when performed in a hemagglutination tray was concluded to be much more economical and less time requiring than the standard method.

15. A RNA containing bacteriophage, M-12, was found to be inactivated by RNAse.

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STANDARDIZATION OF SOME METHODS FOR EXPERIMENTS WITH BACTERIOPHAGE P22 AND SALMONELLA TYPHIMURIUM

by

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The purpose of this work was to standardize methods used with the temperate phage P22 of Salmonella typhimurium. In each of the following experiments an attempt was made to develop a standard method which would include optimal conditions and greater efficiency.

A high frequency recombinant was mixed with each of the preservatives (glyceral, normal horse serum, nutrient broth, and glass beads in the presence of $CaSO_4$) and stored at each of the following temperatures -18 C, 11 C, 25 C, and 37 C for 10 months. The Hfr B3 cells (male Salmonella typhimurium) which were stored in normal horse serum at 11 C showed the highest viable cell count and the highest chromosomal transfer frequency after 10 months.

A test to determine if plates without the previously poured bottom agar layer were as efficient as plates with the bottom agar layer in P22 plaque-count assays and phage P22 stock preparations was carried out. It was found that the use of bottomless agar layer plates yielded as good or better results than those with the bottom agar layer.

Carbowax, a concentrating agent, was tested to determine if after storage at -56 C, -18 C, 11 C, 25 C, and 37 C for 60 days if it in some way decreased the biological activity of the phage (plaque-forming units). The results of the findings were that Carbowax not only did not decrease the biological activity, but it in some way seemed to stabilize the P22 phage plaque-forming units at all temperatures indicated.

To determine if the markers carried on transducing fragments were independent of the method of transduction, three different methods of transduction were performed on the same normal lysate from a wild type Salmonella typhimurium. The results were that of independency; thus, the transducing frequency of markers in a normal lysate was shown to be independent of the

method used.

After incubation of transducing phage P22 and Salmonella typhimurium at 37 C up to 95 minutes, the frequency of the transductants for each locus tested showed no significant increase or decrease.

A comparison of bacteriophage concentrating agents, revealed saturated ammonium sulfate solution in a concentration of 1 part crude phage P22 lysate and 9 parts saturated ammonium sulfate to be the best phage concentrating agent. It gave about 50% recovery of viable phage P22. In contrast, acetone and methanol revealed inactivation characteristics in all concentrations tested.

Phage P22 and Salmonella typhimurium were shown to be present but in extremely low numbers in the air of the microbial genetics laboratory where these experiments were conducted.

Since sterile toothpicks are used frequently by geneticists to pick plaques, a determination was made of the expected average number of phage P22 obtained on the end of a toothpick per 30 stabs with separate toothpicks. The number was higher than expected, 202×10^7 phages. The mean number of P22 contained in a single plaque was found to be 57×10^7/ml.

Phage P22 stock preparation plates were shown to be extractable up to five times. Three methods of extracting phage P22 were compared and none of them showed any advantage over the other.

The optimal volume of Phosphate buffer solution which showed the higher titer was 3 ml. The optimal time for allowing the extraction fluid to set on the phage P22 stock preparation plates were revealed to be about 2 hours at room temperature.

Rapid bacteriophage typing in a hemagglutination tray was shown to

require less time and to be economical. RNA containing phage M-12 showed sensitivity to RNAse; thus, RNAse may be used to identify sensitive RNA containing phages.