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A QUANTITATIVE STUDY OF BACTERIOPHAGES FOR "CHEESE STARTER BACTERIA": THEIR OCCURRENCE AND DISTRIBUTION IN THE ATMOSPHERE OF CHEESEMAKING ROOMS

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ABSTRACT

The object of this study was to examine quantitatively the phage distribution in the atmosphere of a cheesemaking room so as to obtain an overall picture of: phage build-up in the air during the cheesemaking process, the areas where phage was most abundant, and the rate at which phage disappeared. A detailed knowledge of the typical fluctuations of phage in the atmosphere of a cheesemaking room throughout the day should provide a basis for intelligent mitigation of the problem of phage infection in cheesemaking.

Several methods for estimating phage in the air were employed. As a rough guide, the well-known sedimentation method of exposure to the air of agar plates spread with the host organism was used throughout the work. Experiments showed that regardless of whether the plates were spread with the host organism shortly before exposure, or held overnight in the refrigerator after being spread, little difference was made to the results. Using another sedimentation method, Petri dishes of peptone-salt solution were exposed to the air. The phage particles in the suspensions were estimated by a plating method. The slit sampler method, where air was drawn over the surface of agar plates spread with the host organism, was used only on some occasions. The most reliable method used was that of filtration of air through calcium alginate wool. In this method a measured volume of air was drawn, by means of a vacuum cleaner motor, through several layers of calcium alginate wool held in a glass tube. The phage which collected in the fibres of the calcium alginate wool was estimated by a plating method. It was at this stage, when developing a suitable method, that grave difficulties were encountered, as the hexametaphosphate solution used for dissolving the calcium alginate apparently sequestered some of the calcium needed for phage proliferation, with the result that plaque counts were adversely affected. Calcium was, therefore, supplied by the addition of $CaCl_2$ but, when calcium alginate and sodium hexametaphosphate were present during estimations, some calcium combined with phosphate, and this resulted in the production of plates which were cloudy and difficult or impossible to read. After much experimental work with different quantities of agar media and of $CaCl_2$, and trials with and without phosphate in certain layers, a four-layer method was eventually devised in

which calcium was supplied by diffusion to the phage-organism layer, and this gave satisfactorily high counts and plates which could be easily read. Experiments were also carried out to determine a suitable age for the culture used as host in the estimations.

During the course of the work it was necessary to prepare the phage aerosols needed for experiments in which the efficiency of the method could be checked. Forcing air through a rubber tube into which phage suspension was injected by means of a hypodermic needle proved to be satisfactory for producing the required aerosol. Investigations of the survival of aerosols were also carried out.

Surveys were made at three commercial factories (two mechanized, one non-mechanized) by carrying out sedimentation tests at various places in the cheesemaking room at regular intervals throughout the day. Air filtration counts were also carried out at regular intervals, and the phage content of the whey released during cheesemaking was determined on occasions.

Results showed that the phage content of the cheese factory atmosphere depended on the amount of phage development in the milk and whey and the extent to which the whey droplets were dispersed into the air during the cheesemaking operations. The times for maximum distribution of phage were after cheddaring (milling, salting, etc.). The whey separator dispersed large quantities of phage into the air. The majority of aerosolized phage particles normally sedimented fairly rapidly. The unwrapping of cheese bandages on the morning following cheesemaking resulted in extra phage being distributed into the air.

The agar plate sedimentation method gave an approximate idea of comparative amounts of phage in the atmosphere.

As a result of the various experiments carried out, an overall picture of phage distribution in the air of the cheesemaking room during the cheesemaking process was secured.

PREFACE

The work described in this thesis was undertaken with the object of obtaining a comprehensive view of the quantitative distribution of airborne phage in the making rooms of cheese factories during the manufacturing process. It was not intended to define the level of airborne phage which would be critical to the making process but rather to investigate the variations in levels of airborne phage at different times, in various areas near the cheese vats, and under varied conditions of manufacture.

As the methods for evaluating airborne phage available hitherto were qualitative at best, the development of a more precise method formed an important part of the work.

There already exists some knowledge regarding the quantities of phage in the curd, whey and atmosphere during cheese manufacture. Amplification of this knowledge should be of value in dealing with phage-contamination problems which are ever likely to occur in the cheesemaking industry.

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INTRODUCTION

INTRODUCTION

Although the intermittent occurrence of "slow vats" in cheesemaking had been recognized for many decades, it was not until the early 1930s that the problem was seriously investigated. Single strain starter cultures were introduced into commercial use in New Zealand in 1933. Subsequent to the introduction of single strain cultures, bacteriophage (or phage) was discovered (Whitehead & Cox, 1935) to be a potent cause of starter failure. With a mixed starter, if phage attacked one or more strains there were still left other strains which could produce sufficient acid for cheese manufacture albeit slowly, but when a single strain starter culture was attacked by phage the effect was, on occasions, almost a complete cessation of acid production in the cheese vat. Since the discovery of the importance of phage in cheesemaking a great deal of work has been carried out in examining phages related to a wide range of single strain starter cultures, and recommendations for the use of these starters have been made in the light of their known phage relationships.

There are several sources of infection of starter bacteria by phage, and the infection may vary from slight to highly likely to cause starter failure. Whey taken by farmers from the factory may contaminate cans in which milk is returned to the factory; the bulk starter can be infected during inoculation; phage on incompletely cleaned vats and equipment may infect the cheese milk; phage from the whey separator or whey splashed from vats can result in a phage-laden atmosphere which may subsequently cause further infection.

The extent to which the cheesemaking process is affected by phage contamination depends on several factors, viz. the amount of contaminating phage, the length of any ripening period, the stage of the cheesemaking process at which infection of starter, milk or vat contents occurs, the latent period and burst size of the phage attacking the starter and, when more than one starter strain is used, whether the phage which develops on one strain attacks the other strain or strains or whether the lysin of the first affects another strain. Numerous studies have been made concerning phage contamination in the cheesemaking process, but the work reported in this thesis was specially concerned with airborne phage and the role of the atmosphere, in the cheesemaking room, as a source of phage problems.

The chief source of phage in the atmosphere is the whey produced during the manufacture of cheese. Whey droplets containing phage are dispersed into the atmosphere at various stages of the process. The extent to which phage has been propagated in the vat, i.e. the concentration of phage, and the physical factors tending to distribute phage into the atmosphere or to remove or destroy it, would in sum total determine the quantity of phage in the air at any one time. Under physical factors could be listed: splashing of whey, agitation and running of vats, the atomizing action of the whey separator, violent movement of curd particles at milling, salting and hooping (due to manual or mechanical manipulations), squeezing out of whey (and air) during pressing, air movements due to draughts or fans, and factors affecting the sizes of whey droplets. Vigorous handling of cheese cloths during and after removal could also contribute to the atmospheric phage.

The remaining pages of this introductory section provide a background to the topic studied and outline the methods and results obtained by others working on subjects relevant to the present investigation.

Phage in the Atmosphere

For many decades phage had caused much trouble to the cheesemaking industry, but it was not until some years after the discovery of phage for lactic streptococci that it was recognized that phage was actually present in the atmosphere of the cheese factory. It is relevant to briefly outline the position before and after the recognition of this fact.

Whitehead & Cox (1935) were the first to demonstrate the existence of phage for bacteria of industrial importance other than species of clinical significance. They established the occurrence of phage in a culture of lactic streptococci used as cheese starter. Reference to "flaring up" of phage upon aeration of milk medium (Whitehead & Cox, 1936) did not connect any such apparent effect with airborne phage, although at the time phage was known to be a cause of slow starters. In 1937 Whitehead & Hunter postulated that phage was a product of the organism and that contamination from outside sources was unlikely. In a publication by Whitehead & Hunter (1939) there was no reference to airborne contamination, but in 1941 these authors, when investigating

the cause of starter failure in two well-separated areas of New Zealand, concluded from their experiments that the only explanation must be the presence of phage in the air, and they were able to prove experimentally that phage was present in the atmosphere of one of the places, viz. a large commercial cheese factory. The whey separator was a major source of phage distribution into the atmosphere. Methods suggested for combating phage failure were: (a) the use of disinfectant spray for destroying airborne phage, (b) culture protection in the factory, and (c) the use of a separate building for preparing starters. Mattick, Nichols & Wolf (1944) investigated "slowness" in cheesemaking in two factories in England where there had been manufacturing difficulties due to phage. They showed that by the use of isolated starter rooms, the thorough rinsing of vats with hypochlorite solution, and other precautions, contamination from airborne and other phage was reduced to a minimum, and there was marked improvement in the manufacturing process and in the quality of the final product. Whitehead & Hunter (1945) discussed the points to be considered in siting an isolated starter room. They also compared the dispersal of phage in two districts of approximately the same size, one with 100 cheese factories and the other with five factories. There was a slower spread of phage in the district with the fewer factories. In this connection it must be realized that small particles can be transferred over great distances in the atmosphere. It has been pointed out (Ludlam, 1967) that particles of 30 μ radius seldom become airborne, those of radius greater than a few microns do not remain airborne for long, and that a small proportion of the smallest particles (radius about 0.1 μ) may be carried round the hemisphere before being eventually deposited on the earth's surface. Many phages for the lactic streptococci are round about the 0.1 μ size, so it can readily be understood that single phage particles or even small clusters can be transported in the atmosphere from district to district, or even further. Whitehead (1953) suggested that the protection of cultures from airborne phage was a more practicable procedure than eliminating airborne phage itself. Robertson (1966a) discussed methods for the inoculation of factory starter in a phage-laden atmosphere, and also described a method for providing phage-free air in the head-space of a bulk starter machine. The same author (Robertson, 1966b) pointed out that airborne phage was one of the factors contributing to phage contamination of the farm milk supply.

Quantity of Phage Needed to Cause Slow Vats

At this point the question may be raised as to what actual quantities of phage have been found to affect the cheesemaking process. It has been shown that the critical time for phage contamination is before the addition of rennet. Hunter in 1944 found that 2×10^6 phage particles per ml of milk gave a slow vat when using 1% starter (HP) and ripening for half an hour before renneting. Czulak (1952) demonstrated that 6×10^5 phage particles per ml caused slowness in the vat, with ¼-½-h ripening period and 1% starter. Whitehead & Harkness (1952), using 2.1% starter and a ½-h ripening period, showed that 1.4 phage particles per ml gave a slow vat. It is axiomatic that phage in the starter could have critical results on occasions. On the other hand, phage added later in the process has no measurable effect on the particular day's cheesemaking. It has been demonstrated that large amounts of phage added after cutting the curd have had little effect on the production of acid (Czulak, 1952). Naturally it has been recommended that the ripening period should be kept to a minimum and preferably eliminated.

Phage Aerosols Produced Artificially

When studying phage in the atmosphere it is often necessary to be able to produce at will a phage aerosol in order that experiments may be carried out, such as checking methods for phage estimation or testing methods for phage destruction. Zentner (1961) summarized known methods of aerosol formation under the following headings:

1. condensation processes - by condensation of vapour;
2. (a) hydraulic - by forcing liquid through an aperture,
   (b) air-blast atomizers - by shattering drops of liquid with a violent blast of air,
   (c) centrifugal action.

Wolf, Nichols & Ineson (1946) investigated the effectiveness of hypochlorite aerosol for destroying phage in the air. The aerosols were prepared by means of a Dynalysor, an apparatus consisting of a spraying machine, a compressed air container, and a container for the liquid preparation. Czulak (1953) used a hand-operated spray gun to prepare a fine mist of phage when investigating the use of ultra-violet irradiation as a method for controlling airborne phage. Bennett & Nelson (1954) distributed phage for lactic streptococci

into a closed room by means of atomizers. They found that most phage was deposited or disappeared in 1-2 h, but occasionally there was the recovery of some after a longer time. Ehrlich, Miller & Idoine (1964) studied the effect of relative humidity and other factors on the survival of E. coli phage T_3 aerosol. They found that there was a significant reduction in survival at humidities lower than 70%. Decay was due to settling of particles and loss of vitality. Harstad (1965) found that there was greater stability with T_1 phage aerosol than with T_3. This was more marked at lower relative humidities.

Estimation of Atmospheric Phage

The principal methods for estimating phage in the atmosphere are: sedimentation onto a solid or liquid surface, suction of air through fibrous material (cotton wool, alginate wool) or through liquid, suction of air in such a way that it impinges on the surface of an agar plate spread with the host organism, and suction of air combined with electrostatic precipitation.

In the sedimentation method using a solid surface, an agar medium spread with the host organism is exposed to the atmosphere for a specific time, and is then incubated. Plaques are subsequently counted. If a liquid collecting agent is used, the numbers of phage particles which have fallen into the liquid may be estimated by the soft agar layer method. The sedimentation method gives only rough, qualitative results. Particles settle at rates depending on their size and density, and air currents may greatly affect the results. Depending on initial drop size and phage concentration, droplets of whey could contain several phage particles. Wolf et al. (1959) have pointed out that a particle:

0.1 µ	in diameter could settle at a rate of	2.9 mm/h
0.4	"	23.8 "
1.0	"	128.0 " .

The figures given by Wolf et al. confirm the fact that settling rates for different sized whey droplets could vary considerably. Nevertheless the sedimentation method has some merit and has been useful in obtaining a rough idea of phage levels in the air of cheesemaking rooms.

The earliest tests for atmospheric phage (Whitehead & Hunter, 1941) were done by exposing sterilized skim milk and inoculated agar surfaces to the atmosphere, and also by aspirating air through sterile water. Phage was detected in air by all these methods. Whitehead & Hunter

(1945) discussed the value of phage tests in which sterile milk was exposed in Petri dishes in the surroundings of a cheese factory. Czulak (1953) estimated phage in an artificially produced aerosol using the sedimentation method by exposing tryptone-yeast-extract agar plates to each of which was added 10 ml of culture-agar-$CaCl_2$-water mixture. Bennett & Nelson (1954) estimated phage in an artificially produced aerosol by aspiration of a measured volume of air through distilled or buffered distilled water or by sedimentation onto stainless steel discs or into buffered distilled water contained in Petri dishes. Ehrlich et al. (1964) estimated phage in an artificially produced aerosol of T_3 coliphage by means of an all-glass impinger using tryptose saline. Plaque counts were carried out on the liquid suspension. Decker, Geile, Harstad & Gross (1952) estimated coliphage T_3 particles by sieve sampling and impingement of particles on tryptose phosphate agar plates. The plates were covered with melted tryptose phosphate agar containing the host organism. After incubation of the plates, plaques were counted. Morris, Darlow, Peel & Wright (1961) estimated phage in coliphage aerosol by electrostatic precipitation. They found that the collection of particles by the slit sampler method gave less than 1% of the counts obtained by the electrostatic precipitation method. McDougall (private communication) estimated poliomyelitis virus aerosol by filtration through calcium alginate wool.

The Role of Calcium in Phage Estimation

The importance of inorganic ions and organic cofactors has been increasingly realized in connection with the part they play in phage replication. The factors which are necessary, and the stage at which they act, have been investigated for a fairly wide range of genera. The role of calcium in phage development has been of special interest. Anderson (1948) noted that previous work had indicated that some viruses were activated by adsorption cofactors.

Delbruck (1948) showed that calcium ions could be required at some stage before phage multiplication. Shew (1949) discussed the effect of calcium on the development of lactic streptococcal phages, and Reiter (1949) used $CaCl_2$ when studying lysogenic strains of lactic streptococci. Cherry & Watson (1949) found that certain electrolytes stimulated cellular lysis and virus adsorption when working with the lactic streptococci. Collins, Nelson & Parmelee (1950) investigated

the relation of calcium and other constituents of a defined medium to the development of phage for lactic streptococci. The presence of phosphate and citrate in media inhibited phage increase, possibly due to the effect on calcium. In a particular defined medium in which 10 strains of lactic streptococci could grow, fortification with calcium was necessary for the multiplication of 8 out of 10 homologous phages. Two other experiments suggested that calcium was not needed for phage adsorption. Turner & Nelson (1951) counted phages for lactic streptococci using a 2-layer plate method. It was suggested that the calcium in the milk may assist phage proliferation. Potter & Nelson (1952a) used a 2-layer plating medium with $CaCl_2$ incorporated in the top layer. Potter & Nelson (1952b), working with lactic streptococcal bacteriophage, showed that the adsorption of phage on their host bacteria did not require calcium, although calcium caused a slight increase in the rate of adsorption, but that calcium was needed for phage multiplication. Doull & Meanwell (1953) stated that for lactic streptococci phage adsorption was unaffected by lack of calcium, but that after adsorption a certain amount of calcium (depending on the strain of phage) was necessary for proliferation. Lark & Adams (1953) suggested that in coliphage T_5 "the tip of the tail was composed of a calcium-activated enzyme which was necessary for the ingestion of nucleic acid". Luria & Steiner (1954) pointed out that calcium was needed "so that the coliphage T_5 tail could penetrate the organism and also in order that maximum phage production might be obtained in a synthetic medium". Rountree (1955) stated that for streptococci divalent cations were needed for phage adsorption and possibly calcium was needed later in the process. Adams (1959) drew up a list showing whether calcium had effect on adsorption or after adsorption for a large number of organisms. Mikolajcik (1963) gave the composition of a broth suitable for lactic cultures and phage. Sterile $CaCl_2$ was added to give a concentration of 0.33%. Shafia & Thompson (1964) pointed out that small amounts of citrate could interfere with steps in phage proliferation which were dependent on divalent ions (phage $\phi\mu$-4).

The need for the presence of calcium in phage estimations has thus been well established. It appears that phages for the lactic streptococci need calcium for a stage or stages after adsorption of the phage particle to the host organism.

PART I

SAMPLING, METHODS AND PLATING OF PHAGE

A. SAMPLING AND METHODS OF ESTIMATION

A. SAMPLING AND METHODS OF ESTIMATION

(i) SEDIMENTATION USING AGAR PLATES FOR PHAGE DETECTION

Quantities of 10-15 ml of lactose yeast phosphate agar (LYPA) were poured aseptically into sterile Petri dishes and allowed to harden. The plates were dried in the 37°C incubator for approximately 1 h. One drop (approximately 0.05 ml) of a 24 h clotted milk culture of a phage-sensitive strain of Streptococcus was added to each plate, and this culture was spread over the surface of the plate using a sterile glass spreader. These plates were exposed to factory atmospheres as a means of estimating the numbers of droplets containing phage falling onto the plates per unit of time.

Comparison of Cooler-kept and Freshly Spread Plates

During investigations of the phage present in the atmosphere of a cheese factory it is not always convenient to spread plates with a sensitive culture immediately prior to exposure of the plates. A more convenient method is to take to the factory a number of plates already spread with the culture.

Hence, experiments were carried out to compare freshly spread plates with plates prepared a considerable time before use. Plates were prepared as follows:

Plates to be kept in the cold room were spread with culture in the afternoon and then left in the cold room (4°C) overnight in a portable ice box (with the lid off). Next morning 1-2 frozen Slikka pads were placed in the ice box, the lid was placed in position, and the plates were transported to the factory where the exposures were to be carried out. Slight variations in the method were made on occasions.

Examination of an ice box treated in this way and containing two Slikka pads showed temperatures of 8.5°C, 10°C and 11°C at 9.30 a.m., noon, and 12.30 p.m. The room temperature was 22.2°C at 9.20 a.m. The ice box had been removed from the cold room at 9.00 a.m.

Freshly spread plates (F) were prepared a short time (a few min to 1 h) before use.

Comparisons were made by exposing cooler-kept plates and freshly spread plates (either singly or groups of each) in close proximity to one another near a cheese vat. The lids were removed for 10 min and then replaced. The Petri dishes were then transferred to the laboratory at approximately room temperature for incubation at 30°C overnight. The plaques were counted subsequently.

Several experiments were carried out using a total of 10 different phage-sensitive starter bacteria. Most of the experiments were carried out in the New Zealand Dairy Research Institute cheesemaking factory, but experiments were also carried out at the Mangawhata dairy factory and at the Belvedere factory. In all, the comparisons were carried out on 20 different days. Plates were exposed at the times most likely to secure high plaque counts, i.e. near milling time. To get high plaque counts in the atmosphere is a matter of chance, especially in a factory such as that of the Dairy Research Institute where the amount of cheese made is relatively small. Even in a commercial cheese factory with many large vats there can be wide variations in the amount of phage in the air on different days, depending on factory technique and other factors.

Results are given in Table I. In many experiments low comparative figures were obtained, and sometimes groups of cooler-kept (C) and freshly spread (F) plates did not show any plaques at all. In cases where corresponding cooler-kept and freshly spread plates showed all negative results, entries were not made in the Table. Comparisons showing very low figures would not be as reliable as when the figures were higher. Results are grouped together under particular starters irrespective of the days on which the comparisons were made. Figures are tabulated in ascending order of numbers of plaques. Individual plaque counts are given on the left, and average figures on the right, in the columns. In those cases were single-plate comparisons were made, the figures are placed on the right-hand side of the column, or in single columns.

Examination of figures (averages where possible, otherwise individual counts) shows that the cooler-kept plates showed fewer plaques than the freshly spread plates on 59 occasions, and that the freshly spread plates showed fewer plaques than the cooler-kept plates on 49 occasions. In five comparisons the figures were the same.

Table I Plaque counts using cooler-kept and freshly spread plates.

	HP (DRI)			ML_8 (Bel.)	ML_8 (Bel. cont'd)	ML_8 (Bel. cont'd)		KH (DRI)	
C		0		1	15		331	0,0,0,0	0.0
F		2		0	11		232	1,0,1,0	0.5
C		0		1	11			0,1	0.5
F		2		0	17	C_{13} (DRI)		0,0	0.0
C		0		1	16	1,0	0.5	0,1,1,0	0.5
F		2		1	16	0,0	0.0	2,0,1,2	1.3
C		0		0	14	0,1,1,0	0.5	1,3,1,1	1.5
F		4		2	13	0,0,1,0	0.3	4,1,5,6	4.0
C		7		2	17	0,1	0.5	4,3,2,0	2.3
F		19		0	18	2,0	1.0	6,4,5,3	4.5
C		8		0	18	2,3,1,1,0	1.4	3,3,7,3	4.0
F		26		2	18	1,0,0,0,1	0.4	3,8,3,0	3.5
C		18		3	12	1,1,0,0,3	1.0	1,3,3,4	2.8
F		40		0	27	0,0,0,2,0	0.4	3,3,7,6	4.8
	ML_8 (Mangawhata)		C	0	17	2,3,3,7	3.8	6,-,1,4	3.7
			F	3	31	1,0,0,0	0.3	4,3,8,11	6.5
C	0,1,0,0	0.3		4	26	3,5,5,2,3	3.6	7,4,3,3	4.3
F	0,1,0,0	0.3		0	23	4,1,2,2,3	2.4	6,12,7,7	8.0
C	0,1,0,2	0.8		4	30	11,6,4,3,2	5.2	1,13	7.0
F	1,3,-,0	1.3		0	25	1,3,5,4,5	3.6	6,9	7.5
C	0,4,4,1	2.3		4	27	22,19,27,17	21.3	8,6	7.0
F	5,2,6,1	3.5		1	30	4,30,32,18	21.0	8,7	7.5
C	4,5,5,3	4.3		6	27			11,3,5,11	7.5
F	6,6,4,3	4.8		14	50	E_8 (DRI)		7,14,6,14	10.3
C	8,3,5,8	6.0		7	52	1,2,0,1	1.0	21,22	21.5
F	3,13,5,4	6.3		14	40	0,0,1,0	0.3	14,23	18.5
C	5,11,7,9	8.0		11	41	1,0,1,1	0.8	52,72	62.0
F	10,10,5,10	8.8		11	60	1,1,1,1	1.0	121,114	117.5

C = Plates cooler-kept
F = Plates freshly spread
Bel. = Belvedere
DRI = New Zealand Dairy Research Institute

(cont'd)

Table I (cont'd) All at DRI.

	SK_1		SK_1 (contd)		US_3 (contd)		H_2 (contd)	
C			18,11	14.5		2	0,1	0.5
F			29,19	24.0		0	3,2	2.5
C	0,0,0,0	0.0	20,20	20.0	6,1,1,2	2.5	0,1,0,0,3	0.8
F	0,1,0,0	0.3	21,21	21.0	1,1,1,2	1.3	1,3,1,3,3	2.2
C	0,0,0,0	0.0	38,49,51,34,33	41.0	3,5,7,4	4.8	2,0,5,0,3	2.0
F	0,1,0,0	0.3	36,50,37,31,31	37.0	4,2,5,1	3.0	0,1,3,0,1	1.0
C	1,1,-,0	0.7	TR		5,6,1,1	3.3	1,3	2.0
F	0,-,1,-	0.5			6,6,9,2	5.8	3,3	3.0
C	1,2	1.5	0,0,0,0,0	0.0		10.0	2,1,1,1,1,2,1,2	1.4
F	1,0	0.5	0,0,1,0,0	0.2		1.0	2,5,3,5,7,9,2,-	4.7
C	6,3,3,5	4.3	1,1,1,0,0	0.6		23.0	10,4	7.0
F	3,4,2,5	3.5	0,0,0,0,0	0.0		4.0	6.5	5.5
C	4,2,6,5	4.3	2,2,8,4,3	3.8	10.13,15,21	14.8	5,12	8.5
F	4,3,8,7	5.5	0,3,2,4,3	2.4	13,10,16,11	12.5	5,11	8.0
C	10,9,5,6,3	6.6	3,102,20,50,23	39.6		29.0	8,3	5.5
F	4,5,4,3,1	3.4	19,21,14,33,31	23.6		9.0	17,7	12.0
C	3,7	5.0	R_1			39.0	(188,256)	
F	7,5	6.0				10.0	C (130,104)	149.4
C	3,7	5.0	0,1	0.5	94,111,92,124	105.3	(116,204)	
F	11,9	10.0	2,0	1.0	129,89,127,89	108.5	(100, 97)	
C	9,8,11,13,5	9.2	0,20	10.0	H_2		(100,101)	
F	9,9,12,8,7	9.0	7,1	4.0			F (296,364)	201.1
C	15,11,5,7,9	9.4			1,0,0,0,0	0.2	(232,246)	
F	4,13,11,11,9	9.6	US_3		0,1,0,3,2	1.2	(130,140)	
C	6,16,11,9	10.5		1.0	0,1,0,0,1	0.4		
F	5,9,12,11	9.3		0	1,3,0,1,3	1.6		
C	14,10	12.0		1.0	3,1,0,0,1,0,9,1	1.9		
F	12,20	16.0		0	1,1,2,1,0,1,0,0	0.8		

HP C_{13} E_8 KH SK_1 TR R_1 US_3 H_2 were strains of Str. cremoris
ML_8 was a strain of Str. lactis

Figures are presented in tabular form below (Table II) for the sake of clarity.

Table II Comparison of plaque counts for cooler-kept and freshly spread plates.

	No. of occasions	Average % by which lower
Cooler-kept plates lower	59	38% (48*)
Freshly spread plates lower	49	40% (37*)
Cooler-kept, freshly spread, plates the same	5	

* Occasions

The percentage reductions were arrived at as follows: the amount by which a group of cooler-kept plates was lower than its corresponding group of freshly spread plates was calculated as a percentage, and the average of all such figures was calculated as an average percentage reduction. In this way the cooler-kept plates showed an average reduction of 38% compared with the freshly spread plates. This figure represents the average for 48 occasions out of 59. On the other 11 occasions the figures for cooler-kept plates were zero, so in such cases the percentage reduction could not be calculated.

In a similar way the average percentage reduction for freshly spread plates compared with cooler-kept plates was obtained from the respective figures.

It is well known that the estimation of phage in the air by sedimentation is a very rough method, with factors such as variation in rates of settling of droplets due to varying densities, air movements which upset the even sedimentation in different places, and different numbers of phage particles per droplet, all contributing their part in introducing inaccuracies. The results discussed above indicate that keeping spread plates in a cooler overnight is also a source of error, but not of such magnitude as to preclude its use in a method used for making only approximate estimations of the phage droplet level of the atmosphere.

Comparison of Clotted Milk Cultures and Broth Cultures for Spreading Plates

Plates were generally spread with a clotted milk culture (0.05 ml), but a broth culture method has been used (Pearce, 1967). A broth suspension of organisms can be added to a plate, spread over the surface by tilting the plate, and the excess liquid can then be drained off. The method using broth is quicker than the method of spreading with a clotted milk culture. Experiments were carried out to compare the two methods of spreading plates, and results are shown in Table III.

In Expt A (Table III) plates were spread with clotted milk culture (0.05 ml) and were kept overnight in the cooler. Other plates were spread (next morning) with broth. Both lots of plates were put in an ice box containing Slikka pads. The plates were opened in the factory at the times given and at the places indicated in the Table. Results showed that plates spread by the two methods gave figures which agreed reasonably well.

In Expt B (Table III) broth plates were prepared by centrifuging three tubes of broth culture, decanting, and taking up the residue in 0.33 ml of broth. One drop per plate was used. Reference to the Table shows that results were about the same for freshly prepared plates, but that where plates were spread early, the broth plates showed nil counts. The plates spread early had been kept at room temperature for approximately 5 h and on the warm day (temperature up to 27°C) considerable growth would have occurred. The effect of this was more apparent with the broth culture-spread plates than with the clotted milk culture-spread plates.

In view of the foregoing results it was decided to use plates spread with clotted milk culture. Subsequent work has shown that broth cultures can give variable results depending on conditions.

Effect of Age of Mat and of Amount of Inoculum on Plaque Counts

The age of the mats (where plates have been spread for short periods before exposure) and the amount of inoculum are factors which could influence the apparent count to some extent. A plate spread

Table III Comparison of growth media of cultures for spreading plates (clotted milk culture and broth culture). Plaque counts with exposure times of 10 min.

EXPT A

Mangawhata Dairy Company

Time Plate Opened	Medium for Culture	Press Vat 1	Agit. Vat 2	Agit. Vat 4	Near Vat 6
a.m.					
11.30	Milk	0,1	0,0	1,0	1,3
	Broth	0,1	2,0	2,0	1,-
12.00	Milk	0,0	1,1	1,0	7,9
	Broth	1,1	1,1	0,2	25,1
p.m.					
1.00	Milk	4,5	12,9	2,6	2,0
	Broth	4,4	21,-	5,4	3,5
2.00	Milk	47,-	59,47	62,74	98,57
	Broth	46,27	32,29	58,68	82,55
3.00	Milk	1,1	16,9	1,0	6,4
	Broth	1,0	5,9	5,2	5,9
4.00	Milk	3,5	10,17	12,7	16,12
	Broth	6,4	11,10	13,6	18,12

EXPT B

N.Z. Dairy Research Institute Dairy Factory

Time Plate Opened	Spread Early 9-10a.m. Clotted Milk Culture	Spread Early 9-10a.m. Broth Culture	Freshly Spread Clotted Milk Culture	Freshly Spread Broth Culture
p.m.				
2.10	8	0	14	3
2.20	2	0	7	10
2.30	21	0	33	45
2.40*	37	0	56	40
3.00	10	0	16	30

Press = Plates located on press
Agit. = " " " agitator beam
Near = " " in various places near vat

Starter bacteria in vats = ML_8 AM_2
" " on plates = ML_8

* Plates exposed 20 min
Starter bacteria in vats = HP US_3
" " on plates = US_3

with 0.05 ml culture may, on standing at ambient temperature, become the equivalent of a plate spread with 0.1 ml culture. However, the stage of growth may have an effect on the readiness with which the cells are lysed by phage particles. The amount of inoculum generally used (0.05 ml) gives reasonably sized plaques with the majority of the starters in common use in factories. When a phage particle attaches itself to a growing bacterial cell on an agar medium it will eventually lyse the organism, producing a crop of phage particles. These in turn lyse the bacterial cells in the immediate neighbourhood, and this process continues until bacterial growth ceases, resulting in the production of a clear area or plaque. An increase in the amount of inoculum results in possibly the same amount of phage being produced, but the area of clearing is more limited, i.e. increased inoculum gives smaller plaques.

Experiments were carried out to find the effect of keeping spread plates at room temperature for various short periods and of varying the amounts of inoculum. The results are shown in Tables IV and V.

Table IV shows that the time of keeping spread plates before use did not produce any consistent effect. Variations in figures are of the order expected of the sedimentation method. Similarly, Table V shows that 0.05 ml of clotted milk culture is a suitable quantity for inoculating a spread plate.

Table IV Effect of age of mats on plaque counts.

Starter Bacteria	Time spread until Opened h/min	Time Plate Open min	Counts: Individual Plates	Counts: Averages	Starter Bacteria	Time spread until Opened h/min	Time Plate Open min	Temp. after Spread °C *	Counts
KH	3.35	10	3,11,11	8.3	C_{13}	6.20	20	22	211
	0.10	"	5,0,2	2.3		6.20	"	30	199
	0.05	"	1,2	1.5		0.05	"		148
Z_8	1.10	"		4.0		6.10	10	22	421
	0.05	"		4.0		6.10	"	30	507
	1.00	"	0,0,3	1.0		0.05	"		276
	0.05	"	0,0,1	0.3		6.00	"	25	1
	0.50	"	3,3,7	4.3		6.00	"	30	6
	0.05	"	7,3,4	4.7		4.00	"	22	2
	0.40	"	2,0,4	2.0		4.00	"	30	3
	0.05	"	1,2,1	1.3		0.05	"		6
H_2	2.20	"	11,4	7.5					
	0.05	"	4,3	3.5		5.50	"	22	16
	2.10	"	16,7	11.5		5.50	"	30	-
	0.05	"	7,10	8.5		3.50	"	22	16
	2.00	"	0,3	1.5		3.50	"	30	41
	0.05	"	8,4	6.0		0.05	"		17
SK_1	2.20	"	0,5	2.5					
	0.05	"	5,4	4.5		5.30	"	22	19
	2.10	"	9,8	8.5		5.30	"	30	21
	0.05	"	6,11	8.5		3.30	"	22	30
	2.00	"	5,6	5.5		3.30	"	30	24
	0.05	"	5,6	5.5		0.05	"		23

*Plates were kept at room temperature for the last 2-3 h (approx.)
Freshly spread plates were sometimes kept longer than 5 min before exposure

Table V Effect of amount of C_{13} inoculum on plaque counts.

Treatment of Plates	Plaque counts at stated times† 1.50pm	2.00pm	2.10pm	2.20pm
Spread several h before exposure	-	136	29	6
" 1 drop (0.05ml)	3	248	40	4
" 2 drops (0.10ml)	4	123	59	5
" 3 drops (0.15ml)	7	196	21	9

† Plates were exposed for 10 min

(ii) SEDIMENTATION USING LIQUID FOR PHAGE COLLECTION. LIQUID FOR DILUTIONS

A suitable liquid was required for exposure to the atmosphere in order to collect phage by sedimentation. A suitable diluent was also required for the preparation of serial decimal dilutions when estimating phage quantitatively in samples of whey and in calcium alginate wool pads (alginate method discussed later). A liquid suitable for one purpose could be suitable for other purposes. This section deals mainly with the effect of various liquids on phage.

For many years, phage dilutions had been made in water or in Ringer's solution at the New Zealand Dairy Research Institute and sometimes resultant suspensions were kept in the refrigerator for several h. Comparisons were made of water, Ringer's solution, and peptone-salt solution as diluents, and the effects of light and darkness, temperature, and pH on phage determination were investigated.

Effect of Keeping Phage in Ringer's Solution at 30°C

An experiment was carried out (Table VI).

In this experiment phage (diluted as detailed below) was added to Ringer's solution and the mixture was kept at 30°C. Plaque counts were carried out at zero time and at intervals, using a 2-layer plate method. (A concentration of phage suitable for experimental purposes was prepared by diluting a whey suspension of hp phage with water and Ringer's solution to approximately 10^{-4}. Phage hp attacks strain HP.)

Table VI Effect of keeping phage in Ringer's solution at 30°C

	Plaque Counts			
Time	Zero	0.5 h from zero	1 h from zero	Overnight
Counts	48	37	27	12
	44	44	47	9
	39	40	38	7
Av.	44	40	37	9

Note: Throughout the investigation Ringer's solution was ¼-strength whether specified or not.

Results showed that the phage count decreased progressively with length of time of keeping. In the experiment an initial average count of 44 plaques decreased to 40 plaques in 0.5 h, to 37 plaques in 1 h, and to 9 plaques after holding overnight. Ringer's solution was obviously unsuitable as a liquid in which to keep phage for any considerable time at 30°C.

Effect of Keeping Phage in Water Under Various Conditions

The results of three experiments are shown in Table VII.

Dilutions of phage were made in distilled water and the suspensions were kept under varying conditions, viz. light* and dark**, temperature (30°C, room, 4°C cold-room) and in some cases with added milk. Comparisons of mixing phage by pipetting and shaking plus pipetting were also made. Plaque counts were made on 0.1 ml amounts.

In Expt (a) plaque counts decreased to some extent as a result of storing at 30°C (3 days), and to an extreme extent when held in light at room temperature. Comparisons of mixing by pipetting and shaking plus pipetting showed that shaking plus pipetting resulted generally in slightly higher counts than pipetting alone. Phages in the cold-room kept better than at room temperature (in light) and at 30°C (in bath).

In Expt (b) the counts were slightly higher in water plus milk than in water alone. In this experiment there was not much alteration in counts on keeping samples overnight, except in case of 10^{-4} dilution (light) and 10^{-5} dilution in cold-room (both without milk).

In Expt (c) there was a marked decrease as a result of keeping samples overnight in the cold-room, room (light) and room (dark). In the case particularly of the sample kept at room temperature (light) overnight, the phage count had decreased to almost nil.

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* Light = daylight as applicable
** Dark = in closed cupboard

Table VII Effect of keeping phage in water under various conditions.

Expt	Holding time of phage suspension	Plaque Counts: Phage dilution 10^{-4} Cold-room	Light (room temp.)	Dark (room temp.)	30°C bath	Phage dilution 10^{-5} Cold-room	Light (room temp.)	Dark (room temp.)	30°C bath
a	Start		675(P) 840(S)				61(P) 61(S)		
	4 h					44(P) 82(S)	19(P) 32(S)		42(P) 64(S)
	3 days	425(P) 448(S)	<1(P) 44(S)		123(P) 316(S)	53(P) 40(S)	0(P) 1(S)		8(P) 6(S)
b	Start		521 804(+)				57 76(+)		
	2 h	524 692(+)	365 600(+)	505 729(+)		40 59(+)	44 68(+)	61 70(+)	
	Overnight	801 994(+)	313 1021(+)	805 1104(+)		9 135(+)	54 110(+)		
c	Start						177		
	2 h					92	37	93	
	Overnight					25	1	21	

Phage dilutions prepared in distilled water for each experiment from a suspension of hp phage in whey. Most counts averages of 3 plates.

Expt (a) P = dilutions mixed by pipetting S = dilutions mixed by shaking plus pipetting.

Expt (b) + = milk addition (1 ml skim milk added to 9 ml suspension).
Counts for suspensions without milk were multiplied by 0.9 to correct for non-addition of 1 ml water to 9 ml suspension.

Effect of pH on Phage when Kept in Water and in Water plus Milk

An experiment was carried out in which phage was added to distilled water and to distilled water plus milk, with pH values ranging initially from 7.5-5.6. Samples were kept at 30°C and at 4°C and plaque counts were done at zero, 2 h and 20 h. No consistent effect due to pH was found. Variations were in part possibly due to the use of water and of water plus milk as diluents.

Effect of Keeping Phage in Peptone-salt Solution

Two experiments were carried out using peptone-salt solution as a medium in which to keep phage. Serial decimal dilutions of an hp phage in whey were made in peptone-salt solution to 10^{-4} x 0.7. Quantities of 0.1 ml of this phage dilution were added to McCartney bottles, each containing 0.04 g calcium alginate wool. Nine ml peptone-salt solution were then added. The suspensions were kept at 22°C, and 4°C, for specific periods of time. For analysis, 1 ml sodium citrate (10%)* was added to each McCartney bottle to dissolve the calcium alginate wool, and the phage was estimated by a 4-layer plating method. Results are shown in Table VIII.

The initial phage count was an approximate figure for the hp phage which had been used as standard over a long period. The results showed that in Expt (a) the counts did not diminish in approximately one and two days at 22°C and 4°C, and also that in Expt (b) the counts did not diminish in three and six days at these temperatures. Storage at 4°C gave higher counts than at 22°C. It was therefore concluded that peptone-salt solution was a suitable medium in which to make dilutions and also in which to collect phage by sedimentation and thereafter in which to keep the phage until it was estimated quantitatively.

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* Throughout this thesis the percentage concentration of a solution means w/v unless otherwise stated

Table VIII Effect on plaque counts of keeping phage in peptone-salt solution.

Expt	Holding temp.	Initial	Held 18 h		Held 42 h	
		Plaque Counts				
a	Room 22°C	150*	143	Av.	172	Av.
			158	148	163	160
			144		145	
	Cold-room 4°C	"	158		157	
			158	156	211	187
			151		192	
		Initial	Held 72 h		Held 144 h	
b	Room 22°C	150*	203	Av.	202	Av.
			192	203	187	206
			214		229	
	Cold-room 4°C	"	230		257	
			212	222	256	248
			223		230	

* Many counts had been done on an equivalent quantity of phage, and these counts varied within a wide range. A representative value is given by a count of 150.

Effect of the Quantity of Sodium Chloride in Peptone-salt Solution on Phage

An experiment was carried out to find whether the large amount of sodium chloride in normal peptone-salt solution had any effect on the phage. Results of an experiment are shown in Table IX. Analyses were done by method (2). Details of method (2) will be given later.

Table IX Effect on plaque count of varying the salt content of peptone-salt solution.

Peptone-salt solution		Plaque counts (av. of 3)		
% Peptone	% Salt	Start	1 day	3 days
0.1	0.88	155	197	191
0.1	0.4	177	129	124
0.1	0.1	171	163	143
0.1	0.0	224(1)	201	172

In this experiment four peptone-salt solutions were made up with sodium chloride concentrations varying from 0-0.88%. Phage dilutions were prepared in each of the four liquids and were kept under cold conditions ($4^{\circ}C$). Phage estimations were done at the commencement of the experiment and after the suspensions had been kept for one day and then for a further two days.

The results showed that whereas with 0.88% salt the counts were low initially, nevertheless with this amount the counts were high at one and three days. Whereas cold conditions may have helped in the phage preservation, nevertheless the high amount of salt in the 0.1% peptone and 0.88% salt solution did not have any adverse effect on the results over the relatively extended test period.

Discussion

Various materials have been used as diluents for bacteria and phages.

The most commonly used diluents for bacteria have been distilled water, Ringer's solution, and peptone water. Cohen (1922) used distilled water as a diluting medium for Bact. coli and Bact. typhosum, as tests had shown that it was satisfactory for his purposes. Winslow & Brooke (1927) suggested that the beneficial effect of peptone and meat extract in diluents was analogous to the operation of a protective colloid. Wilson (1935) discussed the relative values of distilled water, saline, and Ringer's solution as diluents for milk in bacteriological investigations, and concluded that Ringer's solution was preferable. Baird-Parker & Davenport (1965) used 0.1% peptone water for making dilutions of Staphylococcus aureus.

In phage work diluents such as water, Ringer's solution, and peptone-salt solution have been used.

Turner & Nelson (1951) showed that when five phage strains were diluted with water instead of milk the counts were lower with water in three out of five cases, but that addition of milk to the Petri dishes raised these counts. They considered that the raising of the counts was due to the calcium supplied by the milk. Lark & Adams (1953) pointed out that, in dealing with the effect of heat on phages, the phages were much less stable in dilute saline than in broth or in balanced salt solution. Meynell & Meynell (1965) pointed out that phages are rapidly killed by dilution and shaking in protein-free salt solutions without special precautions.

The need for protein in phage dilutions is thus obvious. In the present investigation peptone-salt solution has been found satisfactory for keeping a typical lactic streptococcus phage for several days - it was concluded therefore that it would not affect phage during preparation of serial decimal dilutions. It was also used as a medium for collecting phage by sedimentation and for storage until examined quantitatively.

(iii) SLIT SAMPLER (CASELLA)

Description of Instrument

Some aspiration methods which are used for estimating bacteria in air can also be used for estimating atmospheric phage, e.g. by drawing known volumes of air through liquid media in suitable containers, or over the surface of Petri dishes containing nutrient agar media. The Casella Airborne Bacteria Sampler is an example of equipment for the latter method. In this instrument a measured volume of air can be drawn through the apparatus at a constant rate, viz. 28 l./min. This air passes through a narrow slit and impinges on a rotating Petri dish containing sterile nutrient agar medium. The plate is enclosed in a metal box with glass door and can be adjusted to a known distance from the slit. By means of a motor the turntable on which the plate sits can be made to rotate completely in 0.5, 2, or 5 min. In this way the bacteria from 14, 57, or 142 l. of air, respectively, are collected on the plate. After incubation of the plate at a suitable temperature, the bacterial count per unit volume of air may be calculated. For phage estimations the technique is similar but the agar medium is spread with a culture of the host organism. The phage particles in a specific volume of air are collected on the surface of the medium and, on incubation at the appropriate temperature, plaques are formed.

Comparison of Counts by Sedimentation and Slit Sampler

On several occasions experiments were carried out in which the atmosphere in a cheese factory was examined for phage content by the slit sampler method and the sedimentation method, and the counts obtained by the two methods were compared. Table X shows the results of such experiments carried out at the Belvedere cheese factory.

In these experiments sedimentation plates were opened to the air for 10 min near the slit sampler, at various times throughout the day, and the slit sampler was run for 0.5 min (one revolution) during the 10-min period of its corresponding sedimentation plate.

The Table shows that there was a rough correlation between the counts by the two methods. However, this correlation does not prove the accuracy of the slit sampler for phage estimation; it simply shows

Table X Estimation of phage in atmosphere - comparison between counts by sedimentation method and slit-sampler method (Belvedere).

EXPT A (ML_8)			EXPT B (ML_8)		
	Sedimentation	Slit Sampler		Sedimentation	Slit Sampler
Time plates opened	No. plaques per plate in 10 min	No. plaques per plate in 0.5min	Time plates opened	No. placues per plate in 10 min	No. plaques per plate in 0.5min
10:50 AM	0,0	0,0	11:00 AM	0	0
11:30	4,4	1,0	11:15	1	1
12:00	5,3	1,0	11:40	22	0
12:30 PM	7,0	0,3	12:00	34	18
1:00	9,4	2,2	12:20 PM	95	19
1:30	52,60,49	34,50,39	12:35	22	18
2:00	80,82,44	53,48,70	12:50	88	28
2:35	27,25,21	42,28,20	1:25	83	127
2:45	15,21,21	10,21,19	1:55	167	144
3:00	18,25,29	26,21	2:05	>1375	
3:30	795, >500, >500	54,40,437	2:15	1589	>500
			3:20	380	97
			3:40	79	296
			3:50	126	712
			4:00	410	909

that, as the amount of phage in the atmosphere increases, a larger amount of phage is deposited on the plate in the slit sampler, but no indication is given of how many phage 'droplets' bounce off the plate or are otherwise lost.

It was pointed out by Boudillon, Lidwell & Thomas (1941) that for collecting bacteria under varying conditions the ratio of slit sampler to Petri dish collection varied from 5.5 : 1 to 1,000-5,000 : 1, and they suggested that the use of Petri dishes could therefore give results at least 200 times greater for large droplets than for finer dispersion resulting from washed bacteria. A somewhat parallel inaccuracy arises in the rate of settling of phage droplets when dispersed into the atmosphere by whey splashed from cheese vats or, for example, by dispersal from the whey separator. Large droplets settle more quickly than finer droplets.

Value of Slit Sampler for Phage Estimations

Wolf, Nichols & Ineson (1946) discussed the value of the slit sampler and the sedimentation method for phage estimations. They found that when agar was used in the slit sampler there was sometimes complete lysis of the plate, but that gelatine was suitable as it could be liquefied and the number of phage particles could then be estimated. Percentage recoveries by various methods are selected from their initial experiments and are presented in Table XI.

Table XI † Recovery of phage aerosol by different methods.

(Determinations made as soon as phage spraying ceased)

Method of estimation	Time for which exposed (min)	Volume (l.)	% of theoretical recovery
Sedimentation (agar plate)	5-10		0.015-0.5
" (basin - subsequently rinsed with milk)	30-45		0.32 -3.2
" (basin - subsequently rinsed with ¼-Ringer)	11-30		0
" (milk-Petri)	5-10		0-0.19
Slit sampler (agar plate)		28.3	0.5-1.9
" " (gelatine plate)		85.0	3.4-90
Aspiration (broth)		2.5-4.0	0.03-27.6

† from Wolf *et al.*

In their subsequent investigations on the use of hypochlorite mists for destroying phage, Wolf et al. (1946) considered it necessary and used three methods for phage estimation, viz. sedimentation on agar plates, aspiration through slit sampler onto agar plates, and aspiration through slit sampler onto gelatine plates.

It seems that the slit sampler method has obvious inaccuracies. Phage particles may bounce off the surface of the medium, or may be carried through the apparatus in pockets of air. According to the work of Wolf et al. (1946) the slit sampler using agar gave a very low percentage recovery, but using gelatine was the best of the methods tried.

Morris, Darlow, Peel & Wright (1961) stated that "numbers of coliphage collected with the slit sampler were less than 1% of those collected with an electrostatic sampler and the slit sampler was therefore not used for phage aerosols".

In the present work it was desired to retain the use of sedimentation on agar plates as a rough sorting method, but another method was required which would give reasonably accurate results. This led to the consideration of a filtration method.

(iv) FILTRATION THROUGH CALCIUM ALGINATE WOOL

Description of Apparatus and Results of Tests Carried Out

The apparatus for collecting phage from measured volumes of air comprised several pieces of equipment, viz. a flowmeter with an attached tube for holding calcium alginate wool, a Variac transformer, and a vacuum cleaner unit for drawing air through the apparatus (Fig 1). The apparatus was initially mounted on a bench and used for aerosol experiments, but was subsequently fitted up on a table with screw-on legs, and was so arranged that the complete unit could easily be transported and set up in dairy factories. A detailed description is given of the various pieces of apparatus.

(a) Flowmeter

(b) Flowmeter and lid

(c) Vacuum cleaner unit,
Variac transformer and flowmeter

Fig 1. Apparatus used for filtration of air through calcium alginate wool.

Flowmeter

An orifice plate with a central opening was placed between the flanged ends of two stainless steel pipes 2.54 cm in diameter and was clamped in position. On each side of the orifice plate a small diameter tube was connected to a stainless steel tank or reservoir and the tanks were connected with an inclined-tube manometer fitted with a calibrated scale (Linford (1961), Owen & Pankhurst (1966)). The lower tank contained water coloured with the dye fluorescein.

Two spirit levels were attached to the top of the apparatus and screws for adjusting the level of the whole instrument were provided. This apparatus was fitted into a wooden box with clip-on lid and handle. Glass tubes containing calcium alginate wool pads were attached to one end of one 2.54 cm diameter tube by a plastic tube, and the end of the other main tube was connected to the hose of a vacuum cleaner unit for drawing air through the apparatus. The scale of the manometer was graduated in l./min (maximum 57 l.) and the calibration checked with a large flowmeter (Parkinson Cowan volumetric gas meter, accuracy $\pm$ 1%).

Motor and transformer

A vacuum pump or its equivalent was needed for drawing air through calcium alginate wool. Such a pump needed to be light in weight, easily transportable, and suitable for use near a cheese vat. Laboratory vacuum pumps are somewhat weighty, and the smallest commercial pump available (Massport Junior) weighed 59 lb. The requirements were met by the use of a household vacuum cleaner (minus dust collection bag), which proved to be effective for the job.

The air flow was regulated by means of a Variac auto transformer voltage regulator, 1 KW, load rating 600 VA. A watch was kept for motor over-heating, as the air flow was small compared with the large volume of air which normally passes through a vacuum cleaner during household use and which exerts some cooling effect. Temperature readings were taken by a thermometer placed inside the casing against the Bakelite where air comes off the motor.

Table for housing apparatus

A wooden table, 76 cm high, with a top 48 cm x 69 cm, was fitted with four screw-in legs. This table housed the flowmeter (on a stand), transformer, and vacuum cleaner unit. Such a table could be put together and fitted with the apparatus very quickly, and was suitable for placing at the end of a vat, or elsewhere, in a cheese factory.

Tubes, internal supports for calcium alginate wool, and quantities of calcium alginate wool

The calcium alginate wool for collecting phage was placed on a metal support in a glass tube, and this was attached to the tube of the flowmeter by a plastic sleeve. It was necessary to decide upon a suitable internal diameter for the glass tube, to find the correct type of internal support, and to determine the amount of calcium alginate wool required. Too great an amount of calcium alginate wool in a narrow tube would unduly restrict air flow and render difficult filtration of a considerable volume of air, besides possibly causing overheating of the motor. Too small an amount of calcium alginate wool would allow an unduly large proportion of phage particles to pass right through.

Various diameters of glass tubes were tried and eventually a size (for internal diameter) was decided upon which gave suitable filtration. After preliminary experiments a stainless steel tube fitted with fine cross-wires was adopted as a support for the calcium alginate wool. This metal support was kept in position on projections in the walls of the glass tube. A suitable amount of calcium alginate wool was determined experimentally.

Experiments carried out in determining a suitable size for internal diameter of the tube and the amount of calcium alginate wool required are detailed below.

Several trials were conducted with various combinations of tubes and quantities of calcium alginate wool. Phage was distributed into the air, measured volumes of air were drawn through the calcium alginate wool pads, and the quantities of phage in the different layers were determined. Table XII shows experiments which were carried out in investigating these factors.

Table XII Filtration through calcium alginate wool. The relation between tube size, quantity of alginate wool, air flow, voltage and temperature of motor, and phage counts.

Internal diameter of tube	Layers, pads Alginate wool No. g		Air flow	Transformer Time from start of filtering	Transformer Volts	Heating of motor Time from start of filtering	Heating of motor Temp °C	Phage counts per layer First	Second	Third
cm			l./min	min		min				
1.78	3 layers		57		Low			4.9×10^{6}	2.9×10^{5}	1.9×10^{4}
	2 "	†	57		Low			7.1×10^{4}	3.0×10^{3}	
0.76	2 "		28		210	(5min total)	Hot	2.2×10^{5}	6.0×10^{2}	
1.27	2 "		28		200	(8 " ")	Warm	4.2×10^{6}	3.2×10^{4}	
	3 "	†	<28		200	(4 " ")	Hot	1.8×10^{6}	4.5×10^{4}	5.0×10^{2}
1.27	3 "		57		145-150	6-10	43-45	2.6×10^{6}	9.6×10^{5}	1.7×10^{5}
	2 "	†	57		108	3-10	42-54	3.6×10^{6}	3.4×10^{5}	
1.27	2 x 0.04		57	5	186	5-10	28-54	3.1×10^{6}	2.9×10^{4}	
	3 x 0.04	†	<57	2-4	200-180	(4min total) 2-4	46-66	9.7×10^{5}	3.8×10^{4}	6.0×10^{2}
1.27	* 2 x 0.04		57	0-4	120-130	0-10	22-49	1.3×10^{6}	2.0×10^{4}	
	* 2 x 0.04	†**	57	0-9	140	2-9	37-60	9.6×10^{5}	5.0×10^{2}	
1.27	* 1 x 0.04		57	0-10	75-80	0-10	23-36	1.0×10^{6}		
	* $1\frac{1}{2}$ = 0.04 + 0.02	†	57	0-10	120-126	1-10	29-53	1.5×10^{6}	8.2×10^{4}	
1.78	* $1\frac{1}{2}$ = 0.04 + 0.02		57	0-5	35-38	3-10	23-26	7.8×10^{5}	1.2×10^{4}	
	* 2 x 0.04	†	57		40	5-10	28-29	1.7×10^{6}	2.0×10^{5}	

Temperatures and voltages were not recorded completely for all air filtrations.

(cont'd)

Table XII (cont'd)

Internal diameter of tube	Layers, pads Alginate wool No.	g		Air flow	Transformer: Time from start of filtering	Transformer: Volts	Heating of motor: Time from start of filtering	Heating of motor: Temp °C	Phage counts per layer: First	Second	Third	Fourth
cm				l./min	min		min					
1.78	‡ 1	x 0.04		57	0	33	4	22				
	2	x 0.04	†	57	5	41	7-9	23-24				
	3	x 0.04	†	57	9	52	14	27				
	4	x 0.04	†	57	14-19	68-70	18-24	30-33				
1.78	* 3	x 0.04		57	0-9	48-51	3-10	25-31	1.0×10^{6}	1.3×10^{5}	1.6×10^{5}	
	* 4	x 0.04	†	57	2-10	72-79	2-10	26-39	1.8×10^{6}	1.0×10^{5}	5.1×10^{3}	4.0×10^{2}
1.85	* 4	x 0.04		57	1-10	58-58	1-10	25-31	2.4×10^{6}	3.0×10^{4}	2.2×10^{3}	1.0×10^{3}
1.85	* 3	x 0.04		57	1-10	49-51	2-10	25-29	2.1×10^{6}	1.0×10^{4}	1.2×10^{4}	
1.85	4	x 0.04		57	3-75	51-59-57	3-75	23-37				
	g	No.	g									
1.85	* 0.08	+ 2 x	0.04	57	1-10	60-59	1-10	22-29	7.5×10^{5}	4.2×10^{4}	1.7×10^{3}	
1.85	* 0.08	+ 2 x	0.04	57	2-10	58-62	2-10	24-29	3.5×10^{6}	6.6×10^{4}	9.4×10^{3}	
	* 0.12	+	0.04 †	57	0-10	57-63	2-10	30-35	3.1×10^{6}	5.5×10^{4}		
1.85	* 0.12	+	0.04	57	1-7	37-38	4	23	1.6×10^{6}	1.1×10^{5}		
	0.12	+	0.04 †	57	0-10	32-36	6-10	25-29	3.0×10^{6}	2.4×10^{5}		
1.85	* 0.12	+ 2 x	0.04	57	1-10	40	5-10	23-24	9.8×10^{5}	5.0×10^{4}	8.0×10^{2}	
	* 0.12	+ 2 x	0.04 †	57	3-10	38-39	3-10	23-27	3.2×10^{6}	5.8×10^{3}	8.0×10^{2}	

† Experiment soon after previous experiment
* Pads consolidated before recorded readings
** Pads separated by wires
‡ Pads added at 5, 9, 14 min from zero to give totals of 2, 3, 4, respectively

In this Table results are shown in the order in which the experiments were carried out. In most cases the air was drawn through the calcium alginate wool at the rate of 57 l./min. This rate was decided upon as giving reasonable accuracy of measurement and a fairly large total volume of air, viz. 566 l. in 10 min, and it was a rate which did not cause too much heating of the motor. Voltage readings and motor temperatures were recorded.

Tubes of 0.76 cm, 1.27 cm, 1.78 cm, and 1.85 cm internal diameter were used for the trials. The tubes were drawn out slightly at one end for insertion into the plastic tube connected with the metal flowmeter tube. Projections (three per tube) for holding the metal supports were made by pressing heated spots with a metal instrument.

After a few initial experiments in which layers or pads of calcium alginate wool were not weighed, the necessity for weighing became apparent, and 0.04 g quantities (or multiples) were always used. This wool was teased out and moulded into a flat pad by the fingers and was then placed in the glass tube on the metal support. Several pads could be placed one on top of the other. Later in the work a stainless steel pestle and mortar were used to shape the pads.

Deliberate dispersal of phage into the air was effected by introducing phage-laden whey into a rubber tube by means of a hypodermic syringe while forcing air through the tube fitted with a restricted opening. Details of the method are given later. Phage particles trapped by the calcium alginate wool were estimated after dissolution of the pads in sodium hexametaphosphate solution and preparation of serial decimal suspensions in $\frac{1}{4}$-strength Ringer's solution. Quantities of 0.1ml of resultant phage suspension, 0.2 ml broth culture of the host organism, and 2.5-3.0 ml of soft agar were mixed and poured over the surface of a prepared lactose yeast phosphate agar plate. Plates were incubated overnight at 30°C and plaques were then counted.

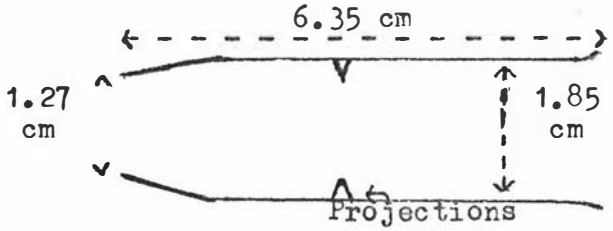
Air drawn through a tube 0.76 cm diameter, containing two unweighed layers of alginate wool, at the approximate rate of 28 l./min, required a voltage of 210, much too high a figure if over-heating were to be avoided. This tube was obviously too narrow. Tubes 1.27 cm diameter required higher voltages and hence temperatures for two or three 0.04 g pads than 1.78 cm or 1.85 cm diameter tubes using three or four pads. With 1.78 cm and 1.85 cm diameter tubes air sampling was

satisfactory at 57 l./min.

Most of the phage was collected on the first layer (0.04-0.12 g) throughout the series of experiments. When using 0.2 g alginate wool (equivalent of five 0.04 g pads) with the 1.85 cm diameter tube, the amount of phage on the (equivalent) fifth pad was less than 1/1,000 of the amount on the first layer (0.12 g).

Table XII clearly shows that a large volume of air can be filtered through five 0.04 g pads and a 1.85 cm diameter tube at a reasonable voltage and with maintenance of a reasonable temperature. The cross-sectional area of a 1.85 cm diameter tube is seven times that of a 0.76 cm diameter tube. Obviously the largest tube tested (1.85 cm diameter) was the most suitable under the circumstances, and this size of tube was used in subsequent work. Glass blowing and other problems made the use of larger sizes impractical and they were not necessary for satisfactory working.

Later in the work the tubes were standardized to the following dimensions:



Main internal diameter:	1.85 cm
Overall length:	6.35 cm
Internal diameter, tapered discharge end:	1.27 cm
Distance in of projections (from inlet end):	3.3-3.8 cm

Inlet end slightly flared outward.

A series of experiments established that the diameter to which the tube was tapered at the discharge end did not significantly affect motor heating as measured by voltage requirements. (Internal diameters of 1.19 cm, 1.17 cm, and 0.96 cm were compared.)

Dissolving of Calcium Alginate

Some difficulty was experienced in dissolving calcium alginate in a mixture of sodium hexametaphosphate solution and Ringer's solution due to the use (by mistake) of 1% instead of 10% sodium hexametaphosphate solution. Even warming the materials up to 45°C did not improve solubility much. The initial examinations led eventually to a survey of the solubility of varying amounts of calcium alginate in mixtures of sodium hexametaphosphate solution and Ringer's solution, where the sodium hexametaphosphate solutions had been previously subjected to different treatments, viz. non-heating, steaming for periods of time up to 1 h, and autoclaving at 15 lb pressure for 20 min. Results of experiments are shown in Table XIII.

One ml of sodium hexametaphosphate solution was mixed with 9 ml of ¼-strength Ringer's solution, and the solubility of alginate wool in the mixture was examined. The quantities of alginate wool are shown in the left-hand columns. In some cases 0.08 g calcium alginate was added to 10 ml solution, and the solubility noted. Then two 0.04 g pads were further added, observations of solubility being made after each 0.04 g addition. In the footnote are explained symbols for solubilities of the pads, as well as symbols for efficiency of pipetting. Narrow-pointed pipettes were easily blocked by undissolved threads or gelatinous masses of calcium alginate.

Expt 1. This experiment was to find the effect of the non-heating and heating of calcium alginate, and the non-heating and heating of sodium hexametaphosphate solution on the solubility of calcium alginate. Quantities of 0.08 g of calcium alginate were soluble in mixtures containing unheated and 1-h steamed sodium hexametaphosphate solutions, but were partly insoluble in mixtures containing autoclaved sodium hexametaphosphate solution.

The treatment of the pads themselves (non-heating, autoclaving, and autoclaving [dissolved in two portions]) did not affect the results.

Expts 2 and 3. Up to 0.12 g calcium alginate could (mostly) be dissolved quite satisfactorily in solutions containing unheated and steamed (1 h) sodium hexametaphosphate solution, but solubilities were unsatisfactory in mixtures containing autoclaved sodium hexametaphosphate solution.

Table XIII The relationship between the heat treatment of sodium hexametaphosphate solution and the quantity of calcium alginate which can be dissolved in mixtures of sodium hexametaphosphate solution and Ringer's solution.

Expt	Calcium alginate g: Portions added	Total	Effect on solubility: 1 ml 10% sodium hexametaphosphate solution + 9 ml ¼-strength Ringer's solution					
			Hexa. Unheated		Hexa. Steamed (1 h expts 1-3)		Hexa. Autoclaved (15 lb, 20 min)	
1		0.08 Unheated	S	P	S	P	X	PX
		0.08 Autoclaved	S	P	S	P	X	PX
		0.08 Autoclaved*	S	P	S	P	X	PX
2	0.08	0.08	S	P	S	P	X	PX
	+0.04	0.12	S	P	S	P	X	PX
	+0.04	0.16	S	P	X	X	X	not tried
3	0.08	0.08	S	P	S	P	X	PX
	+0.04	0.12	S	P	S	P_	X	not tried
	+0.04	0.16	S_	P_	S__	PX	X	not tried
4		0.12	S					
		0.12			S	5 min		
		0.12			S	15 "		
		0.12			S__	30 "		
		0.12			X,PX	60 "		

S = soluble
S_ = almost soluble
S__ = partly soluble
X = partly insoluble gelatinous mass
* = dissolved in two portions

P = satisfactory for pipetting
P_ = slightly difficult to pipette
P__ = difficult to pipette
PX = impossible to pipette
\+ = additions to give total in parallel column
Hexa. = sodium hexametaphosphate

Expt 4. In this experiment sodium hexametaphosphate solution was used unheated and also heated for various periods of time. Calcium alginate dissolved well in a solution containing unheated sodium hexametaphosphate solution and almost as well in solutions containing sodium hexametaphosphate solutions steamed for 5 min and 15 min, respectively. Solubilities were unsatisfactory in solutions containing sodium hexametaphosphate steamed for 30 min and 60 min, respectively.

While the results were not always consistent, it is obvious that too much heat could upset the usefulness of sodium hexametaphosphate solution when used for dissolving calcium alginate in the presence of Ringer's solution. Autoclaved sodium hexametaphosphate solution was unsatisfactory. Ten per cent sodium hexametaphosphate solution steamed for $\frac{1}{4}$ h was satisfactory, and up to 0.12 g of calcium alginate could be satisfactorily dissolved in a mixture of 1 ml of 10% sodium hexametaphosphate solution thus treated and 9 ml of $\frac{1}{4}$-strength Ringer's solution.

Consequently the standard practice was to prepare batches of tubes of 10% sodium hexametaphosphate solution, steam for $\frac{1}{4}$ h, and keep until required for analyses. This heat treatment is more than adequate to 'kill' all lactic streptococcal phage races which have been tested by other workers.

Serial decimal dilutions in $\frac{1}{4}$-strength Ringer's solution were prepared from the solutions containing dissolved calcium alginate.

Quarter-strength Ringer's solution was replaced by peptone-salt solution during the course of the work.

Phage Survival in Undissolved Calcium Alginate and in Solution of Calcium Alginate

When calcium alginate wool is used for collecting phage it is sometimes necessary to keep the alginate wool pads for a time before estimating their phage content. Experiments were therefore carried out to investigate the effects of various keeping conditions on phage survival. A summary of results is shown in Tables XIV and XV.

In the initial experiments pads containing phage were prepared by drawing air from a phage-laden atmosphere (aerosol) through several calcium alginate wool pads held in a glass tube. In later work the experimental pads were prepared by adding small, measured amounts of a diluted whey suspension of phage to alginate pads. After preparation, phage was estimated in representative pads, and the remainder were kept under different conditions and then examined. In this way it could be seen whether the phage count remained constant or not.

Table XIV deals with pads impregnated with phage from aerosols.

In Expt 1, phage-laden air was drawn through two 7-pad tubes. Analyses were made on alternate pads, and the remaining pads were left overnight in the refrigerator, and were then analysed. (There should be a regular fall-off in amount of phage from first to last pads (1-7). Keeping in the refrigerator seemed, if anything, to increase the count. This method of holding alternate pads seemed unsatisfactory, and was discarded.

In Expts 2 and 3, the pads, after aerosol treatment, were divided into approximately equal parts. One-half of each was analysed on the day of treatment. In Expt 2, corresponding halves were kept overnight in the refrigerator (dry). In Expt 3, alternate pads were kept in peptone-salt solution at $4^{o}C$, and at $22^{o}C$, overnight. Keeping by both methods seemed satisfactory.

In Expt 4, pads were divided after aerosol treatment. One lot was analysed the same day and the other lot was kept in peptone-salt solution overnight. There were discrepancies which may have been due to uneven distribution of phage on the pads.

In Expt 5, after aerosol treatment of two tubes of 7 pads each, the pads were divided. Half the pads from both tubes were analysed immediately, and the remainder were kept in bottles with lids on at

Table XIV Factors affecting the survival of phage in alginate wool pads after exposure by drawing phage-laden air through 7 pads per tube.

In the first experiment whole pads were used. In all other experiments half of each pad was assayed fresh and the balance after the treatment indicated.

Expt	Treatment of alginate wool pads	No. of phage particles per pad or half pad ‡						
		1	2	3	4	5	6	7
1	Fresh *	1.8×10^{5}		1.2×10^{4}		7.0×10^{2}		3.0×10^{2}
	Overnight in refrigerator		3.2×10^{5}		1.1×10^{4}		1.1×10^{4}	
	Fresh *	7.4×10^{4}		1.3×10^{3}		1.0×10^{2}		1.0×10^{2}
	Overnight in refrigerator				4.5×10^{3}		2.1×10^{3}	
2	Fresh *	5.4×10^{4}	1.0×10^{4}	3.9×10^{3}	1.1×10^{3}	4.0×10^{2}	4.0×10^{2}	4.0×10^{2}
	Overnight in refrigerator	6.6×10^{4}	1.8×10^{4}	2.8×10^{3}	9.0×10^{2}	2.0×10^{2}	$<1.0\times10^{2}$	2.0×10^{2}
3	Fresh *	9.7×10^{3}	1.9×10^{3}	8.0×10^{2}	4.0×10^{2}	$<1.0\times10^{2}$	1.0×10^{2}	2.0×10^{2}
	Overnight in pep.-s.s. Storage temp.	9.0×10^{3} 4°C	2.1×10^{3} 22°C	2.0×10^{2} 4°C	1.0×10^{2} 22°C	1.0×10^{2} 4°C	1.0×10^{2} 22°C	$<1.0\times10^{2}$ 4°C
4	Fresh *					6.0×10^{4}	4.2×10^{4}	1.8×10^{4}
	Overnight in pep.-s.s. 22°C					5.8×10^{4}	7.1×10^{3}	2.8×10^{3}
5	Fresh *	3.9×10^{7}	1.6×10^{7}	6.6×10^{6}				
	2 days at 22°C in McCartney bottles	3.7×10^{7}	9.0×10^{6}	4.1×10^{6}				
	Fresh *	3.0×10^{7}	9.7×10^{6}	3.1×10^{6}				
	2 days at 22 C in desiccator	2.4×10^{7}	5.1×10^{6}	3.5×10^{6}				
6	Fresh *		7.4×10^{5}	1.1×10^{6}	4.6×10^{6}			
	Fresh *		5.4×10^{6}	1.5×10^{6}	1.5×10^{5}			

* Analysed same day, as soon as practicable after Expt set up.

No. 1 = first pad, No. 7 = last pad.

Pep.-s.s. = peptone-salt solution.

‡ Each figure is the average count for 2,3 or 4 plates.

Table XV Factors affecting the survival of phage in alginate wool pads after adding 0.1 ml volumes of phage dilution in peptone-salt solution and storing as indicated.

Expt	Treatment of alginate wool pads		No. of phage particles per pad*	
			18 h	42 h
1		22°C	1.1×10^4	5.5×10^3
	Dry	22°C desiccator	6.2×10^2	4.7×10^2
		4°C	1.2×10^4	1.1×10^4
	In pep.-s.s.	22°C	1.5×10^4	1.6×10^4
		22°C & 4°C	1.6×10^4	1.9×10^4
	In pep.-s.-citrate s.	22°C	1.5×10^4	1.4×10^4
		22°C & 4°C	1.6×10^4	1.8×10^4
			72 h	144 h
2		22°C	9.2×10^3	8.4×10^3
	Dry	22°C desiccator	6.0×10^2	4.0×10^2
		4°C	2.0×10^4	2.2×10^4
	In pep.-s.s.	22°C	2.0×10^4	2.1×10^4
		22°C & 4°C	2.2×10^4	2.5×10^4
	In pep.-s.-citrate s.	22°C	1.8×10^4	2.1×10^4
		22°C & 4°C	2.0×10^4	2.4×10^4

* Each figure is the average count for 3 or 6 plates. For analysis, volume made to 10 ml where not already done, and 0.1 ml used for plating.

22°C, and in bottles in a desiccator with lids off at 22°C, respectively. Variations may have been due to uneven phage distribution in the pads.

In Expt 6, after aerosol treatment, pads were divided, and all halves were analysed immediately. Results showed that divided pads were unsuitable, apparently due to uneven phage distribution.

To sum up, the results of Expts 1-6 (Table XIV) show that the use of alternate pads, and division of pads, as investigational methods were unsatisfactory. Another more accurate method was required.

The practicability of direct addition of a small, measured amount of phage to each pad was tested. Table XV is concerned with pads impregnated in this way. Phage dilution (0.1 ml) was added to each pad. Pads were kept dry (at 22°C, in desiccator at 22°C, and at 4°C), in peptone-salt solution (22°C, and 4°C), and in peptone-salt-citrate solution (22°C, and 4°C). Analyses were done at 18 and 42 h, and at 72 and 144 h, respectively, from commencement of each experiment.

Both Expts 1 and 2 show that phage dies off in the dry state, more especially at room temperature (22°C) and especially so in a desiccator.

Peptone-salt solution and peptone-salt-citrate solution were suitable for phage preservation, especially in the cold.

The method therefore adopted for any necessary holding of calcium alginate wool pads was to immerse the pads in peptone-salt solution and then maintain the samples at a low temperature where possible.

Effect of (a) Moisture and (b) Compression on Air Flow Through Calcium Alginate Wool Pads

Calcium alginate wool pads used for filtration of phage in dairy factories are liable to exposure to conditions of widely varying relative humidities. The pads as normally prepared and sterilized are relatively dry, and any remaining trace of moisture that may be present is removed by air drawn through the apparatus. Experiments were carried out to find the effects of moisture on pads by measuring the voltage required to draw air through pads at the rate of 57 l./min. At the same time the effect of compressing the pads by pressure (i.e. repeated tapping applied with a glass rod)was examined. Results of

experiments are shown in Table XVI.

Table XVI The effects of moisture and pressure on calcium alginate wool pads as reflected in the voltage required to draw air through them at 57 l./min.

Expt	Pestle and mortar treatment	Condition of pad after autoclaving	State of pad	Volts to reach 57 l./min	Pressed with rod for	Volts to reach 57 l./min	0.5 min at 113 l./min	Volts to reach 57 l./min
1	+	Dry	Fairly springy	96	1 min	116	"	110
2	++	Dry	Compact	112	1 "	112	"	113
3	+	Wet	Compact Then *	184	1 min	160	85 l./min at max. voltage †	156‡
4	+	Wet	Compact Then *	180	1 min	153	85 l./min at max. voltage †	156‡

\+ Pad pestled a little

++ Pad pestled thoroughly

† Maximum voltage gave only 85 l./min

* Motor run to take off some water

‡ Outer surface appears dry

In the experiment outlined in Table XVI alginate wool pads (each 0.04 g) were prepared and put in tubes, 7 pads per tube. Some pads were subjected to a little, some to a greater amount, of pestle compaction. Autoclaved 'dry' pads were autoclaved at 15 lb for 10 min and the autoclaved 'wet' pads were autoclaved in such a manner that the resulting pads were soaking wet. The other details of experiments are recorded in the Table. The final result was that it took 110-113 volts to draw 57 l./min through the 'dry' pads, and 156 volts through the 'wet' pads.

An experiment was carried out in which comparatively large amounts of water were added to calcium alginate wool pads. Results are shown in Table XVII.

Table XVII Effect of comparatively large amounts of water on voltage required for drawing air through calcium alginate wool pads.

(a) Dry		(b) Wet		(c) Wet		(d) Wet	
		0.1 ml water		0.5 ml water		1 ml water	
	Volts		Volts		Volts		Volts
	138		150		189		230
AP	150	AP	162	AP	154	AP	184

Rate of air movement = 57 l./min

AP = after pressing 20 times with rod

In this experiment a circular piece of blotter (approx. 1.8 cm d) was fitted on top of the pads (one set of 7 pads in a glass tube). The experiment was carried out by adding successive amounts of water and finding the voltage required to draw air through the flowmeter at 57 l./min. The blotter itself restricted the free flow of air onto the pads.

From the foregoing experiments it may be concluded that wet pads are entirely unsuitable for filtration purposes because of the necessity for a high voltage. Some of the fibres of the calcium alginate wool may have joined together and conditions for filtration may have been somewhat altered.

It is therefore necessary to use dry pads which need to be pressed together. The air travelling through the pads compacts the pads to some extent and the additional pressure can be applied by tapping the first pad with the flattened end of a glass rod. In this way a relatively compact mass of fibres is available for efficient filtration.

Method Adopted for Preparation and Use of Calcium Alginate Wool Pads

Weigh out 7 pads of calcium alginate wool, 0.04 g each.

Tease out pads until of fairly uniform fibre distribution and pestle in a stainless steel mortar until compact. (A pestle and mortar of correct diameter were available.)

Fit pads on top of each other on metal support in glass tube.

Place weight on top of pads, wrap tube in paper, and autoclave at 15 lb for 20 min.

When required for experiment, remove cover and weight, and fit tube to flowmeter by plastic sleeve.

Start motor, adjust flow to about 57 l./min, and consolidate pads by tapping about 40 times with a sterile glass rod.

Adjust flow to 57 l./min.

After filtration has finished, remove tube, and place each pad in a McCartney bottle containing 9 ml of sterile peptone-salt solution (0.9% NaCl, 0.1% peptone).

Add 1 ml 10% sodium citrate solution (boiled $\frac{1}{4}$ h) to each McCartney bottle and mix thoroughly by shaking. (Reference to sodium citrate and sodium hexametaphosphate solutions will be made later.)

Where considered necessary, prepare serial decimal dilutions in McCartney bottles containing 9 ml peptone-salt solution.

The suspensions are now ready for plating.

(v) SUMMARY OF METHODS ADOPTED FOR PHAGE ESTIMATION IN DAIRY FACTORIES

The calcium alginate wool filtration method was adopted for estimation of phage in the air, and the slit sampler method was discarded.

Two sedimentation methods were used:

(a) for estimating phage falling onto an agar medium containing the host organism (a rough comparative method); and
(b) for estimating phage falling into a peptone-salt solution.

It was realized that sedimentation methods could be very inaccurate, due to the effects of air currents, to the different rates of settling

of various sized whey droplets, and possibly to other factors. The sedimentation methods, however, served certain useful purposes.

B. PLATING OF PHAGE

B. PLATING OF PHAGE

(i) INTRODUCTION

This section of the work deals with the plating of phage suspensions, and embraces work done in connection with plating difficulties encountered when various phosphate, hexametaphosphate or citrate, calcium and alginate ions were present in the medium. The section also deals with the amount of medium in the different agar layers, the condition of the bacterial culture used for the analyses, the use of trypticase soy agar medium, and the effect of calcium borogluconate on plaque production. A summary of methods is included.

The medium used for plating was lactose yeast phosphate agar. This medium had been developed at the New Zealand Dairy Research Institute (Hunter, 1946; modified Robertson, 1960) and had been used for over 20 years for growing a wide range of Strep. lactis and Strep. cremoris cultures.

Plating difficulties were encountered when phage was estimated in the presence of disodium hydrogen phosphate, calcium chloride, and solution of calcium alginate in sodium hexametaphosphate or sodium citrate.

Outline of Problems Overcome in Securing Satisfactory Plaque Count Plates

During the course of the examination of phage aerosols for phage content, difficulties arose when analysing phage suspensions prepared by dissolving the calcium alginate wool pads in sodium hexametaphosphate solutions. Anomalous results were obtained when using different amounts of the suspensions for analysis, viz. the greater the amount of phage suspension used the lower the calculated amount of phage obtained per pad. It appeared that the sodium hexametaphosphate might affect the result because of sequestering calcium ions which are necessary for propagation of the phage under test. Experiments were carried out mixing sodium hexametaphosphate and phage in the absence of calcium alginate, and it was shown that hexametaphosphate did lower plaque counts.

At this stage, investigations were made to find whether the addition of calcium to the medium would prevent the lowering of phage counts by hexametaphosphate. Trials were made of various combinations of calcium chloride, sodium hexametaphosphate, and calcium alginate.

A method was worked out for securing maximum plaque counts when using calcium chloride without any hexametaphosphate present. When, however, a solution of calcium alginate in sodium hexametaphosphate solution was now added with the calcium chloride present, results were greatly upset, very poor quality plates were obtained, frequently with precipitates present, and the counts were appreciably lowered.

The next problem therefore was to adjust conditions so that plates with optical properties appropriate to plaque enumeration and of correct plaque count could be obtained when calcium chloride, calcium alginate and sodium hexametaphosphate were all present. Various experiments were tried, viz. increasing numbers of agar layers from two to three, centrifugation, various combinations of agar with and without disodium phosphate, alteration of quantity of media in some layers, the use of a fourth layer of agar medium, and replacement of centrifugation by filtration. Eventually a method was worked out by which satisfactory plates could be prepared.

This portion of the work will therefore be dealt with more or less chronologically so as to explain the steps by which satisfactory plates were finally obtained. The investigations outlined here are now dealt with in detail under the various headings.

(ii) STEPS TAKEN TO OBTAIN SATISFACTORY PLATES

Anomalous Results when Sodium Hexametaphosphate Present

In the brief survey just given, reference was made to anomalous results obtained when plating different quantities of neat suspension of phage obtained by dissolving calcium alginate wool pads in sodium hexametaphosphate solution. Experiments were carried out by preparing a phage aerosol and, several hours later, drawing a large volume of air through 7 calcium alginate wool pads. These pads were analysed in triplicate for phage content. Pads were numbered from the top,

1 to 7. In the tabulated results, letters a, b, c, refer to order of triplicate analyses. Results of experiments are shown in Table XVIII. Selected figures are given. In nearly every case the greater the amount of neat suspension the lower was the calculated count per pad, i.e. 1 ml neat gave lower pad counts than 0.5 ml, and 0.5 ml neat gave lower counts than 0.1 ml neat. Even though results were complicated by the fact that Ringer's solution lowered phage counts, it appeared that the extra amount of sodium hexametaphosphate in the larger quantities of phage suspension used for analyses may have had some effect in reducing plaque counts.

Experiments were carried out to find if sodium hexametaphosphate did indeed cause a reduction in plaque counts in the absence of calcium alginate, in order to confirm the suggested effect of hexametaphosphate (Table XVIII). To McCartney bottles were added 8 ml Ringer's solution. At 5 min intervals 1 ml phage and 1 ml sodium hexametaphosphate solution were added, and plates were prepared so that platings proceeded from a series of sodium hexametaphosphate concentrations ranging from 0.25-2.0%, including a control without hexametaphosphate. Further plates were prepared at $\frac{1}{2}$ h and 1 h from zero, and plates were also prepared after the mixtures had stood overnight. The experiment was carried out in a water bath held at 30°C. Results are shown in Table XIX.

Table XVIII Relationship between the quantity of phage suspension used for analysis and the calculated plaque count per pad. (Neat phage suspension was prepared by placing 0.04 g calcium alginate pad in 9 ml ¼-strength Ringer's solution and adding 1ml 10% sodium hexametaphosphate solution.)

		Plaque Counts 0.1 ml		Plaque Counts 0.1 ml		Plaque Counts 0.5 ml		Plaque Counts 0.5 ml		Plaque Counts 1 ml		Plaque Counts 1 ml	
Expt	Pad	N	Pad	N/10	Pad	N	Pad	N/10	Pad	N	Pad	N/10	Pad
1	a 4	2	200			22	440	7	1,400	10	100	11	1,100
	b 4	5	500			5	500	1	200	7	70	6	600
	c 4	2	200			9	180	0	<200	11	110	6	600
	a 4	59	5,900			127	2,540	19	3,800	103	1,030	267	26,700
	a 5	67	6,700			48	960			33	330		
	a 6	9	900			33	660			46	460		
2	a 7	6	600			26	520			22	220		
	b 4	34	3,400			78	1,560	30	6,000	38	380	52	5,200
	b 5	19	1,900			35	700			33	330		
	b 6	5	500			6	120			4	40		
	b 7	0	<100			2	40			1	10		
	c 4	27	2,700			13	260	97	19,400	61	610	49	4,900
	c 5	18	1,800			39	780			65	650		
	c 6	12	1,200			17	340			10	100		
	c 7	1	100			10	200			7	70		

N = neat phage suspension (see heading)

Table XIX Influence of the concentration of sodium hexametaphosphate on plaque counts when phage, Ringer's solution and sodium hexametaphosphate solution were used together.

Time of plating	Plaque Counts (Each figure represents sum of counts for three plates) Concentration of sodium hexametaphosphate in mixture				
	2.0%	1.0%	0.5%	0.25%	0
Zero	53	78	80	125	131
0.5 h	50	104	138	142	121
1.0 h	48	72	74	105	112
Overnight	22	11	6	46	28

Changes in plaque count totals could be the result of:

(a) any reaction while Ringer's solution, hexametaphosphate solution and phage suspension stood together in McCartney bottles;

(b) any factors affecting phage multiplication during incubation and growth of culture in agar medium;

(c) any effect of Ringer's solution on phage destruction.

Results showed that at zero and 0.5 h the higher concentrations of hexametaphosphate reduced the plaque count considerably, so that this was an effect related to (b). Survey of Table XIX as a whole shows that in addition to the (b) effect there was a general progressive lowering of results due to standing in Ringer's solution, effect (c).

It therefore appears that phage development in the presence of hexametaphosphate is progressively reduced by progressively larger concentrations of hexametaphosphate, and that this is expressed during proliferation in the agar medium.

Additional Experiments Showing Effects of Sodium Hexametaphosphate

In all experiments shown in Table XX, plaque counts are averages of two plates unless otherwise noted.

Expt 1. The object of this experiment was to compare the effects on plaque counts of 0.1 ml and 1.0 ml quantities of hexametaphosphate and Calgon Ringer Tablet solutions in neat suspensions and in dilutions made from them.

Neat suspensions were prepared as designated under (i), (ii) and (iii) in Table XX. The 0.1 dilutions of (i) were made:

1 ml neat + 9 ml ¼-strength Ringer's solution.
1 ml neat + 8 ml ¼-strength Ringer's solution,
+ 1 ml 10% hexametaphosphate solution.

The 0.1 dilutions of (ii) were made similarly.

The 0.1 dilutions of (iii) were made:

1 ml neat + 9 ml ¼-strength Ringer's solution. (†)
1 ml neat + 9 ml ¼-strength Ringer's solution,
+ 1 Calgon Ringer Tablet. († †)

Analyses were carried out initially and at ½ h from zero.

Results showed that 0.1 ml neat gave counts of the same order as 1.0 ml due to the carry-over of a large amount of hexametaphosphate with 1.0 ml. Neat suspension of 0.1 ml was equivalent to 0.1 ml of 1% hexametaphosphate and was the normal amount used for analysis. Solutions prepared from Calgon Ringer Tablet(s) gave slightly higher results than other solutions except in († †). Preparation of 0.1 dilution in hexametaphosphate (giving a 1% hexametaphosphate solution approx.) gave very low results in (i) and (ii) in most cases. (Calgon is the trade name for sodium hexametaphosphate.)

Expt 2. The object of this experiment was:

(a) to compare neat and diluted phage, to find whether any phage particles were trapped in the neat suspension; and
(b) to compare hexametaphosphate solution with Calgon Ringer Tablet solution.

Neat suspensions were prepared by mixing

in column (i) 0.04 g calcium alginate wool, 8 ml Ringer's solution, 1 ml 10% hexametaphosphate solution, 1 ml phage suspension.

(ii) 0.04 g calcium alginate wool, 9 ml Ringer's solution, 1 Calgon Ringer Tablet, 1 ml phage suspension.

The phage was added to the calcium alginate wool in each case. The 0.1 dilutions were prepared in Ringer's solution.

Results showed that 0.1 ml neat and 1.0 ml of 1/10 dilution gave approximately the same results. It appeared that phage particles were not trapped in clumps in the neat suspension. Admittedly, in this experiment, phage would not be attached as firmly perhaps to the wool as would apply in air filtration. Calgon Ringer Tablet solution gave approximately the same result as hexametaphosphate.

Expts 3 and 4. There was no calcium alginate present in these experiments.

Expt 3. The object of this experiment was to find the effect of using volumes of 1% hexametaphosphate solution for analysis above the usual 0.1 ml. The experimental details were as follows:

Culture and phage (0.1 ml, 1.0 ml) and soft agar were mixed on the bottom layer of the plate with 0.1, 0.5, or 1.0 ml of 1% hexametaphosphate solution plus 1.0 ml of water, respectively, as in column (a). Alternatively, the ingredients were mixed in a test tube before pouring onto the bottom layer of the plate, as in column (b).

Expt 4. The object of this experiment was:

(a) to find the effect on plaque count of various amounts of hexametaphosphate over a wide range; and

(b) to find whether order of mixing the ingredients in the test tube had any significant effect.

The experimental details were as follows:

A range of concentrations of hexametaphosphate was prepared by adding portions ranging from 0.05 ml to 1.0 ml so that these plus water and phage (0.1 ml, 1.0 ml) gave total volumes of 2.0 ml.

These amounts were mixed with culture and soft agar. Remaining details are shown in Table XX.

Results of Expts 3 and 4 showed that progressively increasing the amount of hexametaphosphate, progressively lowered the counts. Even 0.05 ml of 1% hexametaphosphate solution (half the amount used in normal analysis for dissolving calcium alginate) lowered the count below the control.

No definite conclusions could be drawn from varying the order of mixing the constituents of the soft agar layer.

From the foregoing it was concluded that the outstanding cause of anomalous results was due to sodium hexametaphosphate which probably sequestered some of the calcium. The next problem therefore was to investigate the role of calcium in estimating phage by the plaque count method, especially when calcium alginate filter pads were being analysed.

Effect on Plaque Count of Calcium Chloride Added to Phage Suspension Mixture

A series of experiments was carried out to find the effect of added calcium on plaque counts. Initially calcium (as $CaCl_2$) was added to the phage suspension mixture, and results of experiments carried out are shown in Table XXII.

In these experiments $CaCl_2$ solutions and phage suspensions were made in McCartney bottles to approximately 10 ml with $\frac{1}{4}$-strength Ringer's solution and 10% sodium hexametaphosphate solution. A wide range of $CaCl_2$ strengths was used. Comparisons were made with and without calcium alginate. The top three lines of the Table refer to quantities of ingredients used in Expts 1, 2a and 2b. Plaque counts were carried out in the usual manner, and the figures given represent counts on single plates. In some cases pH values were adjusted, but in these cases no allowance was made for slight alterations in volumes. Part of Expt 2a was repeated another day. In Expt 3 the sodium hexametaphosphate in normal use was compared with Calgon Ringer Tablet solution.

The calculated quantities of calcium in soft agar resulting from

Table XX Effects of sodium hexametaphosphate on plaque counts.

Expt		PLAQUE COUNTS (i) (0.04 g Ca alg. (8.0 ml ¼ Ringer (1.0 ml 10% Hexa. (1.0 ml phage		(ii) (8.0 ml ¼ Ringer (1.0 ml 10% Hexa. (1.0 ml phage		(iii) (0.04 g Ca alg. (9.0 ml ¼ Ringer (1 Calgon Ringer Tablet (1.0 ml phage	
		Analysis		Analysis		Analysis	
1		0.1 ml	1.0 ml	0.1 ml	1.0 ml	0.1 ml	1.0 ml
Zero	(Neat	32	30	23	20	52	76
	(0.1 dilution in ¼ Ringer	3	36	6	27	7†	54†
	(0.1 dilution in Hexa.	1	0	2	5	1††	4††
½ h	(Neat	23	84	22	25	45	141 (1)
	(0.1 dilution (above Ringer)	5	25	2	22	6†	54†
	(0.1 dilution (above Hexa.)	3	0	1	4	3††	1††

		(i) 0.04 g Ca alg. (Hexa.) Analysis		(ii) 0.04 g Ca alg. (Calgon Tablet) Analysis	
2		0.1 ml neat	1 ml 0.1 diln.	0.1 ml neat	1 ml 0.1 diln.
	Zero	12	8	12	15
	1 h	11	14	13	15

		Mixed on plate (a) PHAGE FOR ANALYSIS		Mixed in tube (b) PHAGE FOR ANALYSIS	
3		0.1 ml	1.0 ml	0.1 ml	1.0 ml
	(0.1 ml 1% Hexa.	>578 (1)	*	>592	*
	(0.5 ml "	>271	*	169	1,252 (1)
	(1.0 ml "	91	504 (1)	29	191

(cont'd)

Table XX (cont'd)

Expt.	PHAGE FOR ANALYSIS		Culture / Hexa. / Agar in test tube before phage: PHAGE FOR ANALYSIS		Culture / Hexa. / Phage in test tube before agar: PHAGE FOR ANALYSIS	
4	0.1 ml	1.0 ml	0.1 ml	1.0 ml	0.1 ml	1.0 ml
Control	118	889				
0.05 ml. 1% Hexa.			63	593	20	567
0.1 ml "			29	509	17	398
0.5 ml "			14	195 (1)	15	160
1.0 ml "			5	31	2	31

* Plates clear or partly clear.

Hexa. = sodium hexametaphosphate

(1) = one plate only

† = 0.1 dilution of iii, Expt 1 (1 ml neat + 9 ml ¼-strength Ringer's solution)

†† = 0.1 dilution of iii, Expt 1 (1 ml neat + 9 ml ¼-strength Ringer's solution +1 Calgon Ringer Tablet)

specific additions of $CaCl_2$-phage-etc. mixture are shown in Table XXI.

Table XXI The concentration of calcium in soft agar after the addition of $CaCl_2$ solution of specific strength in the mixture containing phage etc.

$CaCl_2$ solution		Concentration of calcium in soft agar in ppm	
		$CaCl_2$-phage-etc. mixture in soft agar	
Preparation	Concentration of Ca in ppm	0.1 ml	1.0 ml
0.02 ml 0.25M $CaCl_2$ in 10 ml liquid	20	0.7	5
1.0 ml 0.25M $CaCl_2$ in 10 ml liquid	1,000	36.0	270
2.0 ml 1.25M $CaCl_2$ in 10 ml liquid	10,000	357.0	2,703

Results (Table XXII) showed that in Expt 1 the count was raised to about double the control by 1 ml $CaCl_2$ in phage suspensions, i.e. 270 ppm calcium in soft agar. A somewhat similar result was obtained in Expt 2a, where 270 ppm calcium in soft agar raised the count to about that of the control. Expt 3 shows that the repressive effect of sodium hexametaphosphate on the plaque counts could be overcome by sufficient calcium addition, and that in some cases greater amounts of calcium could then raise the plaque count beyond the control figures. Calgon Ringer Tablets sometimes gave higher counts than the sodium hexametaphosphate in use.

Overall the results in Table XXII show the need for the addition of calcium. The addition of sufficient calcium not only corrected the low counts caused by sodium hexametaphosphate but could sometimes raise counts above the control.

Table XXII Effect on plaque count of calcium chloride added to phage suspension mixture.

Expt		Control							Calcium alginate 0.04 g per 10 ml phage suspension mixture					
	¼-strength Ringer, ml	9	8	8	8	8	8	7	8	8	8	8	8	7
	10% Hexa., ml		1	1	1	1	1	1	1	1	1	1	1	1
	0.25M $CaCl_2$, ml		0	0.02	0.1	0.2	0.5	1.0	0	0.02	0.1	0.2	0.5	1.0
	Phage 10^{-5}, ml	1	1	1	1			1	1	1	1			1
1	pH		6.4	6.4	6.4			5.7	6.2	6.1	6.1			5.5
	Plaque counts using 0.05 ml of above mixture		9	5	11			6	0	5	5			8
	Plaque counts using 0.1 ml " " "	2	19	16	16			12	5	11	7			7
	Plaque counts using 1.0 ml " " "	48	10	9	18			103	29	40	18			119
	Phage 2 x 10^{-5} ml	1	1			1	1	1	1			1	1	1
2a	pH		7.3			8.0	8.1	8.0						
	Plaque counts using 0.1 ml of mixture	19	10			13	14	27	5			15	20	6
	Plaque counts using 1.0 ml " "	210	13			25	63	234	14			27	77	14
	pH		8.4			8.3	8.1	8.0	6.1					5.4
	pH adjusted to		6.9			7.0	6.8	6.7	7.1			6.8	6.9	6.6
	Plaque counts using 0.1 ml of mixture		13			4	13	5	15			11		0
	Plaque counts using 1.0 ml " "		23			19	51	176						
	Phage 2 x 10^{-5} (approx) ml	1							1				1	1
2b	pH								6.1				5.7	
	pH adjusted to								6.5				6.8	6.5
	Plaque counts using 0.1 ml of mixture	49							33				0	48
	Plaque counts using 1.0 ml " "	512							59				0	360

(cont'd)

Table XXII (cont'd)

Expt		Control	Calcium alginate 0.04 g per 10 ml phage suspension mixture									
3	Besides quantities of $CaCl_2$ solution and phage suspension given below, 1 ml of 10% Hexa. solution and sufficient ¼-strength Ringer's solution to give total 10 ml in McCartney bottles and 1 tablet and sufficient water to give total 10 ml in McCartney bottles, were added, respectively.											
	0.25M $CaCl_2$, ml		0	0.5	1.0	1*	2*	0	0.5	1	1*	2*
	Phage 2 x 10^{-5} ml	1**	1	1	1	1	1	1	1	1	1	1
	Plaque counts using Hexa.	0.1 ml mixture 42	21	32	16	26	21	32	37	49†	43†	146†
		1.0 ml " 426	4	56	120	331	17	67	159	450†	669†	350†
	Plaque counts using Tablet	0.1 ml mixture	12	31	24	103	123	19	29	47	87†	138†
		1.0 ml "	19	99	68	1186	1200	52	184	>466	1126†	938†

* = $CaCl_2$ 1.25 M

** = Control 1 ml phage to 9 ml ¼-strength Ringer

Tablet = Calgon Ringer Tablet

Phage = dilution prepared in ¼-strength Ringer's solution from whey suspension

† = calcium alginate insoluble or partly insoluble

Expt 2a - Hexa. or sodium citrate for portion without calcium alginate

In above experiments "mixture" does not refer to control column.

Effect on Plaque Counts of Various Amounts of Calcium in Soft Agar

It was decided to investigate the addition of calcium to the soft agar layer, and a series of experiments was carried out (Table XXIII). Phage suspensions in broth were prepared, as shown in the left-hand column of Table XXIII, so that phage, phage plus sodium hexametaphosphate, and phage plus sodium hexametaphosphate and calcium alginate, could be compared. A specific phage dilution was prepared for each experiment, i.e. Expts 1,2 and 3. In Expt 2, 3.3% sodium hexametaphosphate solution was added to the mixture instead of 10%. Soft agar analyses were carried out, using duplicate plates, and results are shown in the Table. The calcium content of the soft agars ranged from 10 to 10,000 ppm.

In Expt 1 the phage content of the phage suspension mixture was obviously too high. The presence of sodium hexametaphosphate (1.0 ml) (b) restricted counts until the calcium content of the soft agar medium reached about 500 ppm.

In Expt 2 (0.1 ml) (d) increasing the calcium progressively raised the counts for phage from 12 to 86 (1,000 ppm calcium), showing that calcium was needed in the medium. At this calcium level, counts for 1.0 ml were approximately 10 times counts for 0.1 ml. Sodium hexametaphosphate (e) had a restrictive effect on the counts. With sodium hexametaphosphate and calcium alginate (f) both present, there was some precipitate in the plates with 1,000 ppm calcium.

In Expt 3 (g) increasing the calcium progressively raised the count from 37 to 148 (1,000 ppm calcium) for phage alone, but 5,000 and 10,000 ppm calcium caused cloudy plates. Sodium hexametaphosphate (h) somewhat lowered the counts and sodium hexametaphosphate and calcium alginate (i) resulted in plates difficult to read at 500 and 1,000 ppm calcium, and caused cloudy plates at 5,000 and 10,000 ppm calcium.

In summary, the results in Table XXIII showed that calcium was needed in the medium, and that the maximum practical level for calcium addition for phage estimation when sodium hexametaphosphate and calcium alginate were not present lay between 1,000 and 5,000 ppm. The method was not satisfactory for securing maximum counts when sodium hexametaphosphate and calcium alginate were both present. At high calcium

Table XXIII Effect on plaque count of various amounts of calcium in soft agar.

Phage suspension	Calcium in soft agar ppm	Plaque Counts (Av. 2 plates) Expt 1 Analysis 0.1 ml	1.0 ml	Expt 2 Analysis 0.1 ml	1.0 ml	Expt 3 Analysis 0.1 ml
		(a)		(d)		(g)
	0	370	Cl	12	118	37
	10	403	Cl (1)			
9 ml broth	40		∞(1)			51
1 ml phage	50	418	Cl			
	100	444	Cl	25	224	72
	200					91
	500	573	Cl	62	592	131
	1,000	>485	Cl	86	706	148
	5,000					Cloudy
	10,000					"
		(b)		(e)		(h)
	0	98	18	7	29	16
8 ml broth	10	115	29			
1 ml phage	40					28
1 ml 10% Hexa.	50	154	35			
	100	218	55	12	66	36
In Expt 2 only	200					43
3.3% Hexa.	500	332	approx. 606(1)	35	276	82
	1,000	377	Cl	57	457	122
	5,000					Cloudy
	10,000					"
		(c)		(f)		(i)
	0	198	106	13	127	15
8 ml broth	10	190	113			
1 ml phage	40					29
1 ml 10% Hexa.	50	294	146			
0.04 g Ca. alg.	100	292	244	19	209	41
	200					68
In Expt 2 only	500	approx. 256(1)	Cl	57	509	96 ↧
3.3% Hexa.	1,000	approx. 184(1)	Cl	43 ↧	442 ↓	78 ↧
	5,000					Cloudy
	10,000					"

Cl = Plate clear or almost
(1) = one plate only
Hexa. = sodium hexametaphosphate
∞ = assumed > 500

↓ Precipitate
↧ Precipitate difficult to read.

levels complex reactions between reactants gave plates difficult to read.

Trial of Sodium Citrate etc. for Dissolving Calcium Alginate

Sodium citrate was suggested as an alternative to sodium hexametaphosphate for dissolving calcium alginate, and its use was investigated. Experiments were carried out to find the effect on the plaque count of citrate (of different concentrations), with and without calcium alginate, when using a range of calcium concentrations in the soft agar, including agar without calcium addition. Results of experiments are shown in Table XXIV. Phage for each particular day's experiment (i.e. Expts 1, 2 and 3) was used from a specific dilution for that day.

Expt 1. Quantities (0.25-2 ml) of 10% sodium citrate, 1 ml of phage dilution, and sufficient $\frac{1}{4}$-strength Ringer's solution to make a total of 10 ml, were prepared in eight McCartney bottles, and calcium alginate (0.04 g) was added to each of four bottles. A control of phage and Ringer's solution was also prepared. Phage counts on these mixtures (single plates) by the soft agar method showed that citrate lowered the counts below the control and that the greater the amount of citrate present, the more the count was lowered. However, when calcium alginate was present, the counts were higher.

Expt 2. Mixtures of 1 ml quantities of 2.5% and 10% sodium citrate solutions, respectively, 1 ml of phage dilution, and broth to make totals to 10 ml, were prepared in four McCartney bottles. Calcium alginate (0.04 g) was added to two lots. A control of phage and broth was also prepared. Plaque counts (duplicate plates) were done by the soft agar method, using a range of calcium concentrations in the soft agar. Results showed that calcium up to 1,000 ppm increased the plaque count, and that citrate, and alginate plus citrate, did not greatly alter the counts.

Expt 3. Mixtures of 1 ml of 2.5% and 10% sodium citrate solutions, respectively, 1 ml of phage dilution, and broth to make totals to 10 ml, were prepared in two McCartney bottles.

Table XXIV Investigation of the effect on plaque count of the use of sodium citrate for dissolving calcium alginate under various conditions.

	Control*							
Expt	Soft agar Ca ppm	Plaque count	% citrate in phage mixture	Soft agar Ca ppm	Plaque count	% citrate in phage + Ca alg. mixture**	Soft agar Ca ppm	Plaque count
1	-	94	2	-	18	2	-	59
			1	-	46	1	-	86
			0.5	-	65	0.6	-	102
			0.25	-	79	0.25	-	84
2	-	5	1	-	11	1	-	13
	200	73	1	200	48	1	200	62
	500	84	1	500	77	1	500	112 †
	1000	120	1	1000	127	1	1000	84 †
			0.25	-	5	0.25	-	16
			0.25	200	51	0.25	200	65
			0.25	500	74	0.25	500	101 †
			0.25	1000	97	0.25	1000	112 †
			% citrate in phage + Ca alg. mixture**					
3	-	24	1	-	21	0.25	-	38
	200	88	1	200	104	0.25	200	78
	500	116	1	500	130	0.25	500	109
	1000	120 (†)	1	1000	111 †	0.25	1000	159
	2000	190 (†)	1	2000	47 ‡	0.25	2000	177 †
	3000	Itr	1	3000	Itr	0.25	3000	Itr
	4000	"	1	4000	"	0.25	4000	"
	5000	"	1	5000	"	0.25	5000	"

Plaque counts were carried out using 0.1 ml phage suspension in the soft agar.

* No citrate

** 0.04 g Ca alg. added to 10 ml mixture

(†) Some precipitate on one plate

† Some precipitate on plates

‡ Very difficult to count

Itr Impossible to read.

Calcium alginate (0.04 g) was added to each of the mixtures. A control of phage and broth was also prepared. Phage counts (duplicate plates) were done by the soft agar method using a wide range of calcium concentrations in the soft agar.

In the trials without citrate, concentrations of calcium in the soft agar up to a maximum of 2,000 ppm raised the plaque counts, and these figures were somewhat similar to the results where citrate (0.25%) and alginate were present. In the trials where citrate (1%) and alginate were present, the high amount of citrate and the calcium apparently had an effect in causing a low plaque count or obscuring some plaques, at a concentration of 2,000 ppm calcium. Concentrations of 3,000 ppm of calcium and above yielded plates impossible to count both in the absence of citrate and in the presence of citrate plus alginate.

Trial of various compounds for dissolving calcium alginate

Recommended solvents for calcium alginate are sodium citrate, sodium glycerophate, and sodium hexametaphosphate, and almost any other sodium salt provided the calcium salt of the acid radicle is soluble.

An experiment was carried out attempting to dissolve 0.04 g quantities of calcium alginate in broths containing phage and

1% sodium acetate,

1% sodium propionate,

1% sodium salicylate,

respectively, but the calcium alginate was insoluble in each case.

Effect on Plaque Count of Presence or Absence of Phosphate in Bottom Agar Layer, at Various Calcium Levels in Top Agar Layer

It was necessary to further investigate the addition of calcium to the medium to find the amount required for maximum plaque counts. Also the role of phosphate in the medium, and the reason for precipitate formation, required study. Initially work was concentrated on experiments which excluded the use of citrate and calcium alginate.

Expts 1 and 2 were carried out by the two-layer method. Phosphate was added to or omitted from the medium as shown in Table XXV. A range of calcium concentrations in the top agar layer was prepared by adding $CaCl_2$ to the medium, centrifuging, and then using the clear medium. For the top layer 2.5 ml of agar medium, 0.1 ml phage, and 0.2 ml culture were poured onto the agar medium already in the plate. In Expt 2 not only was phosphate omitted from the bottom layer, but previously added $CaCl_2$ removed with the precipitate any phosphate or other substance in the medium which formed an insoluble precipitate with calcium. All phage for one experiment was from the same dilute suspension in peptone-salt solution. The appearance of plaques and media was recorded.

In Expt 1 it was found that maximum plaque counts were secured at 2,000-3,000 ppm calcium. In the second portion of this experiment, where phosphate was not added to the bottom layer, the added calcium reacted with a constituent of the medium (phosphate?) to form a precipitate.

In Expt 2 it was found that there was no advantage in treating the agar used for the bottom layer with $CaCl_2$. This treatment resulted in very faint plaques and there was apparently too much calcium for optimal plaque development in some cases.

The above results confirmed the need for some calcium addition but established that further experiments were necessary to find best combinations of reactants.

Table XXV Effect on plaque count of presence or absence of phosphate in bottom agar layer, at various calcium levels in top agar layer.

		EXPT 1				EXPT 2	
		Soft agar -P (Centrif.) Ca ppm	Plaque count (Av. of 3)	Soft agar -P (Centrif.) Ca ppm	Plaque count (Av. of 3)	Soft agar -P (Centrif.) Ca ppm	Plaque count (Av. of 3)
Top layer	1.	-	6	-	7	-	92
	2.	500	69	500	66	500	67
	3.	1000	86	1000	75	1000	33
	4.	2000	116	2000	129	2000	28
	5.	3000	94	3000	119	3000	52
	6.	4000	34	4000	98	4000	5
	7.	5000	13	5000	95	5000	10
Bottom layer		Hard agar +P		Hard agar -P		Hard agar -P + $CaCl_2$, centrif. used clear medium	

APPEARANCE OF PLAQUES AND PLATES IN ABOVE TABLE

	Plaques	Medium	Plaques	Medium	Plaques	Medium
1.	Clear	Satis.	Clear	Satis.	V.faint	Satis.
2.	"	"	"	White ppt.	"	"
3.	"	"	"	"	"	"
4.	"	"	"	"	"	"
5.	Faint	"	"	"	"	"
6.	"	Sl.different	Faint	"	"	"
7.	V."	"	(Clear(1) (Faint(2)	"	"	"

-P = - phosphate

+P = + phosphate

Further Experiments with Calcium Addition, and Experiments Directed to Securing Plates Free from Precipitate when Calcium Chloride, Calcium Alginate, and Sodium Citrate were Used

The experiments detailed in the last few sections have shown the need for addition of calcium to the medium when used for phage estimations. These experiments have also shown that under certain conditions unsatisfactory plates, e.g. plates showing precipitates, could be obtained. The following section describes experiments carried out to check the quantity of calcium required for maximum plaque counts (without alginate and citrate addition). It also describes experiments carried out, and difficulties encountered, in endeavouring to secure good-quality plates when calcium alginate and sodium citrate were present. Results of the experiments are shown in Tables XXVI-XXX.

The experiments were carried out using the soft agar count thod (0.1 ml phage suspension mixed with 0.2 ml culture and 2.5-3.0 ml soft agar). Initially the mixture was poured onto a layer of medium already solidified in the plate (about 10 ml unless otherwise specified). Later, the phage agar mixture was added to plates bearing two pre-poured layers. Eventually experiments were done in which the phage layer was itself covered with another layer, making a total of four layers in the plate.

When calcium additions were made, measured volumes of $CaCl_2$ solution and melted agar medium were mixed, centrifuged, and the clear liquid was pipetted off for use. Later, filtration was used instead of centrifugation (Table XXX, Expt 4). Calcium chloride solution and hot agar medium were mixed, and the mixture was filtered through blotting-paper mash in a Buchner funnel. The clear medium was kept in the water bath at approximately $46^{\circ}C$ until required. Phage was prepared by making serial decimal dilutions in peptone-salt solution from a stock whey suspension of phage. Generally 0.7 ml of a 10^{-4} dilution of phage suspension in whey and 9.3 ml peptone-salt solution were mixed to give a $<10^{-5}$ dilution, and from this a $<10^{-6}$ dilution of phage was prepared. When using calcium alginate, 1 ml of the $<10^{-5}$ dilution of phage suspension was placed in a McCartney bottle together with 1 ml of 10% citrate

or hexametaphosphate solution, and 8 ml of peptone-salt solution. Adjustments of the proportions of citrate (or hexametaphosphate) solution and peptone-salt solution were made in certain experiments, while still retaining the total of 10 ml. One or more 0.04 *g* pads of calcium alginate wool were added to the mixture, and the bottle contents were well shaken. Phage for each particular experiment was from a specific dilution for use that day.

Counts were done in duplicate or triplicate. Where variations occurred in the numbers of plates counted, the number actually counted is given in parentheses.

Table XXVI Effect of a range of calcium levels on plaque counts (without phosphate addition to agars) and trials of the effect on counts of further phage dilution.

	L1 HA -P	L2 (Phage layer) SA -P	EXPT 1 Counts using diluted phage: Phage suspension	Diluent	EXPT 1 Plaque counts Av. of 3	EXPT 2 Plaque counts Av. of 3
		Ca ppm				
1	"	-			5	4
2(a)	"	2000			115	127
(b)	"	"	3	1	89	91
(c)	"	"	1	1	59	49
(d)	"	"	1	3	28	37
(e)	"	"	1	7	13	17
3	"	2500			154	-
4(a)	"	3000			136	143
(b)	"	"	3	1	108	116
(c)	"	"	1	1	67	69
(d)	"	"	1	3	36	40
(e)	"	"	1	7	16	18
5	"	3500			153	146
6(a)	"	4000			155	143(2)
(b)	"	"	3	1	104	101(2)
(c)	"	"	1	1	76	73(2)
(d)	"	"	1	3	32	36
(e)	"	"	1	7	15	13
7	"	4500			148	114(2)

These experiments are repetitions (with modifications) of Expt 1 (2nd part) in Table XXV. The main object of these experiments was to find the calcium levels giving optimum plaque counts with phage alone, i.e. in the absence of citrate and alginate. A range of calcium additions up to 4500 ppm was made. Results showed that maximum plaque counts were given at 2500-4000 ppm added calcium. Both experiments gave similar results.

Dilutions of the phage-suspension mixture with peptone-salt solution

in the proportions shown in the Table were examined also. The count was linearly related to the dilution.

In Expt 1 plates showed clear plaques, some showed slight opaqueness, and some slight precipitate. In Expt 2 three plates were very poor, due to precipitate formation. Three had faint plaques at 4500 ppm calcium, and one of these was not counted. Characteristically, plates came within the range fair - good in the two experiments.

A list of abbreviations used in the above and following Tables is appended.

Av.	= average	NAC	= no attempt to count
Au.	= autoclaved	N.Cf.	= not centrifuged
Cf.	= centrifuged	+P	= phosphate added
Cit.	= sodium citrate	-P	= phosphate not added
Cult.	= culture	Pep.-s.s.	= peptone-salt solution
HA	= hard agar medium	ppm	= parts per million
Hexa.	= sodium hexametaphosphate	Ppt.	= precipitate
Itr	= impossible to read	SA	= soft agar medium
L1	= bottom agar layer in plate	Satis.	= satisfactory
L2	= 2nd " " " "	Sl.	= slightly
L3	= 3rd " " " "	St.	= steamed
L4	= 4th " " " "		

Table XXVII Effect of calcium alginate and sodium citrate/hexametaphosphate on plaque count when calcium chloride has been added to agar.

EXPT 1

	L1 HA -P	L2 = 2.5ml SA-P Ca ppm	Composition of suspension/mixture from which 0.1 ml added to SA		Plaque counts Av. of 3
1	"	-	Phage $<10^{-6}$		7
2	"	2000			107
3	"	4000			145
4	"	-	Diluent	8.00ml	6
			Ca alg.	0.04g	
5	"	2000	Phage $<10^{-5}$	1 ml	2
			10% Hexa.	1 ml	
6	"	4000			1
7	"	-	Diluent	8.67ml	8
			Ca alg.	0.04g	
8	"	2000	Phage $<10^{-5}$	1 ml	8
9	"	4000	10% Hexa.	0.33ml	15
10	"	-	Diluent	8.00ml	6
			Ca alg.	0.04g	
			Phage $<10^{-5}$	1 ml	
11	"	2000	10% Cit.	1 ml	31
12	"	4000			12
13	"	2000	Diluent to make 10 ml		41
			Ca alg.	0.04g	
14	"	2000	Phage $<10^{-5}$	1 ml	-
			10% Hexa.	1 ml	
15	"	2000	$CaCl_2$ (see note below)		5

L2 In each case 2.5ml agar were used - where $CaCl_2$ had been added, the clear centrifuged medium was used.

EXPT 2

	L1 HA P	L2 = 2.5ml SA P	Ca ppm	Composition of suspension/mixture from which 0.1 ml added to SA		Plaque counts Av. of 2	Appearance of plates
	-	-		Phage $<10^{-6}$		7	Satis.
	-	-	2000	"		125	"
	-	-	4000	"		158(1)	" (1)
	-	-	8000	"		168	Sl. faint
	-	-	-	Pep.-s.s.	8.67ml	12	Satis.
				Ca alg.	0.04g		
	-	-	2000	Phage $<10^{-5}$	1 ml*	16	Ppt.
				10% Hexa.	0.33ml		
	-	-	4000	"		28	"
	-	-	6000	"		42	"
	-	-	8000	"		28(1)	Ppt.(1),other Itr
	-	-	10000	"		30(1)	" " " "
	-	-	-	Pep.-s.s.	8.67ml	18	Satis.
				Ca alg.	0.04g		
	-	-	2000	Phage $<10^{-5}$	1 ml†	65	Ppt.
				10% Cit.	0.33ml		
	-	-	4000			80	"
	-	-	6000	"		83	"
	-	-	8000	"		70	"
16	-	-	10000	"		101(1)	Ppt.(1),other Itr
17	+	-	-	"	*	13	Satis.
18	+	-	6000	"	*	Itr	Ppt. & creamy
19	+	-	10000	"	*	0	"

(cont'd)

Table XXVII (cont'd)

EXPT 2

	L1 HA	L1 P	L2 = 2.5ml SA	L2 = 2.5ml P	Ca ppm			Plaque counts Av. of 2	Appearance of plates
20	+	+			-		*	10	Satis.
21	+	+			-		†	14	"
22	+	+			4000		*	Itr	Ppt. & creamy
23	+	+			"		†	9	"
24	+	+			2000	Cf.	*	Itr(1)	Ppt. & creamy (1)
25	+	+			"	N.Cf.	*	115(1)	Uniform ppt. no problem (1)
26	+	+			"	Cf.	†	NAC(1)	Creamy (1)
27	+	+			"	N.Cf.	†	124(1)	V. faint plaques Ppt. (1)

Note: Mixture for 0.1 ml addition to soft agar in Nos. 13, 14 and 15 contained 0.25, 1.0 and 2.0 ml, respectively, of $CaCl_2$ solution in the 10 ml.

It was shown in Table XXVI that maximum plaque counts were obtained with 2500-4000 ppm calcium in the top, soft agar layer. It was now necessary to show whether the presence of calcium alginate and sodium citrate/hexametaphosphate would yield satisfactory counts at these high calcium levels. Table XXVII shows the results of experiments in which various phage suspension mixtures were tested. These phage suspensions contained (apart from controls) one pad (0.04g) of alginate wool. The citrate/hexametaphosphate varied from 0.33-1.0%. In Expt 1, phosphate was absent from the bottom layer of agar medium, and in Expt 2 phosphate was present in some of the bottom and in some of the top layers.

The control experiments showed large increases in plaque count with calcium addition. Striking results were obtained where calcium alginate was present in that most of the counts were extremely low and that many of the plates showed precipitate or (a few) a creamy appearance. A clue to a possible method of obtaining satisfactory plate counts was given by the plates showing counts of 115 and 124 (lines 25 and 27). The precipitate present as a consequence of not centrifuging or due to chemical reaction slightly obscured the plaques but, for no apparent reason, cleared later in the day. Did culture produce more acid and affect the precipitate?

Table XXVIII Investigation of factors affecting plaque counts - trials with two- and three-layer plates using various combinations of calcium and phosphate (citrate and alginate present).

EXPT 1

	L1 HA P	L1 P	L2 Ca ppm	Composition of mixture from which 0.1ml added to culture layer	L3 P	L3 Ca ppm	Plaque counts Av. of 2
1	-	-	-	Pep.-s.s. 10^{-5} 8.67ml; Phage <10^{-5} 1 ml; Na cit.10% 0.33ml; Ca alg. 0.04g			28
2	-	-	2000	"			50
3	-	-	4000	"			78
4	-	-	8000	"			0(1)
5	+	+	-	"	+	-	19
6	+	+	2000	"	+	-	>21
7	+	+	4000	"	+	-	Itr
8	+	+	8000	"	+	-	"
9	+	+	-	"			7
10	+	+	2000	"			51
11	+	+	4000	"			48
12	+	+	8000	"			17
13	-	-	10000	"	-	-	0(1)
14	+	-	"	"	-	-	0(1)

EXPT 2

L1 HA P	L2 SA+P Ca ppm		L3 SA +P	Plaque counts Av. of 2
+	-		"	5
+	2000		"	84
+	4000		"	104(1)
+	-	+ SA+P		1
+	2000	+ "		109
+	4000	+ "		83

used throughout expt: Pep.-s.s. 10^{-5} 8.67ml; Phage <10^{-5} 1 ml; 10% Cit. 0.33ml; Ca alg. 0.04g

Nos. 1-3 L2 Cf. 7-10ml to plate L3 2.5ml

Nos. 4-6 L2 Cf. 6-10ml mixed with 2.5ml of SA+P (+ culture), giving total of two layers.

Expt 1 Nos. 1-4 L2 2.5ml (Cf. where $CaCl_2$ added) to each plate.
5-8 L2 10ml (N.Cf.) to plate (+P). L3 2.5ml to plate.
9-12 L2 2.5ml (SA+P) + 10ml SA (of Ca content stated, N.Cf.) mixed in test tube and added to plate.
13,14 L2 10ml (N.Cf.) added to bottom layer. L3 culture layer.

(cont'd)

Comment on Table XXVIII

In Table XXVIII results are shown for two- and three-layer experiments. Experiments reported so far in this section have indicated that added phosphate may be needed, but that certain conditions for employment are necessary. In Expt 2, No. 3, one plate showed a count of 104, and the duplicate plate was impossible to count, showing a few faint plaques. Several plates in these experiments showed precipitate or were impossible to count. Possibly non-addition of phosphate to bottom layer and addition of phosphate to top layer might improve results.

Table XXIX Investigation of factors affecting plaque counts - trials with two- and three-layer plates using various combinations of calcium and phosphate and various quantities of agar medium (citrate and alginate present).

EXPT 1

	L1 HA	L2 HA+P Ca ppm	L3 SA	Plaque counts Av.of
1	+P	2000	+P	133(6)
2	"	"	"	130(2)
3	"	"	"	81(6)
4	"	"	"	73(6)
5	"	"+SA(P)		76(3)
6	"	"+ "		95(3)
7	"	"+ "		37(3)
8	"	"+ "		71(3)

Pep.-s.s. 8.67ml
Phage $<10^{-5}$ 1 ml
10% Cit. 1 ml
Ca alg. 0.04g

Cit. 1ml instead of 0.33 .·. counts sl. low.

L2 1. cf. 5-7ml
2. N.cf. 10ml
3. cf. 2.5ml
4. N.cf. 2.5ml
5. cf. 6ml
6. cf. 2.5ml
7. N.cf. 10ml
8. N.cf. 2.5ml

L2 above 1-4 describes treatment & amount to plate. 5-8 describes treatment & amount mixed with the culture layer (nominal L3).

EXPT 2

	L1 HA	L2 SA+P Ca ppm	L3 SA	Plaque counts Av.of2
	+P	-	+P	6
	"	2000	"	134
	"	4000	"	156
	"	-	"	10(1)
	"	2000	"	139
	"	4000	"	Itr
	"	-	"	6
	"	2000	"	196
9	"	4000	"	Itr
10	"	-	"	10
11	"	2000	"	143
12	"	4000	"	169(1)
13	"	-	"	10
14	"	2000	"	140
15	"	4000	"	208(1)

For	0.04g Ca alg.	Cit. ml
1-3	-	1
4-6	1	0.33
7-9	1	1
10-12	2	1
13-15	4	1

All made up to 10ml incl. 1ml phage $<10^{-5}$ to give $<10^{-6}$.
L2 10ml if no Ca(Cf.) Where Ca, Cf., & about 7ml used.

EXPT 3

	L1 HA	L2 SA+P Ca ppm	ml	L3 SA	Plaque counts Av.of3
	+P	0		+P	22
	"	2000	2.5	"	118
	"	2500	"	"	147
	"	3000	"	"	>142
	"	3500	"	"	149
	"	4000	"	"	124
	"	0		"	43
	"	2000	5	"	121
	"	2500	"	"	126(2)
	"	3000	"	"	110(1)
	"	3500	"	"	Itr
	"	4000	"	"	143(1)
	"	0		"	29(2)
	"	2000	8	"	166(2)
	"	2500	"	"	Itr
16	"	3000	"	"	177(1)
17	"	3500	"	"	Itr
18	"	4000	"	"	>189(1)
	HA	SA-P			
19	-P	0	2.5		40(2)
20	"	2000	"		160(1)
21	"	2500	"		166
22	"	3000	"		198
23	"	4000	"		189

L2 Cf.
19-23 2 layers only
1-18 Pep.-s.s. 8ml; Phage $<10^{-5}$ 1ml; 10% Cit. 1ml; Ca alg. 2 x 0.04g
19-23 Phage $<10^{-6}$

EXPT 4

	L1 HA+P ml	L2 SA+P Ca ppm	L3 SA	Plaque counts Av. of 2 1-16	17-34
	0	0	+P	5	7
	0	2000	"	124	87(1)
	0	3000	"	131	86(1)
	0	4000	"	118	Itr
	4	0	"	10	7
	4	2000	"	105	143
	4	3000	"	111	117(1)
	4	4000	"	100	Itr
	8	0	"	11	11
	8	2000	"	101	129(1)
	8	3000	"	103	Itr
	8	4000	"	125	"
	12	0	"	12	10
	12	2000	"	98	133
	12	3000	"	101	Itr
	12	4000	"	113	"
	HA-P*	SA-P			
		2000			119(1)
	" **	3000			171

Nos. 1-16 L2 Cf. 2.5ml
" 17-32 L2 " 6ml
" 1-32 Pep.-s.s. 8ml; Phage $<10^{-5}$ 1ml; 10% Cit. 1ml; Ca alg. 2 x 0.04g
" 33,34 Phage $<10^{-6}$, 2 layers only, L2 Cf. 2.5ml
" 33 *
" 34 **

(cont'd)

Comment on Table XXIX Expts 1-4

In Table XXIX results are shown from several experiments, most being three-layer experiments.

In Expt 1, two- and three-layer plates were compared to find whether $CaCl_2$ should be mixed with the culture layer or should be present in an adjacent layer. The best results were obtained by calcium diffusion from the second layer of three-layer plates, in the cases where 5-10 ml agar medium were used in this second layer. Centrifuging gave higher results than non-centrifuging. Most plates in this experiment were easily read.

In Expt 2, different numbers of alginate wool pads were compared, using different calcium levels. Results showed that from 1-4 alginate pads could be used satisfactorily provided calcium was around the 2000 ppm level, but not at 4000 ppm. In this experiment several of the plates at 4000 ppm calcium were impossible to read.

In Expt 3, the thickness of the agar medium in layer 2 at various calcium levels was investigated. Results showed that increasing the middle layer from 2.5 to 5 or 8 ml caused many poor or impossible-to-read plates. The number of plates impossible to read was very high at 8 ml of medium.

In Expt 4, various quantities of agar were used in the bottom layer. These were tried respectively against a range of calcium levels in the middle layer, using 2.5 ml medium in this layer. The whole experiment was repeated using 6 ml of medium in the middle layer. In results reported in lines 1-4 all plaques were small, as if the lack of medium, and hence possibly of food material, without an underlying layer of medium, had some effect. With the higher quantities of medium in both layers, there were several plates impossible to read.

Table XXX Investigation of factors affecting plaque counts - trials with two-, three- and four-layer plates using various combinations of calcium and phosphate, and various quantities of agar medium (citrate and alginate present with phage in nearly all cases).

EXPT 1

	L1		L2		L3	L4		
	HA	ml	Ca ppm	2.5 ml Cf. SA+P	2.5 ml SA-P	Ca ppm	2.5 ml Cf. SA+P	Plaque count Av. of 3
1.	-P				* "			127
2.	"				** "			180(2)
3.	+P	10	2000	"	+P			97
4.	"	"	4000	"	"			101
5.	"	"	2000	"	"	2000	"	107
6.	"	"	4000	"	"	4000	"	108
7.	"	5	2000	"	"	2000	"	147
8.	"	"	4000	"	"	4000	"	148(2)

Phage $<10^{-6}$ } 0.1ml phage suspension mixed with 0.2ml culture and agar medium in lines 1 & 2.

Pep.-s.s. 8ml
Phage $<10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g
} 0.1ml phage mixture, mixed with 0.2ml culture and L3 in lines 3-8.

* 2000
** 4000 ppm Ca Cf. Nos. 1, 2 cf L2 recorded under L3 heading.

EXPT 2

	L1	L2		L3	L4		
	5 ml HA+P	Ca ppm	2.5 ml Cf. SA	2.5 ml SA	Ca ppm	2.5 ml Cf. SA+P	Plaque count Av. of 3
1.	"	2000	+P	+P	2000	"	173
2.	"	4000	"	"	4000	"	189
3.	"	2000	HA+P	"	2000	HA+P	170
4.	"	4000	"	"	4000	"	186(1)
5.	"	2000	"	"	2000	"	-
6.	"	4000	"	"	4000	"	-
7.	"	2000	"	HA+P	2000	"	178
8.	"	4000	"	"	4000	"	185(2)

L3 Nos. 5,6 + 2.5 ml water
" 7,8 + 1.0 ml water

L4 not added to all Nos. 5,6 plates due to softness of agar.

Pep.-s.s. 8ml
Phage $<10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2x0.04g
} 0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

(cont'd)

Table XXX (cont'd)

EXPT 3

	L1	L2	L3	L4	
	HA+P ml	2.5 ml Cf. HA+P Ca ppm	2.5 ml HA+P	2.5 ml Cf. HA+P Ca ppm	Plaque count Av. of 3
1.	2.5	2000	"	2000	109
2.	"	4000	"	4000	119
3.	5	2000	"	2000	139
4.	"	4000	"	4000	128(1)
5.	7.5	2000	"	2000	104
6.	"	4000	"	4000	>99(1)
7.	10	2000	"	2000	108
8.	"	4000	"	4000	129(1)

Pep.-s.s. 8ml
Phage $<10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

+ 1ml water to phage mixture and culture before mixing with L3 in each case.

EXPT 4

	L1	L2	L3	L4	
	5 ml HA+P	2.5 ml HA+P Ca ppm	2.5 ml SA+P	2.5 ml HA+P Ca ppm	Plaque count Av. of 3
1.	"	2000 Cf.	"	*2000 Cf.	203(2)
2.	"	4000 "	"	4000 "	191(1)
3.	"	2000 St.	"	2000 St.	195
4.	"	4000 "	"	4000 "	211
5.	"	2000 Au.	"	2000 Au.	154
6.	"	4000 "	"	4000 "	**

Pep.-s.s. 8ml
Phage $<10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

* 1 plate 1.5ml only

** no count, plates did not set.

(cont'd)

Table XXX (cont'd)

EXPT 5

	L1	L2	L3	L4	
	HA+P ml	2.5 ml HA+P Ca ppm	2.5 ml SA+P	2.5 ml HA+P Ca ppm	Plaque count Av. of 3
1.	2.5	2000 St.	"	2000 St.	140
2.	"	4000 "	"	4000 "	144
3.	"	2000 Au.	"	2000 Au.	121
4.	5	2000 St.	"	2000 St.	125
5.	"	4000 "	"	4000 "	-
6.	"	2000 Au.	"	2000 Au.	125
7.	7.5	2000 St.	"	2000 St.	113
8.	"	4000 "	"	4000 "	162
9.	"	2000 Au.	"	2000 Au.	115(1)
10.	10	2000 St.	"	2000 St.	86
11.	"	4000 "	"	4000 "	127
12.	"	2000 Au.	"	2000 Au.	74(2)

Pep.-s.s. 8ml
Phage $< 10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

EXPT 6

	L1	L2	L3	
	2000 ppm Ca HA+P ml	2.5 ml SA+P	2000 ppm Ca 2.5 ml HA+P	Plaque count Av. of 3
1.	2.5 St.	"	" St.	87
2.	5.0 "	"	" "	55
3.	7.5 "	"	" "	23
4.	10.0 "	"	" "	38(1)

Phage suspension mixture prepared as in Expt 5.

Medium to which $CaCl_2$ had been added was filtered, then steamed.

0.1ml this phage mixture, and 0.2ml culture, mixed with L2 in all instances.

(cont'd)

Table XXX (cont'd)

EXPT 7

	Medium +$CaCl_2$ Steaming time (min)	L1 5 ml HA+P	L2 2000 ppm Ca 2.5 ml HA+P	L3 2.5 ml SA+P	L4 2000 ppm Ca 2.5 ml HA+P	Plaque count Av. of 3
1.	0	"	"	"	"	121
2.	20	"	"	"	"	96
3.	45	"	"	"	"	108

Pep.-s.s. 8ml
Phage $< 10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

Filtrations varied using filter paper/mash.

EXPT 8

	L1 HA+P ml	L2 2000 ppm Ca HA+P ml	L3 2.5 ml SA+P	L4 2000 ppm Ca 2.5 ml HA+P	Plaque count Av. of 3
1.	0	2.5	"	"	0
2.	0	5	"	"	0
3.	0	7.5	"	"	0
4.	0	10	"	"	0
5.	2.5	2.5	"	"	56
6.	"	5	"	"	61
7.	"	7.5	"	"	29
8.	"	10	"	"	7
9.	5	2.5	"	"	66
10.	"	5	"	"	67
11.	"	7.5	"	"	74
12.	"	10	"	"	47
13.	7.5	2.5	"	"	96
14.	"	5	"	"	87
15.	"	7.5	"	"	119
16.	"	10	"	"	113
17.	10	2.5	"	"	105
18.	"	5	"	"	123
19.	"	7.5	"	"	118
20.	"	10	"	"	111

Pep.-s.s. 8ml
Phage $< 10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

Bulk of agar medium + $CaCl_2$ was steamed >1h.

(cont'd)

Table XXX (cont'd)

EXPT 9

	L1	L2	L3	L4	
	HA+P ml	4000 ppm Ca HA+P ml	2.5 ml SA+P	4000 ppm Ca 2.5 ml HA+P	Plaque count Av. of 3
1.	0	2.5	"	"	42
2.	"	5	"	"	30
3.	"	7.5	"	"	8
4.	"	10	"	"	2
5.	2.5	2.5	"	"	139
6.	"	5	"	"	140
7.	"	7.5	"	"	190
8.	"	10	"	"	123
9.	5	2.5	"	"	133
10.	"	5	"	"	>115
11.	"	7.5	"	"	>132
12.	"	10	"	"	>112
13.	7.5	2.5	"	"	128
14.	"	5	"	"	78
15.	"	7.5	"	"	51
16.	"	10	"	"	125
17.	10	2.5	"	"	134
18.	"	5	"	"	132
19.	"	7.5	"	"	226
20.	"	10	"	"	187

Pep.-s.s. 8ml
Phage $<10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

EXPT 10

	L1	L2	L3	L4	
	HA+P ml	6000 ppm Ca HA+P ml	2.5 ml SA+P	6000 ppm Ca 2.5 ml HA+P	Plaque count Av. of 3
1.	2.5	2.5	"	"	108
2.	"	5	"	"	97
3.	"	7.5	"	"	103
4.	"	10	"	"	101
5.	5	2.5	"	"	96
6.	"	5	"	"	80
7.	"	7.5	"	"	172
8.	"	10	"	"	81
9.	7.5	2.5	"	"	159
10.	"	5	"	"	152
11.	"	7.5	"	"	142 (1)
12.	"	10	"	"	190 (2)
13.	10	2.5	"	"	123 (2)
14.	"	5	"	"	126 (2)
15.	"	7.5	"	"	187 (2)
16.	"	10	"	"	193

Phage suspension mixture prepared as in Expt 9.

0.1ml this mixture, and 0.2ml culture, mixed with L3 in all instances.

(cont'd)

Table XXX (cont'd)

EXPT 11

	L1	L2	L3	L4	Plaque count	
	HA-P ml	6000 ppm Ca HA+P ml	SA+P ml	6000 ppm Ca 2.5 ml HA+P	(4 layers) Av. of 2	(L4 omitted) 1 plate
1.	2.5	2.5	2.5	"	142	187
2.	"	5	"	"	125	118
3.	"	7.5	"	"	131	128
4.	"	10	"	"	146	120
5.	5	2.5	"	"	144	127
6.	"	5	"	"	155	162
7.	"	7.5	"	"	163	187
8.	"	10	"	"	148	147
9.	2.5	2.5	5	"	153	116
10.	"	5	"	"	190	Itr
11.	"	7.5	"	"	150	138
12.	"	10	"	"	168	162
13.	5	2.5	"	"	220	192
14.	"	5	"	"	225	203
15.	"	7.5	"	"	229	205
16.	"	10	"	"	239	193

Pep.-s.s. 8ml
Phage $< 10^{-5}$ 1ml
10% Cit. 1ml
Ca alg. 2 x 0.04g

0.1ml this phage mixture, and 0.2ml culture, mixed with L3 in all instances.

Comment on Table XXX, Expts 1-11

In Expt 1 two, three and four layers were compared. The higher counts recorded in lines 7 and 8, than in lines 5 and 6, may be due to a smaller volume of agar in the bottom layer. Because of this it was not possible to prove conclusively whether the fourth layer was an advantage, though there was a slight indication that it was so. Plates could mostly be read satisfactorily.

In Expt 2 the four-layer method was used for all tests. High counts were obtained and plates were mostly satisfactory in appearance. Dilution of hard agar in layer 3 with water (1 ml), compared with the use of soft agar, made very little difference.

In Expt 3 the effects of different volumes of agar in the bottom layer were investigated, using the four-layer technique. It appeared that 5 ml agar in the bottom layer was best but, as many plates were poor, conclusions were not definite.

Expt 4. Prior to this experiment all agar medium treated with $CaCl_2$ was centrifuged when the clear medium was required. As it was proposed to simplify the clarification by substitution of filtration of agar through blotting-paper mash in a warm Buchner funnel Expt 4 was a comparison of centrifuged medium with filtered medium, part of which was steamed and part autoclaved after filtration. Centrifuged and steamed filtered-medium gave approximately the same results, but autoclaved filtered-medium gave low results at 2,000 ppm calcium, and at 4,000 ppm calcium the plates did not set. Plate appearance was mostly good in the four-layer experiments.

In Expt 5 comparison was made of the effect on the plaque count of various volumes of agar medium in the bottom layer. In layer 2 and layer 4 media of 2,000, 4,000 (steamed after filtration) and 2,000 ppm calcium (autoclaved after filtration) were used for each of the various volumes of medium in the bottom layer. Plates were mostly easily read. Plaque counts generally became less with increasing volumes of medium in the bottom layer. Higher plaque counts were obtained with 4,000 ppm calcium than with 2,000 ppm calcium in layer 2 and layer 4.

Expt 6. The relatively low plaque counts obtained in this

experiment may have been due partly to the absence of a layer below the $CaCl_2$-treated layer. Increase in the volume of the $CaCl_2$-treated layer led mostly to a corresponding decrease in plaque count. Plates showed lack of good culture growth.

Expt 7. In this experiment the mixtures of media and $CaCl_2$ were treated differently after mixing, viz. times for steaming were nil, 20 min and 45 min, respectively, before filtration. Though results were not clear-cut, it appeared that avoidance of steaming after mixing was preferable. All plates were easily read.

In Expts 8, 9 and 10 attempts were made to find the best combinations of volumes of agar in layers 1 and 2 at calcium levels of 2,000, 4,000 and 6,000 ppm. In Expt 8, lines 1-4, in which there was no layer below the lower calcium layer, plaques were not discernable at all, and in Expt 9, also without this layer, there were very low results. In the series, there were many poor plates, and it was impossible to read some plates. The inclusion of only 2,000 ppm calcium did not give as high results as 4,000 and 6,000 ppm calcium. It was apparent that it was necessary to have a correct balance between the volumes of medium in layers 1 and 2 and a suitable calcium content in order to secure maximum counts. It was also necessary to make some adjustment in order to avoid poor plates.

In Expt 11 phosphate was omitted from the bottom layer. Out of 48 plates, 36 were classed as good or nearly so, 11 as fair, and only one as impossible to read. This represented a major improvement over the appearance of plates in some of the previous experiments. In this experiment the fourth layer was omitted from one of each triplicate set of plates. It was found that in 12 out of 15 cases the plaque counts on the four-layer plates (averages of the two) were higher than on the three-layer plates (single counts). Layer 3 varied 2.5 and 5 ml. The highest counts were obtained with 5 ml agar in layers 1 and 3 while changes in the volume of agar in layer 2 had no obvious effect.

Appearance of Plates

Difficulties in securing high plaque counts were due to poor plates (precipitates, opaqueness, faint or small plaques, poor growth) and these points were affected by the amount of calcium, the quantities of medium in the layers, the presence of calcium alginate and sodium hexametaphosphate or citrate, and the presence or absence of phosphate. Table XXXI gives a summary of the quality of the plates in many of the experiments ranging from grades 1-4 (good - impossible or almost impossible to read).

Table XXXI Grading of appearance of plates when using two-, three- or four-layer method. Plates were graded 1-4 (plaques easy to read - plaques impossible to read). The numbers of plates in specific grades are given for selected experiments.

Table	Expt	Appearance, Grade: 1	1-2	2	2-3	3	4
XXVI	1		57*				
"	2		48			2	4
XXVII	1		18		24		
"	2		17		19		13
XXVIII	1	8			13	1	4
"	2		10			1	1
XXIX	1	26		7		1	2
"	2	18		5		1	6
"	3	24		20		3	20
"	4	33		17		9	9
XXX	1	19		3			1
"	2	14		1		1	2
"	3	8		7		5	4
"	4	11		1			1
"	5	27		3			
"	6			6		4	
"	7	9					
"	8	24		24			12
"	9	9		18		26	7
"	10	13		18		11	6
"	11	23	13	11			1

Grade 1 = Good
" 2 = Fair
" 3 = Poor
" 4 = Impossible to read

* Number of plates in this grade for this experiment.

Photographs showing the appearance of typical plates are shown in Fig 2. In good-quality plates plaques are clear and can be counted easily, but counting of poor-quality plates is difficult and inaccurate.

Comments on appearance of plates

A = smallish plaques
B = precipitate
C = smallish plaques, few in number
D = large, clear plaques.

Key showing details of plate preparation

A is a 2-layer plate, 4,000 ppm calcium in top layer, no phosphate in L1 (phage alone).

B is a 2-layer plate, 4,000 ppm calcium in top layer, no phosphate in L1 (phage, calcium alginate and hexametaphosphate).

C is a 4-layer plate, 4,000 ppm calcium in L2 and L4, phosphate in L1 (phage, calcium alginate and citrate).

D is a 4-layer plate, 6,000 ppm calcium in L2 and L4, TSA in L3.

Summary and Discussion for Sub-section

Many difficulties were encountered in using the soft agar method for phage estimation. It was found that, when estimating phage in the presence of calcium alginate wool, the results could be affected by the hexametaphosphate used to dissolve the calcium alginate, and also by hexametaphosphate alone. These observations led to an examination of the role of calcium in phage estimations. It was shown that calcium was in short supply in the lactose yeast phosphate agar, the medium in constant use for phage work. The plaque count of the medium could be increased by supplying calcium, and the quantity required for optimum counts was established

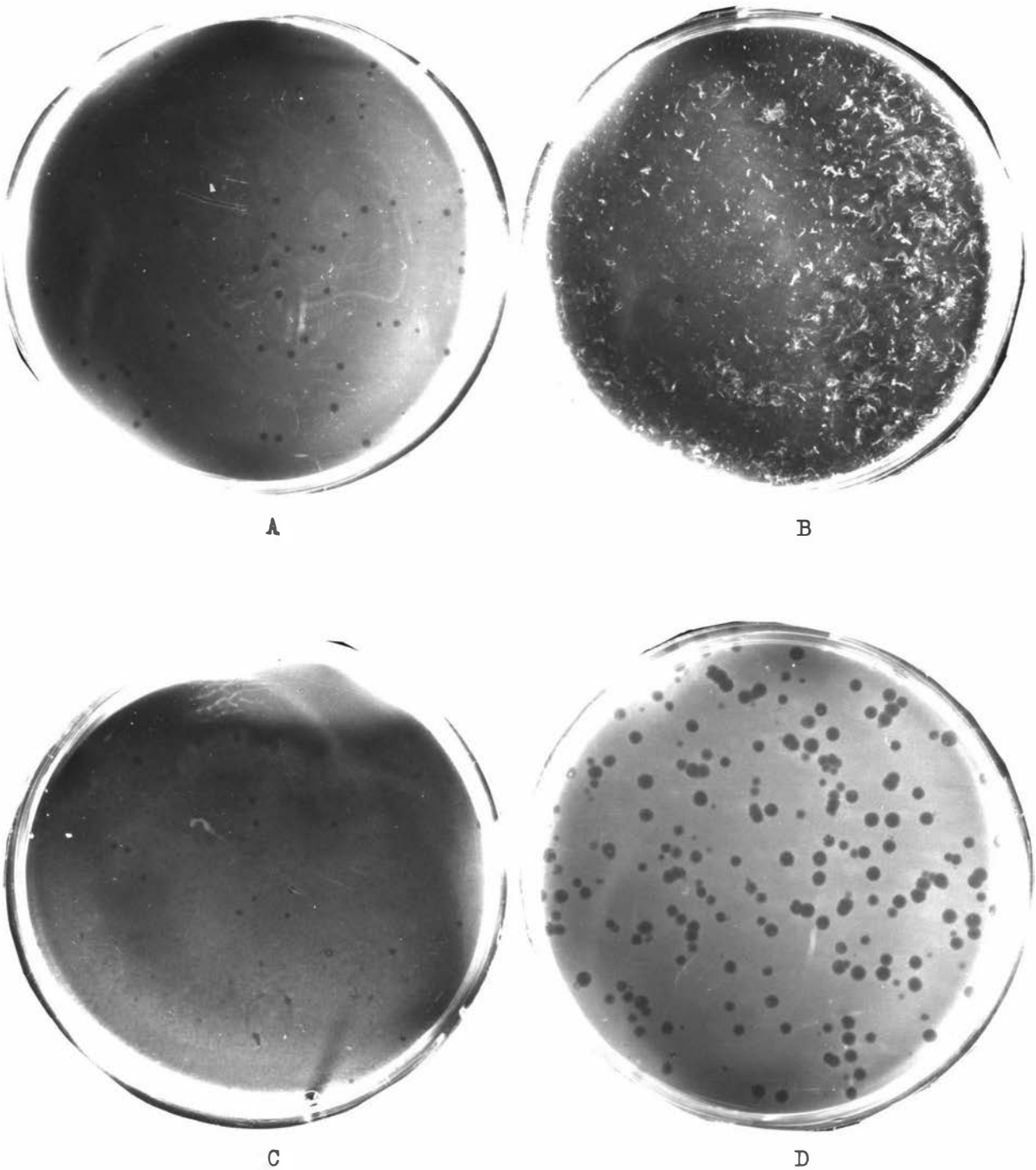


Fig 2. Photographs of typical phage plates showing the relationship between plate appearance and method of plate preparation. (Details of methods for preparing plates are given in key on previous page.)

experimentally. When, however, calcium alginate and sodium hexametaphosphate solution were then included in the improved medium low counts were again encountered, and the ease of counting was markedly impaired. It was therefore necessary to find a way to supply the calcium needed for optimal counts in the presence of calcium alginate and hexametaphosphate, and yet avoid precipitates and other poor plates.

Sodium phosphate is a normal constituent of LYPA medium. For phage estimations, extra calcium is supplied from $CaCl_2$. Calcium alginate is dissolved in sodium hexametaphosphate or citrate solution. Thus with a complex mixture of calcium, phosphate, and hexametaphosphate or citrate ions present, $Ca_3(PO_4)_2$ can be precipitated, and the hexametaphosphate, citrate or phosphate ions can sequester (complex) some of the calcium ions. Too much precipitate can spoil the plates and make impossible the accurate counting of plaques. There must be sufficient available calcium left to secure optimum plaque counts with the phage.

Details of individual experiments in the series have been given along with the various Tables. Two-layer plates were investigated initially, then three-layer plates, and finally four-layer plates. In the two-layer work reported in Table XXVII most plates gave only low plaque counts, and many plates were unsatisfactory, but two plates showing fairly good counts indicated that conditions might be found which give generally satisfactory plates. In the three- and four-layer methods tested, the calcium was supplied by diffusion from the second layer, and the second and fourth layers, respectively.

The change of method (as mentioned in comments on Table XXX) from inclusion of added phosphate to omission of this addition in the bottom layer caused a striking difference in the quality of the plates, and marked a turning point in the quest for easily read plates with relatively high numbers of plaques. However it was clearly necessary to further investigate the concentration of calcium required, the quantities of medium needed in the various layers, and other factors, in order to attempt to secure optimum counts. These investigations are dealt with in later sections of the work.

(iii) THE EFFECT ON PLAQUE COUNTS OF VARIATIONS IN FACTORS CONNECTED WITH THE LAYERS AND ANALYTICAL MATERIAL

Introduction

Following success in developing a method for preparing satisfactory plates, as shown in the previous section, an investigation was made of factors connected with the layers of agar medium, such as quantity per layer, calcium addition, and variation of phosphate. The effect of various amounts of calcium alginate in the phage mixture, the effect of calcium chloride on the keeping of agar medium, and the addition of calcium and citrate to the phage mixture were also investigated. Tables are presented with the minimum of detail. Four-layer plates were used throughout, except in part of Expt 12 (Table XXXII). Unless otherwise stated, phage inoculum was prepared by making 1 ml of $< 10^{-5}$ phage up to 10 ml with peptone-salt solution. If citrate and calcium alginate were used, 1 ml 10% citrate (or amount as stated) was included in the total 10 ml, and the number of calcium alginate pads was specified. The amount of culture used was 0.2 ml unless otherwise stated.

Variations in Quantities of Agar Media, Calcium Chloride, Calcium Alginate and Sodium Citrate

Expts 1-5. Variations in quantities of agar media, calcium chloride and calcium alginate (Table XXXII).

In Expt 1 different quantities of medium in the layers were compared, using 6,000 ppm calcium level in layers 2 and 4. The plates were nearly all of good quality (out of 59 plates, 57 were grade 1, i.e. of good appearance, and two were grade 2, i.e. of fair appearance). A few plates had dark or black areas.

In Expt 2 calcium at 8,000 ppm was used in layers 2 and 4. Many plates were of grade 3 or grade 4 quality, and some were impossible to count. Other results would be unreliable. The general conclusion from this experiment was that the level of calcium was too high.

In Expt 3 calcium at 8,000 ppm was again used in layers 2 and 4.

II Variations in quantities of agar media, calcium chloride, calcium alginate and sodium citrate.

EXPT 2

L1 HA −P ml	L2 HA +P ml	L3 SA +P ml	Plaque count Av.of 3
5	2.5	5	169(2)
"	5	"	206(2)
"	7.5	"	–
"	10	"	–
2.5	2.5	2.5	183
"	5	"	159
"	7.5	"	69(2)
"	10	"	–
5	2.5	"	174
"	5	"	143
"	7.5	"	98
"	10	"	–
2.5	2.5	5	–
"	5	"	180
"	7.5	"	204(1)
"	10	"	139(2)
5	2.5	"	>153(1)
"	5	"	196
"	7.5	"	221(2)
"	10	"	–

L2 8000ppm Ca
L4 (HA+P 2.5ml (8000ppm Ca

Nos. 1–20

(Pep.-s.s. 8ml
(Phage < 10^{-5} 1ml
(10% Cit. 1ml
(Ca alg. 2 x 0.04g

EXPT 3

L1 HA −P ml	L2 HA +P ml	L3 SA +P ml	Plaque count Av.of 3
7.5	2.5	2.5	169
"	5	"	179(2)
"	7.5	"	35
"	10	"	119(2)
10	2.5	"	134
"	5	"	136
"	7.5	"	151
"	10	"	131
7.5	2.5	5	117
"	5	"	147
"	7.5	"	124
"	10	"	125
10	2.5	"	128
"	5	"	145
"	7.5	"	130
"	10	"	112

L2 8000ppm Ca

L4 (HA+P 2.5 ml (8000ppm Ca

Nos. 1–16

(Pep.-s.s. 2ml
(Phage < 10^{-5} 1ml
(10% Cit. 1ml
(Ca alg. 2 x 0.04g

EXPT 4

Ca alg. .04g	L2 HA +P	Plaque count Av.of 3
0	2.5	140
0	5	160
1	2.5	199
1	5	193
2	2.5	206
2	5	207
3	2.5	180
3	5	189
4	2.5	174
4	5	190

L1 HA-P 5 ml

L2 6000ppm Ca

L3 SA+P 5 ml

L4 (HA+P 2.5 ml (6000ppm Ca

EXPT 5

Ca alg. .04g	L2 HA +P ml	Plaque count Av.of 4
0	2.5	116
0	5	129
0	7.5	121
1	2.5	149
1	5	159
1	7.5	148
3	2.5	150
3	5	162
3	7.5	155

L1 HA-P 5 ml

L2 6000ppm Ca

L3 SA+P 5 ml

L4 (HA+P 2.5 ml (6000ppm Ca

EXPT 6

Ca alg. .04g	Plaque count Av.of 3
0	165
1	138
2	148
3	137
4	144
1	139
2	149
3	143
4	132

L1 HA-P 5ml

L2 (HA+P 5ml (6000ppm Ca

L3 (SA+P2.5ml (Cit. 0.5ml

L4 (HA+P2.5ml (6000ppm Ca

Proportionate amounts Cit. in phage mixture.

No.	Citrate ml
6	0.3
7	0.6
8	0.9
9	1.2

No. 1 = control.

(cont'd)

(cont'd)

EXPT 8

g. 4g	L3 5ml SA+P cult. ml	Plaque count Av.of3
	0.2	274
	0.5	231
	0.2	247
	0.5	276
	0.2	231
	0.5	216
	0.2	189
	0.5	210
	0.2	307
	0.5	288
	0.2	243
	0.5	234
	0.2	256
	0.5	214
	0.2	225
	0.5	220
	0.2	328(2)
	0.5	298

HA-P 5ml
(HA+P 5ml
(6000ppm Ca
(HA+P 2.5ml
(6000ppm Ca

pportionate
ts. Cit. in
age mixture.

	Cit. ml.
10	0.3
12	0.6
14	0.9
16	1.2

EXPT 9

Ca alg. .04g	Plaque count Av.of3
0	194
1	215
2	190
3	185
4	133
1	191
2	95
3	176
4	168

L1 HA-P 5ml
L2 (HA+P 5ml (6000ppm Ca
L3 SA+P2.5ml
L4 (HA+P2.5ml (6000ppm Ca

Proportionate amts. Cit. in phage mixture.

No.	Cit. ml
6	0.25
7	0.5
8	0.75
9	1.0

EXPT 10

Ca alg. .04g	Plaque count Av.of3
0	120
1	132
2	152
3	139
4	113
1	141
2	96
3	93
4	89

L1 HA-P 5ml
L2 (HA+P 5ml (6000ppm Ca
L3 SA+P 5ml
L4 (HA+P 2.5 ml (6000ppm Ca

Proportionate amts. Cit. in phage mixture.

No.	Cit. ml
6	0.25
7	0.5
8	0.75
9	1.0

EXPT 11

Ca alg. .04g	L3 5ml SA+P Ca ppm	NaCl 10% .05ml	Plaque count Av.of2
0	0		45
2	0		111
0	2000		76
2	"		7(1)
0	"	+	87
2	"	+	0
0	4000		66
2	"		0
0	"	+	78
2	"	+	0
0	6000		27
2	"		0
0	"	+	12
2	"	+	0

L1 HA-P 5 ml
L2 (HA+P 5 ml (6000ppm Ca
L4 (HA+P 2.5ml (6000ppm Ca

EXPT 12

L2 5ml HA+P Ca ppm	L4 2.5ml HA+P Ca ppm	Plaque count Av.of3
6000	6000	228
"	No layer	202
8000	8000	181
"	No layer	280(1)

L1 HA-P 5 ml
L3 SA+P 5 ml

This time the plates were not as poor in quality as in the previous experiment. Larger volumes of medium in layer 1 may have helped this.

In Expt 4 comparisons were made using 0, 1, 2, 3 and 4 calcium alginate pads (each 0.04 g) in the phage inoculum mixture. In each case 2.5 and 5 ml of agar medium in layer 2 were compared. Plates were mostly good, either grade 1, 1-2 or 2. Counts were somewhat higher with calcium alginate and citrate than without. With more than two pads present there was a slight decrease in plaque counts.

In Expt 5 three quantities of medium (2.5, 5 and 7.5 ml) were compared in layer 2 using 0, 1 and 3 pads in phage inoculum mixture. Plates were grades 1 and 1-2, with one plate in grade 2. One and 3 pads gave counts slightly higher than without pads.

Expts 6-10. Comparison of different proportions of sodium citrate for dissolving calcium alginate pads (Table XXXII).

In Expts 6 and 7 counts from 1, 2, 3 and 4 calcium alginate pads dissolved in 1% citrate were compared with counts from 1, 2, 3 and 4 pads dissolved in approximately proportionate amounts of citrate, viz. 0.3, 0.6, 0.9 and 1.2%. There were no marked consistent differences in results by the two methods. All counts were slightly lower than the control in Expt 6, and slightly higher (except one) in Expt 7; 0.5 ml culture was used throughout the two experiments.

In Expt 8 there was no marked difference between the two methods. With 1% citrate the average counts of 0.2 ml and 0.5 ml inocula dropped steadily with from 2 pads to 4 pads present.

In Expt 9, 4 pads gave low counts in one part of the experiment only. Apart from an anomaly (No. 7) there was no marked difference between the two methods for dissolving pads.

In Expt 10 the results were regular where 1 ml citrate was used for solution except that 4 pads gave a low count. Where proportionate amounts of citrate were used, three out of four counts were low.

Expt 11. Effect of addition of $CaCl_2$ to layer 3 (Table XXXII).

In Expt 11 calcium was added to layer 3 to find the effect of raising the calcium level by direct addition of $CaCl_2$ and filtration.

Trials were made with phage and phage plus two calcium alginate pads. The whole experiment showed unusually poor plates, mostly grades 3-4, and results were unreliable. Nevertheless, the experiment showed that direct addition of $CaCl_2$ was slightly advantageous for phage count alone up to 4,000 ppm calcium, but where two calcium alginate pads were present counts were mostly nil. Additions of 2,000 ppm calcium and more directly to layer 3 were therefore totally ruled out for phage-alginate estimations. A small amount of NaCl had no appreciable effect.

Expt 12. Comparison of plaque counts with and without layer 4 (Table XXXII).

In Expt 12 (using 6,000 and 8,000 ppm calcium) comparisons were made with and without layer 4. All the plates at 6,000 ppm calcium were grade 1 (good), but at 8,000 ppm calcium the four-layer plates were not quite as good, and triplicate three-layer plates had the following appearance:

Plaque count	Appearance of plate
280	Good (grade 1)
142, 80	Poor (" 3) Precipitate on half

With the three-layer method and 8,000 ppm calcium, though one plate had a high count, the three plates varied widely in counts, and on two there were precipitates on half of each plate. Apparently 8,000 ppm calcium was too high for a three-layer plate. It was apparent that a four-layer, 6,000 ppm calcium plate was best.

Conclusions

1. While variations in amounts of agar medium can be used satisfactorily (in different layers) it seems that suitable amounts are 5 ml in each of layers 1, 2 and 3, and 2.5 ml in layer 4.
2. Four layers of medium are better than three layers.
3. For addition to layers 2 and 4, 6,000 ppm calcium is a satisfactory quantity, but 8,000 ppm calcium is too high an amount.
4. High amounts of calcium cannot be added satisfactorily to layer 3 for phage-alginate determinations.

5. One, 2 and 3 calcium alginate pads (0.04 g each) give reasonably satisfactory results in the method in use, but 4 pads tend to give low results.
6. One per cent sodium citrate in peptone-salt solution is satisfactory for dissolving 1, 2 and 3 calcium alginate 0.04 g pads, and it is unnecessary to use proportionate amounts of citrate according to the number of calcium alginate pads present.

Comparison of Different Brands of Ingredients Used in Media for Plaque Counts

As two brands of ingredients (Oxoid and Baltimore Bacteriological Laboratory (BBL)) were available for preparing lactose yeast phosphate agar in this laboratory, comparisons were made of plaque counts carried out using media prepared with the different brands. In Table XXXIII are shown the results of three experiments in which comparisons were made. In Expt 1 media made from the two brands of beef extract were compared using a phage mixture containing 2 x 0.04 g alginate per 10 ml. In this experiment the appearance of the plates were: eight grade 1, five grade 2, and four grade 2-3. The results showed no consistent difference between the two brands of beef extract. In Expt 2 the comparison between the two brands of beef extract was made in the soft agar layer only. Two amounts of culture (0.2 and 0.5 ml) and also phage and phage plus alginate dissolved in citrate, were compared. The Oxoid possibly gave slightly better results. (The plates in No. 3 were all below standard quality, and the count of 120 may have been low.) In Expt 3 Oxoid beef extract and Oxoid yeast extract were compared with the corresponding BBL ingredients in all the media. All were inoculated with phage without alginate. All plates except two were grade 1. Results showed that on two occasions the counts agreed, and on two occasions BBL gave better results.

Overall the results show that there is little to choose between the two brands of ingredients.

Table XXXIII Comparison of different brands of ingredients used in media for plaque counts.

EXPT 1

	In each medium	L1 HA -P ml	L2 HA +P ml	Plaque count Av.of3
	Oxoid			
1	BE	5	5	215
2	"	"	7.5	162
3	"	"	10	171
	BBL			
4	BE	"	5	204
5	"	"	7.5	191
6	"	"	10	172(2)

L2 Ca 6000 ppm
L3 SA+P 5 ml
L4 (HA+P 2.5ml (Ca 6000 ppm
All inoc. with phage mixture containing 2 x 0.04g Ca alg. pads in 10 ml.

HA+P under BBL
BBL beef extract + small amount oxoid.

EXPT 2

	In soft agar +P layer only	† Ca alg. .04g	L3 Cult. ml	Plaque count Av.of3
	BBL			
1	BE	0	0.2	147(1)
2	"	0	0.5	154
3	"	2	0.2	120(1)
4	"	2	0.5	172
	Oxoid			
5	BE	0	0.2	135
6	"	0	0.5	156
7	"	2	0.2	185
8	"	2	0.5	224

L1 HA-P 5 ml
L2 (HA+P 5 ml (Ca 6000 ppm
L3 SA+P 5 ml
L4 (HA+P 2.5ml (Ca 6000 ppm
† in phage mixture

EXPT 3

	In each* medium (Definition terms in last col.)	Time analyses	Plaque count Av.of3	Definition of Oxoid & BBL as used in column*
		Wed.		
1	Oxoid	11.45 am	225	Oxoid
2	BBL	"	223	Peptone BBL
3	Oxoid	2.00 pm	248	BE Oxoid
4	BBL	"	242	YE Oxoid
5	Oxoid	4.30 pm	259(1)	BBL
6	BBL	"	290	Peptone BBL
		Thurs.		
7	Oxoid	10.00 am	256	BE BBL
8	BBL	"	304	YE BBL

L1 HA-P 5 ml
L2 (HA+P 5 ml (Ca 6000 ppm
L3 SA+P 5 ml
L4 (HA+P 2.5ml (Ca 6000 ppm
All inoc. with phage $< 10^{-6}$
Broths for inoculation contained Oxoid and BBL, respectively, as *.

Oxoid = Oxoid Ltd, London
BBL = Baltimore Biological Laboratory
BE = beef extract
YE = yeast extract

Further Experiments with Phosphate in Layers of Agar Medium

In Table XXXIV are shown the results of further experiments carried out in connection with phosphate in the agar medium.

Expt 1 was carried out to find whether the usual phosphate addition could be withheld from the agar medium used in layers 2 and 4. Such a non-addition of phosphate should lessen the $CaCl_2$ addition by the amount required to precipitate the phosphate. Results showed that counts increased with calcium addition up to 4,000 ppm, but that at 6,000 ppm four poor-quality plates (grade 4) out of six were obtained. Normally 6,000 ppm of calcium should give good-quality plates, but the 6,000 ppm added here would result in a higher than usual amount of available calcium due to non-addition of phosphate. The experiment gave reasonably good results, *viz.* eight grade 1 plates, 2 grade 1-2, ten grade 2 (on size of plaques) and four grade 4. As control experiments using phosphate were not included, a strict comparison between the two methods was impossible, but the results were encouraging. All plates were not grade 1 and there was no special reason to change the method. The cause of the small plaques was unknown.

In Expt 2 the objective was to investigate the effect of the addition or withholding of phosphate in layer 3. Counts were lower in Nos. 5-8 (no phosphate) than in Nos. 1-4 (plus phosphate). Growth on the plates was mostly poor (one fair, one fairly good) for 0.2 ml inoculations and good for 0.5 ml inoculations, and of the 0.5 ml inoculations plates 2 and 4 (plus phosphate) showed better growth than plates 6 and 8 (no phosphate). Plaques or plates showing varying degrees of faintness occurred on three plates with phosphate and on eight plates without phosphate. The overall result was that phosphate should be included in layer 3.

In Expt 3 comparison was made between the usual amount and approximately half that amount of phosphate in layer 3. Results showed that at 6,000 ppm calcium the normal amount of phosphate gave higher counts than the smaller amount in three out of four cases. With 7,000 and 8,000 ppm calcium the normal amount of phosphate gave a higher count once out of four times. It was decided to retain the usual amount of phosphate in layer 3. In this experiment all plates were grade 1, but plaques were smallish,

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Table XXXIV Further experiments with phosphate in layers of agar medium.

EXPT 1 Omission of phosphate from layers 2 and 4.

	L1	L2		L3	L4		
	HA −P ml	Ca ppm	HA −P ml	SA +P ml	Ca ppm	HA −P ml	Plaque count Av. of 3
1	5	1000	5	5	1000	2.5	95
2	"	"	10	"	"	"	106
3	"	2000	5	"	2000	"	153
4	"	"	10	"	"	"	150
5	"	4000	5	"	4000	"	182
6	"	"	10	"	"	"	187
7	"	6000	5	"	6000	"	187 (2)
8	"	"	10	"	"	"	–

(2 x 0.04g alginate added to 10 ml of phage mixture.)

5 and 10 ml agar medium in layer 2 gave approx. same results except in Nos. 7 & 8.

EXPT 2 Layer 3 with and without phosphate.

	Ca alg. .04g	L1 HA −P ml	L2 HA +P ml	L3 Cult. ml	L3 SA +P ml	L4 HA +P ml	Plaque count Av. of 2
1	0	5	5	0.2	5	2.5	123
2	0	"	"	0.5	"	"	154
3	2	"	"	0.2	"	"	197
4	2	"	"	0.5	"	"	217
5	0	"	"	0.2	SA −P 5	"	97
6	0	"	"	0.5	"	"	71
7	2	"	"	0.2	"	"	171
8	2	"	"	0.5	"	"	132
9	0	"	"	0.2	SA +P 5	"	121
10	0	"	"	0.2	"	"	147

L2 = 6000 ppm Ca

L4 = 6000 ppm Ca

Nos. 9 & 10 – added extra Ca (by $CaCl_2$) to SA+P medium.

No. 9 75 ppm Ca

No. 10 220 ppm Ca

Plate (No. 9 Grade 2, 1-2
(No. 10 1-2, 1-2

EXPT 3 Variation in amount of phosphate in layer 3.

	Ca alg. .04g	L2 HA+P 5ml Ca ppm	L3 Cult. ml	L3 SA+P Phosphate	L4 HA+P 2.5ml Ca ppm	Plaque count Av. of 3
1	0	6000	0.5	Usual	6000	137
2	0	"	0.4	"	"	124
3	0	"	0.5	$<\frac{1}{2}$	"	115
4	0	"	0.4	"	"	107
5	2	"	0.5	Usual	"	183
6	2	"	0.4	"	"	163
7	2	"	0.5	$<\frac{1}{2}$	"	150
8	2	"	0.4	"	"	172
9	0	7000	0.4	Usual	7000	158
10	0	"	"	$<\frac{1}{2}$	"	128
11	2	"	"	Usual	"	172
12	2	"	"	$<\frac{1}{2}$	"	186
13	0	8000	"	Usual	8000	139
14	0	"	"	$<\frac{1}{2}$	"	140
15	2	"	"	Usual	"	179
16	2	"	"	$<\frac{1}{2}$	"	186

L1 = HA−P 5 ml

Phosphate <half usual = 0.8 of half.

as could be expected with 0.4 and 0.5 ml inocula.

Effect on Plaque Counts of Different Temperature Treatments of Plates after Pouring each Agar Layer

An experiment was carried out to find whether any particular treatment of the plates after pouring each layer was important. To this end two extremes of treatment were tried, *viz.* keeping three plates at 4°C and three plates at 30°C for ½-h periods after pouring each layer. A similar experiment was also carried out in which the time for keeping the plates after pouring was 5 min or less. A control analysis in which plates were treated in the usual manner was also carried out. All plates were inoculated with 0.2 ml of $<10^{-6}$ phage. Results are shown in Table XXXV.

Table XXXV The effect on plaque counts of different temperature treatments of plates after pouring each agar layer.

No.		Time and temp. at which kept after pouring each layer	Plaque counts Av. of 3	Details of layer composition (all plates)
				L1 HA-P 5ml
1	4 layers	Control (room temp. as usual)	188(2) 162(3)	L2 (HA+P 5ml (6000ppm Ca
2	"	½ h at 4°C	177	
3	"	½ h at 30°C	135	L3 SA+P 5ml
4	"	Max. 5 min at 4°C	167	L4 (HA+P 2.5ml (6000ppm Ca
5	"	Max. 5 min at 30°C	138(2)	

All control plates, and all plates in the 4°C experiments, were graded 1 except for one control plate which was poor. Records were not kept of plate gradings for 30°C experiments. Average count of control plates was 162 (one poor, two good plates) or 188 (two good plates only). Plates from the 4°C experiments gave counts approximately the same as the control. Plates for the 30°C experiments gave lower counts than control. The layers for the 30°C experiments tended to slip during handling which may have adversely affected counts.

From the above results it would appear that the method as normally carried out, i.e. standing plates at room temperature between pouring the layers, was satisfactory.

Effect of Storage on Agar Medium Containing Calcium Chloride

Two experiments were carried out to find whether the presence of $CaCl_2$ would adversely affect the keeping qualities of agar medium stored at 45°C and 4°C, and at room temperature, respectively. The medium containing $CaCl_2$ was used in layers 2 and 4. Results of experiments are shown in Table XXXVI.

Table XXXVI Effect of storage on agar medium containing calcium chloride.

	EXPT 1 Medium kept 45°C and 4°C compared with fresh medium	Plaque counts Av. of 3 for 0.2ml inoc.	Plaque counts Av. of 3 for 0.5ml inoc.	EXPT 2 Analyses throughout day using old and fresh medium Time analyses	Medium	Plaque counts Av. of 3 Phage dilution a	b	c
	1st day							
1		*88	257	10.00am	Old	194	176	160
	2nd day							
2	Cold	240	223	"	Fresh	184	180	161
3	45°C	225	232	2.00pm	Old	191	166	160
4	Fresh	197	194	"	Fresh	181	175	150
	3rd day							
5	Cold	231	240	4.30pm	Old	172	181	167
6	45°C	218	243	"	Fresh	191	186	176
7	Fresh	227	214					

* Poor plates - disregard figure

Both expts L1 HA-P 5ml L2 HA+P 5ml 6000ppm Ca L3 SA+P 5ml L4 HA+P 2.5ml 6000ppm Ca Inocs. phage $<10^{-6}$ fresh each day

In Expt 1 media of the same composition and including $CaCl_2$ were used on three consecutive days, analyses being done on the

fresh medium on the first day, and on portions kept at 45°C and 4°C on the second and third days. Fresh medium (+$CaCl_2$) was also made and used on each of the second and third days. No appreciable effect was detected as a result of keeping medium with $CaCl_2$ at 45°C or in the cold room (4°C). All plates were grade 1 except those inoculated with 0.2 ml on the first day.

In Expt 2 analyses were done at 10.00 a.m., 2.00 p.m. and 4.30 p.m. using medium (+$CaCl_2$) one-day-old and comparing it with fresh medium (+$CaCl_2$) prepared before each lot of analyses. Plates were all of grade 1 quality. Results showed that there was no appreciable difference between the different lots of medium. Triplicate phage dilutions were compared in the same experiment.

Summary of results showed that $CaCl_2$ (at 6,000 ppm calcium) did not noticeably affect the medium when stored warm or cold for periods of up to two days or when stored at room temperature for one day.

Effect on Plaque Count of Inclusion of Calcium and/or Citrate in the Phage-culture Layer

Experiments were carried out to find whether the addition of calcium or citrate to layer 3 at about the time of pouring had any effect on the plaque count. Results are shown in Table XXXVII.

In Expt 1 quantities of 2M $CaCl_2$ were added to the tubes which were used for pouring layer 3. If controls from another experiment done the same day are used, *viz.* 123 (phage), 151 (+0.08 g alginate per 10 ml) results show a slight increase (phage), and about the same level of counts (phage plus alginate (2 x 0.04 g alginate per 10 ml)). Some of the plates were poor (No. 3, two plates each part precipitate; No. 5, slight precipitate one plate; No. 6, precipitate all over two plates). The experiment therefore does no more than indicate a possible slight improvement due to the presence of calcium with phage alone.

In Expt 2 mixtures were prepared in McCartney bottles with phage, using 1, 2 and 3 ml of 10% citrate and varied amounts of $CaCl_2$. The control count was slightly above 138. The results

Table XXXVII Effect on plaque count of inclusion of calcium and/or citrate in layer 3.

	EXPT 1			EXPT 2			EXPT 3				
	Addition of $CaCl_2$ to tubes for pouring layer 3			Addition of citrate and calcium to McCartney bottles (no alg.)			Addition of calcium/citrate to layer-3 tubes before pouring				
No.	Ca alg. .04g	2M $CaCl_2$ ml	Plaque counts Av.of2	% Sod. Cit.	2M $CaCl_2$ ml	Plaque counts Av.of3	Ca alg. .04g	Ca ppm	% Sod. Cit.	Cult. ml	Plaque counts Av.of2
1	0	0.01	157	0	0	>138	0	0	0	0.5	125
2	0	0.05	135	1	0	175	0	140	0	"	168
3	0	0.1	138*	2	0	142	0	0	0.034	"	96
4	2	0.01	126	3	0	153	0	140	"	"	196
5	2	0.05	160*	1	1	175	0	0	0	0.2	107
6	2	0.1	95*	2	1	223	0	148	0	"	112
7	0	0	123	3	1	195	0	0	0.036	"	116
8	2	0	151	1	5	205	0	148	"	"	98
9				2	5	187	2	0	0	"	206
10				3	5	192	2	148	0.036	"	191
11				1	6	207					
12				2	6	193					
13				3	6	207					

* Precipitate on some of these plates

Composition of each layer in Table, unless stated otherwise

L1 HA-P 5ml
L2 (HA+P 5ml (Ca 600ppm
L3 (SA+P 5ml (Cult. 0.5ml
L4 (HA+P 2.5ml (Ca 600ppm

indicated that citrate up to 3 ml 10% per 10 ml of phage mixture could be used, showing that with the four-layer method this amount of citrate did not cause problems due to complexing of the calcium. Addition of calcium (as $CaCl_2$) raised counts somewhat, whether compared with No. 1 control or Nos. 2-4 (citrate additions without calcium).

In Expt 3 calcium and citrate were added to the mixture before pouring layer 3. Plate appearance was as follows: Nos. 1-4, mostly grade 1; Nos. 5-8, mostly about grade 2; and Nos. 9-10, grade 1-2. For phage alone results were inconclusive. Where alginate was present the counts were definitely higher than the controls; added calcium and citrate did not show any marked effect.

Small amounts of calcium (75 and 220 ppm) were added to Nos. 9-10 in an experiment shown in Table XXXIV. Counts of 121 and 147, respectively, were compared with control of 123 (148 on a fairly good plate). Therefore no conclusive effect was demonstrated. (Culture 0.2 ml.)

The overall picture was that citrate added to layer 3 had no demonstrable effect, and that calcium added to this layer appeared to sometimes moderately increase the counts.

(iv) CULTURES

The Effect on Plaque Counts of the Method of Preparation of the Cultures used for Inoculating Plates

During the course of this investigation it was noted that one day, and again two days later, abnormally low plaque counts were obtained, although on the day between typical counts were obtained. The variable counts observed raised a question as to whether the broth culture in use (small laboratory) for this study was satisfactory.

It was compared with the broth culture used in the main bacteriological laboratory of the Institute. A striking difference in results was obtained. As is shown in Table XXXVIII, Expt 1, the count given by the main laboratory culture was two to four times

Table XXXVIII The effect on plaque counts of the method of preparation of the broth used for inoculating plates.

EXPT 1

Comparison of small lab. and main lab. cultures

	L3 Cult. ml	L3 SA +P ml	Plaque count Av. of 2 Small lab.	Plaque count Av. of 2 Main lab.
1	0.2	5	111	209
2	"	"	127	200
3	"	2.5	68	157
4	"	"	88	153
5	0.5	5	67	242
6	"	"	76	228
7	"	2.5	40	177
8	"	"	38	151
9	0.2	5	57	127(1)
10	"	"	42	136
11	"	2.5	25	133
12	"	"	26	91
13	0.5	5	23	146
14	"	"	29	161
15	"	2.5	29	111
16	"	"	45	94

L2 & L4 Ca ppm: 6000 — 1-8, 17-24; 2000 — 9-16, 25-32

Additions to L3 (even Nos.) were made to give approx. 140-280ppm Ca & 0.03%-0.07% sod. cit. These did not make much difference.

Nos. 1-16 correspond (small lab. / Nos. 17-32 (main lab.

To 1 tube lab. cult. added 2.5ml broth.

Plates were grades 1, 1-3, 3.

EXPT 2

Comparison of small lab. and main lab. cultures

Source of culture	L3 Inoc. ml	Plaque count Av. of 4
Small lab.	0.2	112
"	0.5	96
Main lab.	0.2	201
"	0.5	257

Expt 2 plates grades 1 and 1-2

EXPT 3

Comparison broths and milks for inoculating experimental broths

	Broths for expt: Inoculation 1 day before expt with: Small lab. culture	Then incub.	Plaque count Av. of 2
	1 drop of broth	22°C (-3 h 4°C)	148
	5 "	" "	140
	1 " milk	" "	41
	5 "	" "	47
	1 " broth	22°C	200
	5 "	"	191
	1 " milk	"	62
	5 "	"	38
	Main lab. culture		
	1 drop of broth	22°C (-3 h 4°C)	105
	5 "	" "	153
	1 " milk	" "	46
	5 "	" "	35
	1 " broth	22°C	146
	5 "	"	238
	1 " milk	"	63
	5 "	"	59

0.5 ml culture in L3

(cont'd)

Table XXXVIII (cont'd)

EXPT 4

Comparison of small lab. and main lab. cultures

No.	(Small lab. broth inoc. from milk) Broth	0.04 g alg.	Inoc. ml	Plaque count Av. of 3
1	Small lab.	0	0.2	136
2	"	0	0.5	121
3	"	x2	0.2	160
4	"	x2	0.5	156
5	Main lab.	0	0.2	244
6	"	0	0.5	265
7	"	x2	0.2	254
8	"	x2	0.5	263

Method (1) for Expts 2-5 detailed on p. in text, but amount of culture varied in expts.

EXPT 5

Comparison broths and milks for inoculating experimental broths

	Inoc.	From small lab.	Amt. ml	L3 cult. ml	Plaque count Av. of 3
	10.15am	Broth	0.05	0.2	189
	"	"	"	0.5	196
	"	"	0.3	0.2	220
	"	"	"	0.5	253
	"	Milk	0.05	0.2	82
	"	"	"	0.5	62
	"	"	0.3	0.2	97
	"	"	"	0.5	107
9	5.15pm	Broth	0.05	0.2	194
10	"	"	"	0.5	197
11	"	"	0.3	0.2	198
12	"	"	"	0.5	190
13	"	Milk	0.05	0.2	117
14	"	"	"	0.5	81
15	"	"	0.3	0.2	122
16	"	"	"	0.5	94
17	Main lab. broth (from milk)			0.2	144
18				0.5	137

EXPT 6

Comparison of broth cultures - few and many transfers in broth

Transfers	Method	Cult. ml*	Plaque count Av. of 3
Many	(1)	0.3	128
Few	(1)	"	127
Many	(2)	"	170
Few	(2)	"	174

(Methods 1 & 2 detailed on p. 147)

* The amount of culture in L3 is 0.1ml more than prescribed in methods (1) and (2).

higher than that given by the small laboratory culture. These results led to further investigation of the matter.

The method of investigation was as follows: a standard phage dilution was prepared, and counts were determined (generally by the four-layer method) using the experimental broth cultures for inoculating the soft agar layer. Various points, such as source of inoculum for cultures, size of inoculum, time of incubation, etc. were studied. The layers were as follows:

L1	HA-P	5ml	
L2	HA+P	5ml	calcium 6,000 ppm
L3	SA+P	5ml	culture 0.2ml, phage suspension 0.1ml
L4	HA+P	2.5ml	calcium 6,000 ppm

Any deviations from these quantities are noted under individual experiments.

The results (Table XXXVIII) of this comparative study are commented upon, in chronological order, as follows:

Reference has already been made to Expt 1 where the outstanding fact noted was that the main laboratory broth culture gave much higher plaque counts for a given phage dilution than the small laboratory broth culture. (In this experiment other factors were also tested, viz.: 6,000 ppm calcium characteristically gave higher counts than 2,000 ppm calcium; 5 ml agar medium in layer 3 generally gave higher counts than 2.5 ml; for inoculation sometimes 0.5 ml gave higher counts than 0.2 ml and sometimes the reverse occurred.)

In Expt 2 the small laboratory culture was prepared by inoculating broth with milk culture: the main laboratory culture was as usual prepared by inoculating broth with a broth culture. The experiment showed that either there was an inherent difference between the cultures or that a difference in propagation was responsible for the effect on plaque counts.

About this time it was realized that the main laboratory broth cultures were regularly prepared by inoculation from broth cultures, whereas the small laboratory cultures were often (or characteristically) inoculated from milk cultures. It appeared that the latter method

could be the cause of the low plaque counts but this, of course, required confirmation.

An experiment was set up to compare broths inoculated from milk cultures and broth cultures, respectively (Expt 3). The results of this experiment showed a striking difference between the broths inoculated from broth, which gave high plaque counts, and the broths inoculated from milk, which gave low counts. The results were not affected by whether the culture for inoculating broths was from the small laboratory or main laboratory. There was no consistent difference between results for broths inoculated with one drop or five drops of culture.

The foregoing results were confirmed in Expt 4 where the main laboratory broth (inoculated broth-to-broth) again gave counts much greater than the small laboratory broth (inoculated milk-to-broth).

Further confirmation was obtained from Expt 5 where broth-to-broth inoculation of experimental tubes gave higher counts than milk-to-broth inoculations. In this experiment inoculations into the experimental broths were made using 0.05 ml and 0.3 ml from broth and from milk both in the morning and again late in the afternoon. The plates for counting were prepared using 0.2 ml and 0.5 ml inoculum in layer 3. Higher counts were obtained with 0.3 ml than with 0.05 ml for inoculation of experimental broths in the morning, but different volumes used for inoculation in the late afternoon did not seem to make much difference. There was no consistent difference in the counts between 0.2 ml and 0.5 ml used in layer 3, but 0.5 ml gave smaller plaques than did 0.2 ml.

In Expt 6 comparisons were made between cultures which had been cultured many times, and few times, respectively, in broth after inoculation from milk. Both cultures gave the same results but method two gave higher counts than method one.

Summary

As some low plaque counts were obtained in certain experiments, comparisons were made between the small laboratory culture and the main laboratory culture. A wide discrepancy was found between the resultant counts. The explanation was thought to reside in the fact that the small laboratory culture was inoculated from a milk

culture on many occasions, whereas the main laboratory culture was regularly inoculated from a broth culture, except on those occasions when a freshly picked-off culture was put into circulation. Experiments proved that inoculation of experimental broth cultures with milk resulted in lower plaque counts than when broths were inoculated from broth cultures. Thus:

Culture used for inoculation		Culture for experiment (for inoculating plates)	Result
Broth	⟶	Broth	High counts
Milk	⟶	Broth	Low counts

Heat Treatment During Preparation of Broth Medium used for Cultures for Plaque Counts

In the three experiments reported in Table XXXIX broths for the culture propagation were subjected to different heat treatments in their preparation in order to find whether there was any subsequent effect on the plaque counts.

A four-layer method was used (method (1), p.147. In Expts 1 and 2 a culture recently revived from freeze-dried storage was compared with a stock small-laboratory culture (HP). Plaque count plates were prepared in the morning and in the afternoon.

Expt 1. Some of the autoclaved media were dark in colour. The results in lines 2-7 (a.m. and p.m.) were approximately the same, and plates were grade 1. Results showed no consistent specific effect due to heating. While plates corresponding to lines 9-14 were mostly below grade 1 in appearance, the results showed no consistent trend.

Expt 2. All a.m. plates were grade 1 and showed no consistent effect due to differences in heat treatment. The p.m. plates were mostly below grade 1.

Expt 3. Again, no specific differences were noted. All plates except one were grade 1 (poor plate count omitted from average).

Conclusion:

The results showed that major differences in heat treatment

Table XXXIX Effect of type and extent of heat treatment during preparation of broth medium used for cultures for plaque counts.

No.	Heat treatment of media	HP	EXPT 1 Plaque counts Av. of 2 a.m.	EXPT 1 Plaque counts Av. of 2 p.m.	EXPT 2 Plaque counts Av. of 2 a.m.	EXPT 2 Plaque counts Av. of 2 p.m.
1	St. ¼ h	Small lab.			215	218
2	" ½ h	"	207	222	215	168
3	" 1 h *	"	223	237	213	169
4	Au. 10 min	"	251	255		
5	" 20 min	"	221	229	271	204
6	" 40 min	"	254	195	254	241
7	**		259	222		
8	St. ¼ h	FD			234	139
9	" ½ h	"	199	172	245	142
10	" 1 h *	"	178	177	230	251
11	Au. 10 min	"	251	191		
12	" 20 min	"	232	195	227	235
13	" 40 min	"	211	203	208	205
14	**		228	192		

No.	EXPT 3	Av. of 3 a.m.	Av. of 3 p.m.
15	**	255	281
16	Au. 20 min	250	236(2)
17	" 40 min	238	247

* Steamed ½ h one day, ½ h after weekend (Expt 1)

** Media as normally prepared for laboratory purposes

N.B. Autoclave pressures not recorded - probably 15 lb/sq in.

during the preparation of broths for growth of cultures did not affect the subsequent plaque counts.

Effect on Plaque Count of Age, Temperature and Quantity of Inoculum in Culture Preparation or Use

Experiments were carried out to find to what extent the history or treatment of the culture used for phage replication affected the plaque counts (Table XL).

In Expt 1 analyses were carried out using cultures of varying ages inoculated from cultures of varying ages. Analyses were done at 9.00 a.m. and noon, in triplicate. The results showed that where the cultures used in plates were 40-51 h old the plaque counts were higher than where 16-27-h cultures were used. For 27-h cultures the history of inoculation did not make much difference.

Expt 2 was carried out to find whether experimental broths are affected by the age and amount of inoculum used in their preparation. These experimental broths were used at different times throughout a day. Results showed that higher plaque counts were often obtained with higher quantities of inoculum. The differences due to the quantities of inoculum were more marked when using the 48-h culture for preparing experimental tubes than when using the 24-h culture. The latter did not consistently show this effect.

In Expt 3 the experimental cultures were grown at 30°C, after inoculation with 0.1 ml, 0.3 ml and 1.0 ml quantities from 22°C cultures of various ages (see Table XL). An experimental culture at 22°C was also used. The results showed that the counts from experimental cultures grown at 22°C were higher than most of the other counts. Highest consistent counts were from 30°C culture inoculated with 1.0 ml of 30½-h, 22°C culture. There was therefore no advantage in growing the latter cultures at 30°C.

In Expt 4 investigation was made of the effect of temperature, age, and size of inoculum on plaque production. Experimental cultures, i.e. those to serve as the source of host bacteria in the third layer of the pour plates, kept at 30°C for 48 and 54 h gave decidedly lower counts than at 24 and 30 h, and lower counts than the corresponding cultures at 22°C at 48 and 54 h. Inoculations

Table XL Effect on plaque count of age, temperature and quantity of inoculum in culture preparation or use.

EXPT 1

Comparison of cultures of varied ages prepared from cultures of varied ages

	Age of culture for inoc. expt. culture h	Age of expt. culture (at analysis h	Plaque count Av. of 3
1	24	48	190
2	32	40	198
3	24	24	141
4	32	16	164
5	16	24	139
6	21	24	137
7	24	24	157
8	24	51	244
9	32	43	185
10	24	27	146
11	32	19	133
12	16	27	144
13	21	27	140
14	24	27	125
15			
16			
17			
18			

Plates good

Nature of layers on plates

Expts 1-4

L1 HA-P 5ml
L2 HA+P 5ml 6000ppm Ca
L3 SA+P 5ml 0.2ml cult., 0.1ml phage suspension
L4 HA+P 2.5ml 6000ppm Ca

EXPT 2

Effect of varying amounts and ages of cultures used in preparing experimental cultures. (Expt. cultures used at different ages.)

	* Age of expt. cult. h	24 h culture for inoc. expt. cult. ml	Plaque count Av. of 3	48 h culture for inoc. expt. cult. ml	Plaque count Av. of 3
1	24	0.1	128	0.1	97
2	"	0.3	99	0.3	176
3	"	1.0	133	1.0	236
4	25½	0.1	150	0.1	147
5	"	0.3	166	0.3	203
6	"	1.0	185	1.0	255
7	27	0.1	169	0.1	159
8	"	0.3	189	0.3	221
9	"	1.0	190	1.0	256
10	28½	0.1	153	0.1	189
11	"	0.3	169	0.3	210
12	"	1.0	166	1.0	249
13	30	0.1	171	0.1	189
14	"	0.3	189	0.3	234
15	"	1.0	163	1.0	282
16	31½	0.1	145	0.1	191
17	"	0.3	174	0.3	218
18	"	1.0	178	1.0	245

Plates good

* This column should logically be placed between the columns in both sets of figures.

EXPT 3

Effect on plaque counts of growth of cultures at 30°C

	Cultures for inoc. expt. tubes: Age h	Temp. grown °C	Amt. to expt. tube ml	Treatment of expt. cultures before use: Age h	Temp. grown °C	Plaque count Av. of 3
1	24½	22	0.1	24	30	111
2	"	"	0.3	"	"	89
3	"	"	1.0	"	"	106
4	30½	"	0.1	18	"	141
5	"	"	0.3	"	"	139
6	"	"	1.0	"	"	173
7	24	"	0.3	24½	22	164
8	As above respectively			26½	30	182
9				"	"	142
10				"	"	155
11				20½	"	210
12				"	"	180
13				"	"	232
14				27	22	206
15	As above respectively			29	30	205
16				"	"	164
17				"	"	151
18				23	"	214
				"	"	196
				"	"	227
				29½	22	221

Plates good

(cont'd)

Table XL (cont'd)

EXPT 4

Effect of temperature of incubation and age of experimental and previous cultures on plaque counts (including amount of inoculum for experimental cultures)

History of cultures before experimental cultures				Experimental cultures		Plaque counts (Av. of 3)			
						Age of experimental cultures at analysis h			
°C	h	°C	h	Amt. for inoc. ml	Incub. at °C	24	30	48	54
22	26 →	22	23½ →	0.3	22	130	107	175	154
				1.0	"	112	107	168	172(2)
				0.3	30	122	106	36	34
				1.0	"	144	142	43	23
30	26 →	30	23½ →	0.3	22	170	180	238	226
				1.0	"	186	194	242	226
				0.3	30	179	139	105	80
				1.0	"	148	151	69	62
			Plus 23½ same temp.	0.3	22	95	91	123	134
				1.0	"	153	153	200	158
				0.3	30	108	126	38	38
			(49½ h between inoculations)	1.0	"	139	152	38	18
			Plus 23½ same temp.	0.3	22	67	119	173	148
				1.0	"	67	123	162	187
				0.3	30	197	208	134	90
			(49½ h between inoculations)	1.0	"	175	233	112	109
						Plates good			

of 1.0 ml and 0.3 ml generally gave about the same results. Experimental cultures incubated at 22°C gave higher counts when 48 and 54 h old than when 24 and 30 h old. Highest counts were obtained at 48 and 54 h by subculturing and incubation at 30°C on two occasions before putting up experimental tubes at 22°C. High counts were also obtained at 24 and 30 h by subculturing and incubation at 30°C for two days before putting up experimental tubes at 30°C. In experimental tubes, at 22°C age improves count, and at 30°C age decreases count.

Conclusion:

Results show that the plaque count is markedly affected by the state of the culture used as host in pour plates. This can be influenced by the culture used for inoculation of the experimental tube, and by the age and temperature of incubation of the experimental tube.

The Effects on Plaque Counts of Cultures when Used in the Log Phase and Resting Phase

Cultures were investigated to find the influence on plaque counts of the use of cultures in the log and resting phases (Table XLI).

In Expt 1, cultures were compared at $3\frac{1}{2}$, $6\frac{1}{2}$ and 24 h of age. Results did not show any consistent advantage from using culture in the log phase.

In Expt 2, comparisons were made of cultures using small and large inocula. The results established that counts decreased to a minimum where the inoculum was 6-8 h old and then rose again when the culture was 24 h old. Due to variations in counts obtained with cultures up to 8 h old, it appears that a 24-h culture would be more satisfactory to use than a culture in the log phase.

In Expt 3, one-day culture was compared with culture $2\frac{1}{2}$ and $5\frac{1}{2}$ h old at time of use. (The one-day culture would be approximately $26\frac{1}{2}$ - $29\frac{1}{2}$ h old at time of analysis.) The day-old culture gave slightly higher counts than the young cultures. In this experiment, comparisons were also made between $CaCl_2$ medium with and without adjustment to pH 7.0. The counts on the adjusted medium were mostly

Table XLI Comparison of the effects on plaque counts from using cultures in the log phase and resting phase.

EXPT 1

Comparison of log phase and resting phase of culture

Plaque count method	Age expt. cult. h	Vol. expt. cult. ml	Plaque count Av.of3
(1)	3½	0.3	135
	"	1.0	143
(2)	"	0.3	161
	"	1.0	181
(3)	"	0.3	173
	"	1.0	201
(1)	6½	0.3	164
(2)	"	"	205
(3)	"	"	236
(1)	24	"	172
(2)	"	"	210
(3)	"	"	212

Plaque count methods in Expts 1, 2 and 3 are detailed on p.

EXPT 2

Comparison of log phase and resting phase of culture

Inoc.	Time from inoc. h	Method (1) Plaque count Av.of3	Method (2) Plaque count Av.of3
S	2	136	165
L	"	160	190(1)
S	3	147	171
L	"	146	161
S	4	131*	169
L	"	-	151
S	5	125	170
L	"	128	170
S	6	117	145
L	"	94	120
S	7	124	143
L	"	115	131
S	8	80	121
L	"	110	156
S	24	130	164
L	"	130	170

Small inoc. 1 ml to 24 ml
Large inoc. 1 ml to 9 ml

Culture in seed agar = 0.3 ml
Plates good

$CaCl_2$ media adjusted to pH 7.0

* 2½ ml agar

EXPT 3

Comparison of log phase and resting phase of cultures

Culture media Age		Method (1) Plaque count Av.of3	Method (2) Plaque count Av.of3
1 day	NA	107	149
"	A	104	141
2½ h	NA	129	148
"	A	91	138
1 day+3 h	NA	122	138
"	A	102	141
5½ h	NA	104	121
"	A	91	136

Plates good

NA = $CaCl_2$ media; pH not adjusted

A = $CaCl_2$ media; pH adjusted to 7.0

(young culture in Expt 3
(1 ml to 24 ml broth

lower than for the unadjusted medium. However, with the adjusted medium the results were more uniform, and this suggests that perhaps pH adjustment should have been investigated more extensively - lack of pH adjustment may account for some of the irregular results reported earlier in this work.

In an experiment shown in Table XLII plaque counts were determined with host culture of increasing age. Twenty-five LYP broth tubes were inoculated with 0.4 ml of strain HP in broth and at intervals these were used for triplicate estimation of phage in a standard phage suspension. On the second day a fresh phage suspension was prepared (comparisons were made with the previous suspension). Counts are averages of triplicate plates. All plates were of good appearance. Analyses were carried out up to 32 h. The results showed that counts rose steadily in the early hours after inoculation. Maximum results were obtained between 24 and 32 h.

Binocular examination of plates under low-power objectives showed a progressive increase in density of growth as age of culture increased. The extent of growth on plates steadied up between 8-24 h. The plaque count was somewhat related to number of bacteria on plates, and therefore to the number of bacteria in the culture. (See Fig 3.)

Conclusion:

According to this and the earlier experiments in this group the use of log phase would not be as suitable for securing maximum counts as are 24-h and older cultures.

Phage Estimations Over Prolonged Periods

The previous section has shown that in this investigation the culture used for plaque counts should be at least 24-32 h old. It was of importance to know whether even older cultures could be suitable, and in this section are presented results of a study of this matter, in graphical form. Methods are as p. 147, method (1), unless otherwise stated.

Table XLII Relationship between plaque count and age of host culture at time of plate inoculation; density of growth on plates.

Age of culture h	Plaque count *	Density of growth on plates	Age of culture h	Plaque count *	Plaque count **	Density of growth on plates
1	76	++	6½	139		+++
1½	96	++	7	145		
2	91	++	7½	155		++++
2½	95	++	8	153		++++
3	124		24	182	199	+++++
3½	117	++	25	177	197	+++++
4	118	++	26		192	+++++
4½	118		29		197	
5	114		30		196	+++++
5½	140	+++	31		210	
6	142		32		202	++++

Plaque counts by method (2) see p. 147

* Phage suspension prepared 1st day

** " " " 2nd "

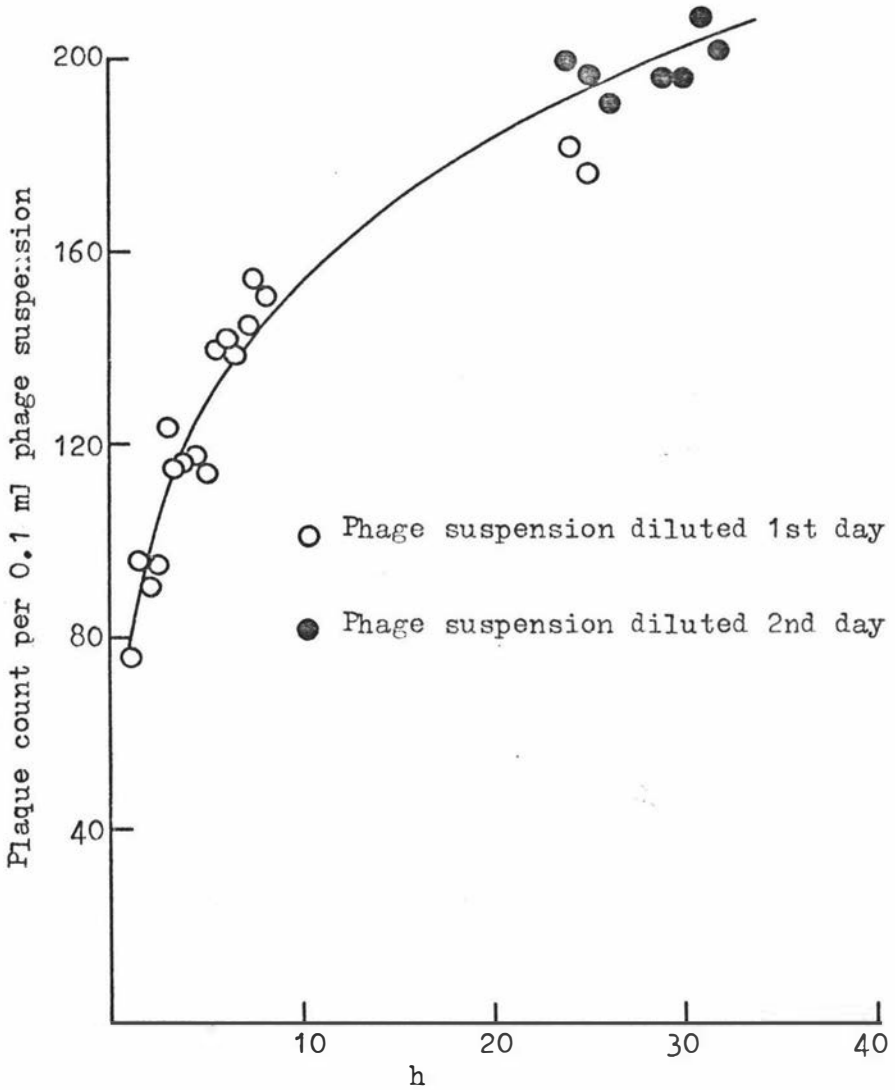

Fig 3. Relationship between plaque count and age of host culture at time of plate inoculation. Culture was grown at 22°C.

In Expt 1 (Fig 4) two flasks of broth were inoculated from broth and milk, respectively, and were subsequently incubated at 22°C. At intervals the flasks were shaken and a 10 ml quantity from each was pipetted into a test tube for use in phage estimation. The results (usually the average of triplicate counts) showed: (1) that broth inoculated from broth gave consistently higher counts than broth inoculated from milk (this further confirms experiments shown in Table XXXVIII; (2) that 0.2 ml of culture for phage estimation gave higher counts than did 0.5 ml; and (3) that low counts were obtained initially (at 12 h), these rose to a maximum between 24 and 39 h, and then fell off to very low counts when the culture was 62 h old.

Expt 2 was carried out to investigate variations in plaque counts over a long period, comparing:

(a) broth cultures which had been prepared by inoculating a flask of broth, mixing, and distributing to test tubes, and

(b) broth cultures which had been prepared by direct inoculation of test tubes of broth.

Results showed that maximum counts were obtained when the culture was between 24 and 53 h old. At 76½ and 95½ h, counts were relatively low. Plates were mostly grade 1 in appearance; results from poorer plates are identified in Fig 4 (Expt 2) by a circle. Most counts were averages of triplicate counts. There was fairly good agreement between the results for the two sets of cultures.

In Expt 3 cultures were put up by inoculating a flask (distributed to tubes) and tubes as in the previous experiment. All plates were grade 1. Results showed a maximum about 16-41¾ h. (Tube for inoculation of experimental broths was used late at night. It was thus about 13 h older than usual.)

Expt 4 was similar to the previous experiment. Maximum counts were obtained at 22½-48 h. All plates were grade 1.

The above four experiments in Fig 4 show results when a specific culture was used over a period of time, showing the effects of aging of culture.

In contrast, Fig 5 shows the results for Expt 5 in which fresh

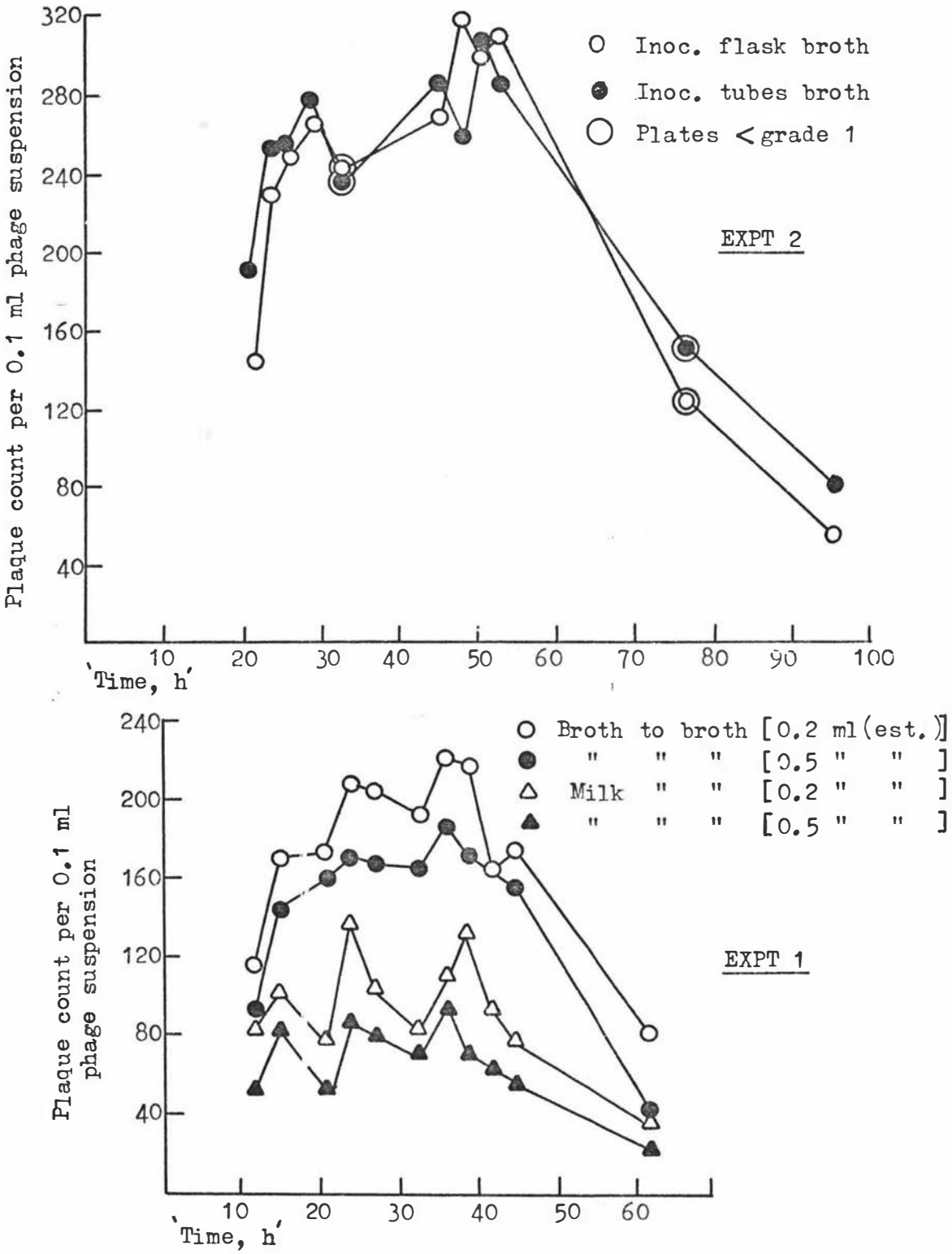

Fig. 4. Graphs showing the effects on plaque counts of using cultures over prolonged periods of time.

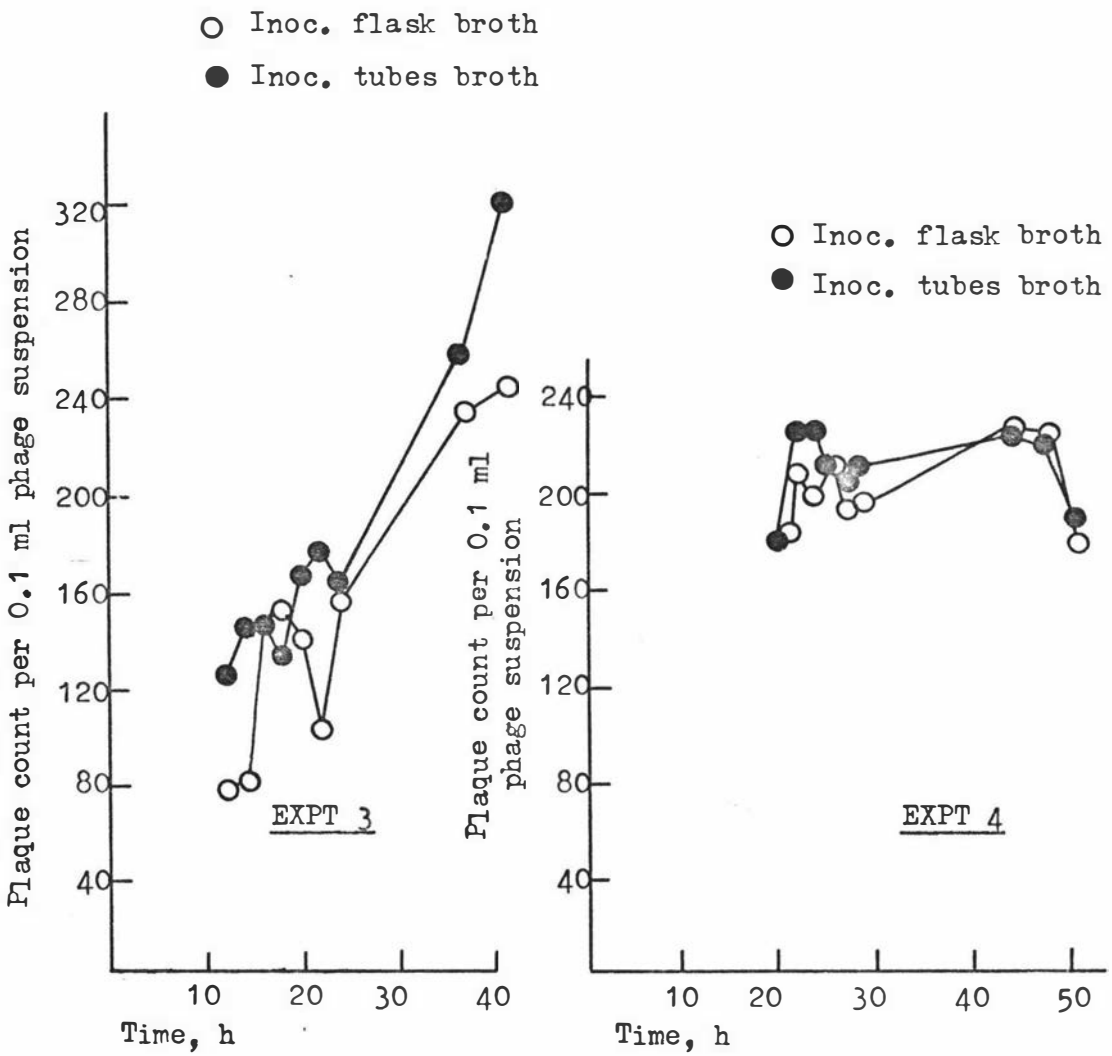
Inoc. flask broth
Inoc. tubes broth
Plaque count per 0.1 ml phage suspension
320
280
240
200
160
120
80
40
EXPT 3
10
20
30
40
Time, h
Inoc. flask broth
Inoc. tubes broth
Plaque count per 0.1 ml phage suspension
240
200
160
120
80
40
EXPT 4
10
20
30
40
50
Time, h

Fig 4. (cont'd)

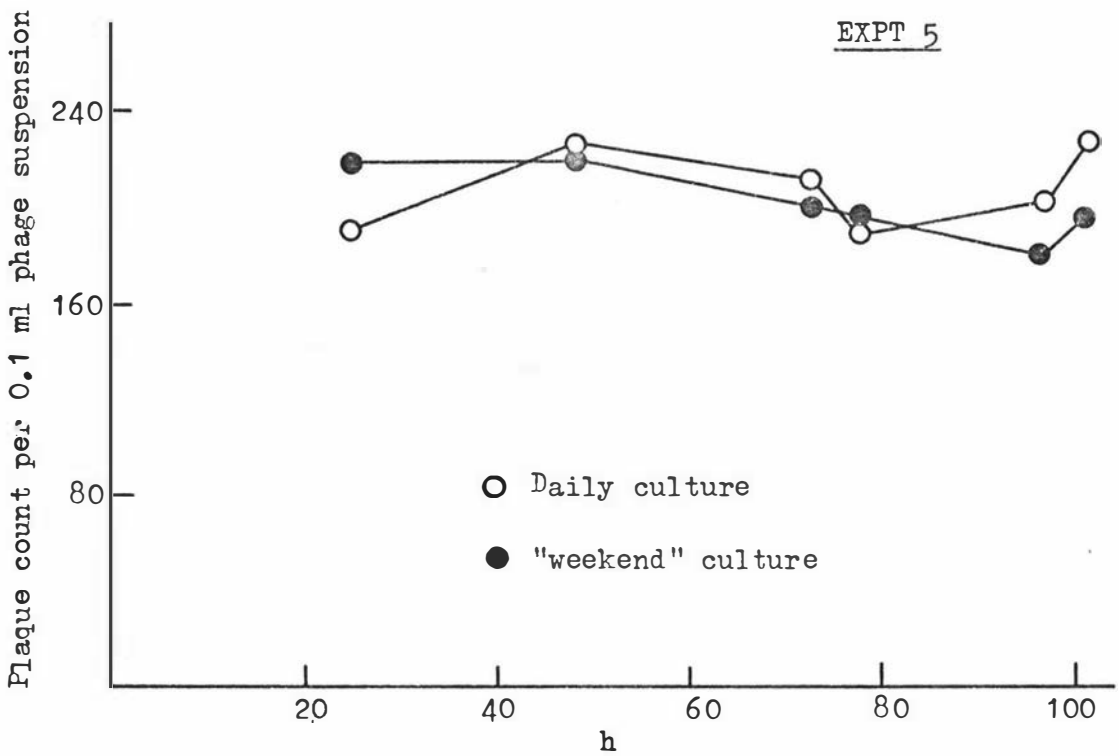

Fig 5. Plaque counts over three-day period using two cultures, one subcultured daily including weekend; and one subcultured daily but only once at weekend.

cultures ($25\frac{1}{2}$-30 h) were used each day. It thus shows the level of counts over an extended period of time. Cultures were from two sources, viz. a culture daily subcultured over the weekend, and a culture subcultured once at the weekend. For the remainder of the week both cultures were subcultured daily.

Each point in Fig 5 was the average of 15 estimations* as, concurrently with the above investigation, comparisons were made of cultures (room temperature) and phages (RT) with cultures ($30^{\circ}C$) and phages ($30^{\circ}C$) in cold tubes and in tubes at $45^{\circ}C$. (There was no consistent effect for the various temperatures, although the cold tube, culture (RT) and phage (RT) combination tended to give generally slightly higher counts than the combination: tube at $45^{\circ}C$, culture ($30^{\circ}C$) and phage ($30^{\circ}C$).)

Most plates were grade 1. Counts from a few plates under this grade were included, but would exert a negligible effect on results. Over a three-day period the average counts were reasonably consistent and even.

Conclusion:

Maximum phage counts are obtained with cultures between about 24 and 39 h old. Sometimes maximum counts are obtained with cultures up to about 50 h old.

Summary for Sub-section on Cultures

Efficiency of plating is greatly influenced by the culture and the presence or absence of certain cofactors. In this investigation it was necessary to explore widely certain conditions or factors which could influence the culture, so as to know the extent of such influences on plaque counts. While it might not be practicable to apply every condition which would tend to a maximum plaque count, it was necessary to know what conditions had a marked effect so as to establish an acceptable and somewhat standardized procedure for culture treatment. For example, in Expt 4, Table XL it was shown that highest plaque counts were obtained with experimental cultures

* Each 15 = average of usual triplicate and four combinations each in triplicate.

48-54 h old and grown at 22°C which had been inoculated from a culture twice subcultured at 30°C. For practical purposes, however, it was more convenient to use for routine analyses a culture propagated daily at 22°C, although this gives somewhat sub-optimal counts. It was necessary to inoculate broth culture media from broth cultures, as it had been shown that preparation of experimental broths from milk cultures gave considerably lower counts than the former. The logarithmic phase of growth was shown to be unsuitable for obtaining high plaque counts, and it was found to be important to use cultures which had been incubated at 22°C for some 24-39 h.

(v) FURTHER FACTORS AFFECTING PLAQUE COUNTS IN THE SOFT AGAR LAYER

A series of experiments was made to investigate the effects on plaque counts of temperature, order of mixing, and time of standing when mixing culture, phage suspension and soft agar medium.

In Table XLIII are shown the results of trials in which the components of the soft agar layer (culture, phage suspension and soft agar medium) were mixed at different temperatures and in different orders.

Two points required clarification:

(1) Would raising the temperature lessen the viscosity and allow greater contact of phage and bacteria and consequently give a higher count?

(2) During mixing of phage and culture, several phage particles might (in some cases) attach themselves to one organism. If the culture were diluted by mixing with agar before the addition of phage, would there be a more uniform distribution of phage particles amongst bacteria and consequently a higher plaque count?

In Expt 1 results showed that with layer components at 46°C and 49°C, culture mixed with agar before phage addition gave higher counts than culture and phage mixed together first. Counts at 49°C

Table XLIII Experiments showing the effects on plaque counts of mixing the components of layer 3 under various conditions.

EXPT 1

Effect on plaque counts of temperature, amount of culture, and order of mixing components of layer 3

	No.	Cult. ml	Phage ml	Soft agar ml		Plaque count Av.of3
46°C	1	0.2	0.1	5.0	Mixed as usual	128
	2	0.2	0.1	5.0	MWV	87
	3	0.4	"	"	"	63
	4	0.8	"	"	"	14
	5	0.2	")M	")M		134
	6	0.4	")W	")W		90
	7	0.8	")V	")V		41
		Soft agar)		Phage)		
	8	0.2	5.0)M	0.1)M		153
	9	0.4	")W	")W		135
	10	0.8	")V	")V		99
49°C	11	0.2	0.1	5.0	Mixed as usual	82
	12	0.2	"	")M		53
	13	0.4	"	")W		32
	14	0.8	"	")V		35
	15	0.2	")M	")M		55
	16	0.4	")W	")W		68
	17	0.8	")V	")V		43
		Soft agar)		Phage)		
	18	0.2	5.0)M	0.1)M		115
	19	0.4	")W	")W		99
	20	0.8	")V	")V		92

Cult., phage, agar & tubes at 46°C & 49°C for lines 1-10 & 11-20, respectively.
MWV = mixed with Vortex mixer, i.e. constituents of L3 to left of MWV in above Expt.

EXPT 2

Effect on plaque counts of temperature during preparation of layer 3. Comparison of cultures also

Cult.	Temp. agar °C	Temp. tube C=cold	Plaque count Av.of3
Daily*	45	C	187
Weekend**	45	C	210
Daily	55	C	233
"	50	C	218
"	47	C	233
"	45	C	233
"	55	45°	135
"	50	"	191
"	47	"	154
"	45	"	167
Weekend	55	C	205
"	50	C	220
"	47	C	206
"	45	C	216

Phage 30°C Culture 30°C
(Tube cold = room temp.
(Tube 45°C taken from bath
(before additions.
* Daily - broths were inoculated daily
** Weekend - broths were inoculated daily except at weekends.

EXPT 3

Effect on plaque counts of order of adding components of soft agar layer

(a)

Cult.	Order of mixing	Method	Plaque count Av.of3
Small lab.	CPAg	(1)	157
"	CAgP	"	185
"	CPAg	(2)	178
"	CAgP	"	162
"	CPAg	(Ord)	41
"	CAgP	"	36
Main lab.	CPAg	(1)	186
"	CAgP	"	163
"	CPAg	(2)	187
"	CAgP	"	196
"	CPAg	(Ord)	14
"	CAgP	"	5

C = culture
P = phage
Ag = agar
Ord method = 2 layers only and no Ca.

(b)

Order of mixing	Method	Plaque count Av.of3
CPAg	(1)	135
CAgP	"	137
CPAg	(2)	157
CAgP	"	191
CPAg	(Ord)	4
CAgP	"	2

Small lab. culture was used in (b).

were generally lower than at 46°C. Increasing the amount of culture generally caused a lowering in the count. Obviously lowered viscosity as a result of higher agar temperature does not need further consideration. Tube contents would be mixed more thoroughly by Vortex mixing than by the usual method of rolling the tube between the palms of the hands.

In Expt 2 phage and culture (both 30°C) were mixed in cold tubes (C. tubes) and in tubes taken from 45°C bath (45°C tubes) before the agar at various temperatures was added. Agar at a temperature as high as 55°C did not lower the count when added to the C. tubes, but counts were generally lowered when agar at various temperatures was added to phage and culture in tubes taken from the 45°C bath. In the case of the C. tubes apparently there would not be much harmful effect from the mixing of the phage and culture, and the cold tube would cool the agar somewhat. In the case of the tubes warmed to 45°C the reduction in count may have been caused by interaction of phage and culture in the warmed tube, aggravated perhaps by the temperature of the agar.

In Expt 3(a) the order of mixing: culture, phage, agar, and: culture, agar, phage, were compared. In methods (1) and (2) there was no marked difference, sometimes one sequence of mixing gave higher counts and sometimes the other.

In Expt 3(b) the order of mixing: culture, agar, phage was slightly better than: culture, phage, agar.

In further experiments, investigation was made to find the effect of temperature on cultures and phage either alone or mixed together. Culture alone, phage alone, or culture plus phage mixed together were kept at specific temperatures for various times before mixing with the other constituents of the soft agar layer.

Brief details of the individual experiments are given below.

Expt	Component	Holding temp.	Mixed at specific times with:	
1	phage	45°C	agar 45°C	culture 30°C
	culture	"	" "	phage 30°C
	culture + phage	"	" "	
2	culture + phage	"	" "	
	" " "	Room temp.	" "	
3	phage	49°C	" 49°C	culture 30°C
	culture	"	" "	phage 30°C
	culture + phage	"	" "	
4	culture + phage	Room temp.	" 45°C	
	" " "	" "	" "	

The results of these trials are presented graphically in Fig 6 and show that:

(1) Phage is fairly stable for 20 min at 45°C and 49°C.

(2) Culture gives progressively lower counts during a 20-min holding period at 45°C and markedly lower counts when held at 49°C for even a few min.

(3) The effects on plaque counts of holding mixtures of culture and phage under various conditions were as follows:

slight (room temperature for 10 min)
marked (45°C for 8 min)
extreme (49°C " 4 ").

Summary:

It appears that while a phage suspension is fairly stable when held by itself for a time even at relatively high temperatures (45°C and 49°C), a mixture of phage and culture tends to give a slightly diminished plaque count when held at room temperature and the decline in count is more rapid at higher temperatures. Phage and culture should therefore not be left long in contact before plates are poured. Mixing of culture with agar before the addition of phage tends to give slightly higher counts than mixing the phage and culture together before the addition of agar.

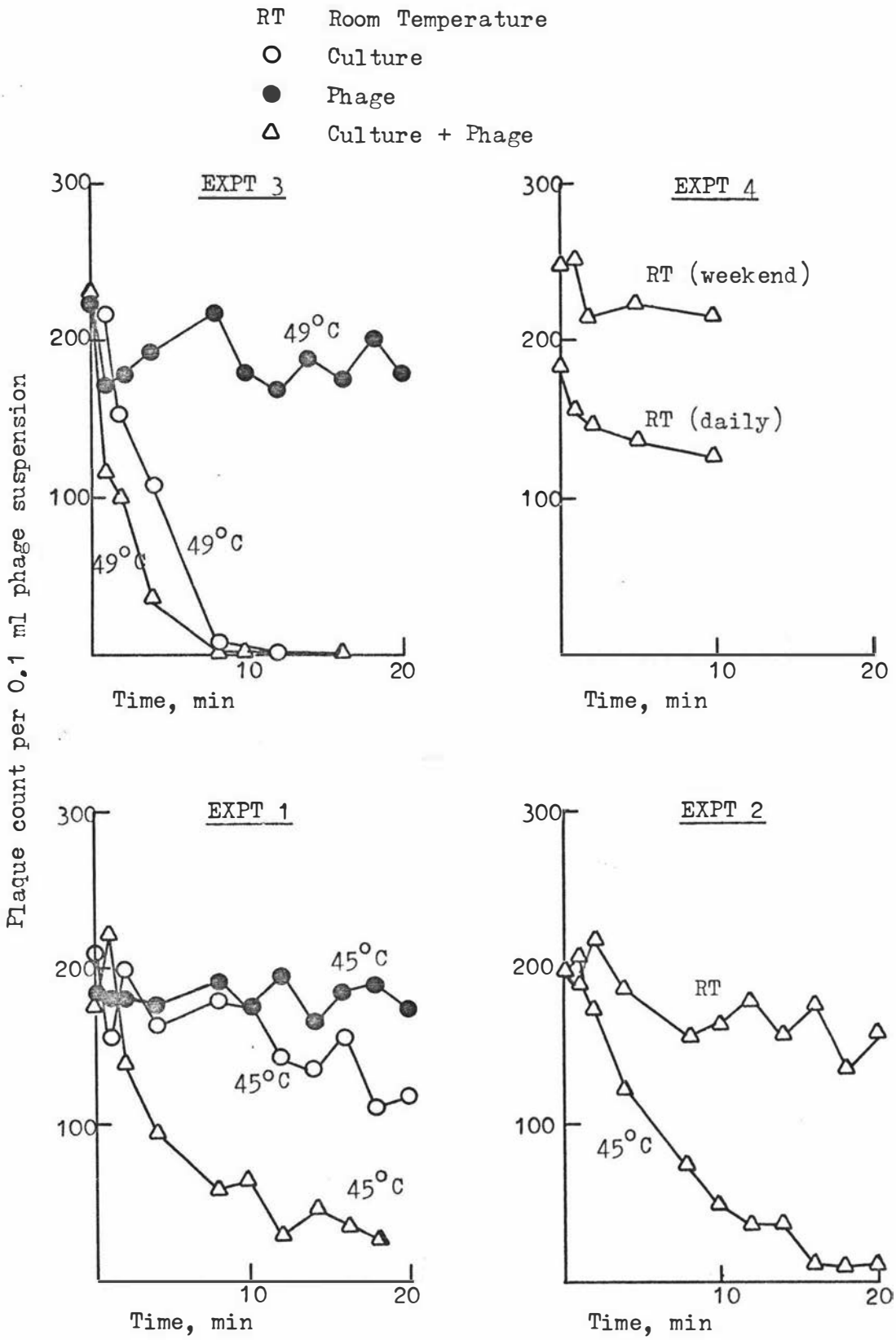

Fig 6. Graphs showing the effects on plaque counts of holding phage, culture, and mixture of phage and culture, at specific temperatures for various times before mixing with the other components of the soft agar layer.

(vi) THE USE OF TRYPTICASE SOY AGAR (TSA) IN MEDIA FOR PHAGE ESTIMATION

Introduction

Because another laboratory worker had found Trypticase Soy Agar (TSA) more satisfactory than Lactose Yeast Phosphate Agar (LYPA) for his particular investigation, it was decided to compare the merits of Trypticase Soy Agar and Lactose Yeast Phosphate Agar as media for phage estimation. The series of investigations made are discussed under appropriate headings.

Experiments with TSA in Layer 3

An initial experiment (Expt 1, Table XLIV) using TSA in place of LYPA was unsuccessful in that counts of 3 and 0 were obtained. In Expt 2 the use of soft TSA in place of soft LYPA in layer 3 was successful. In spite of some poor plates it could be deduced that calcium (2,000 ppm) could be added to layer 3 but not if two calcium alginate pads were present in phage suspension (Part 1). Two layers of TSA were not suitable with the particular amount of calcium tried, *viz.* 6,000 ppm (Part 2).

In Expt 3, 5 ml TSA in layer 3 gave higher counts than 5 ml LYPA, whereas 2.5 ml TSA gave a layer which was too thin.

In Expt 4 soft TSA seemed a suitable medium for layer 3, but 2.5 ml TSA was insufficient and gave unsatisfactory plates. TSA at 6-7.5 ml seemed a suitable quantity and gave higher counts than LYPA (5 ml) in layer 3. The use of 3 x 0.04 g pads of calcium alginate in citrate gave satisfactory results.

In Expt 5 ordinary LYPA counts were high on this occasion. While 1 x 0.04 g calcium alginate pad did not lower the count, 4 x 0.04 g pads lowered the result by about one-third. TSA gave about the same counts with all thicknesses of agar resulting from the use of 5-9 ml medium.

Conclusion:

TSA seemed suitable to use in layer 3 in quantities of about 5-9 ml. TSA gave higher counts than LYPA in layer 3. One or three

Table XLIV Experiments comparing Trypticase Soy Agar with Lactose Yeast Phosphate Agar in layer 3. Method (1), modified as appropriate, used throughout (0.2 ml culture replaced by 0.5 ml in Expt 1), except for one 2-layer trial.

EXPT 1 — Initial use of TSA in layer 3 (of 4-layer method)

No.	Ca alg. pads 0.04g	Vol. TSA in L3	Plaque count Av.of3
1	0	5ml	3
2	2	"	0
		Method (1)	
3	0	"	123
4	2	"	151

EXPT 2 (Part 1) — Replacement of soft LYPA (L3) in method (1) by soft TSA

No.	Ca alg. pads 0.04g	L3 Soft TSA Vol. ml	L3 Soft TSA Ca ppm	Plaque count Av.of2
5	0	5	-	168
6	2	"	2000	174
7	0	"	-	186
8	2	"	2000	36

(Part 2) Use of 2 TSA layers only

No.	L1 TSA Hard ml	L2 TSA Soft ml	Ca ppm	Plaque count Av.of2
9	0	5	0	2
10	0	"	6000	18
11	2	"	0	4
12	2	"	6000	28

Plates were grades: Part 1, 1,2,2-3,4 Part 2, 2,4

EXPT 3 — Comparison TSA and LYPA in layer 3

No.	Age medium LYPA	L3 Usual soft LYPA ml	Plaque count Av.of3
1	0	5.0	123
2	N	"	149
		TSA	
3	0	2.5	118
4	0	5.0	221
5	N	2.5	Itr
6	N	5.0	191

LYPA: 0 = Old (lab. stock); N = New (freshly made)

Where counts were recorded plates were grades 1, 2-3.

EXPT 4 — Comparison TSA and LYPA in layer 3

No.	Age medium LYPA	L3 TSA soft ml	Plaque count Av.of3
1	0	2.5	Itr
2	"	5.0	176(1)
3	"	6.0	209
4	"	7.5	182
5	N	2.5	NVC
6	"	5.0	210 (2)
7	"	6.0	203
8	"	7.5	200
	LYPA soft		
9	0	5.0	126
10	N	5.0	172
	TSA soft		
11	N	5.0	187
12	"	7.5	199

All counts recorded were from grade 1 plates.
Lines 11 & 12, 3 x 0.04g Ca alg. in 10 ml phage suspension.

LYPA: 0 = old agar; N = new agar (1-day old)
Ca agar 1-day old for 0 & N agars.

EXPT 5 — Comparison TSA and LYPA. Effect of pads when using TSA

No.	No. Ca alg. pads 0.04g	L3 Soft LYPA +Phos. ml	Plaque count Av.of3
1		5.0	238
2		5.0	231
		Soft TSA	
3		5.0	238
4		6.0	268
5		7.5	253
6		9.0	254
		ml	
7	1(.25)	5.0	255
8	1(1.0)	5.0	287
9	4(1.0)	5.0	181
10	1(.25)	7.5	273
11	1(1.0)	7.5	281
12	4(1.0)	7.5	181

In 1st column, amt. Cit. is shown in ().
All plates grade 1 approx. except 1 each in lines 3 & 7, respectively.
Lines 7,9,10,12 showed strands in plates (? fibres alginate).

calcium alginate pads could be used with TSA in layer 3, but four pads lowered the count by about one-third, when 5-7.5 ml TSA were used.

TSA in Various Layers Instead of LYPA

An experiment was carried out to investigate for a four-layer method the use of TSA in various layers instead of LYPA. (Table XLV.)

Comparisons made with daily culture showed:

(1) TSA (5.0, 7.5, 9.0 ml) in layer 3 gave higher counts than LYPA in layer 3;

(2) TSA in layers 1 and 3, and in layers 2, 3 and 4 gave good counts.

The counts for media containing TSA were slightly lower than for the average control count for daily and weekend cultures.

Experiments with Single-layer TSA Medium

Experiments reported in Table XLVI were designed to find whether a single-layer TSA medium could be prepared in a way suitable for phage estimation in place of multilayer LYPA or multilayer LYPA (TSA layer 3).

In Expt 1 TSA with various calcium additions (0-10,000) was used as a single-layer medium, using phage, and phage plus three pads of calcium alginate. Many of the plates had a glazed appearance. None of the plaque counts was as high as on the control plates.

In Expt 2 some smaller amounts of calcium were added than in Expt 1, with 2,000 ppm added calcium giving highest counts, but, using the one-layer method, counts were always lower than for controls. Counts were lower with calcium alginate than without it. The one-layer method was not satisfactory.

In Expt 3 pH values of the one-layer medium were adjusted to 6.9 when they were below this figure. The adjustment of pH values to 6.9 of the medium containing 6,000 and 10,000 ppm added calcium

Table XLV TSA in various layers instead of LYPA.

No.	Culture	L1 LYPA H-P ml	L2 LYPA H+P ml	L3 LYPA S+P ml	L4 LYPA H+P ml	Plaque count Av.of3	No. of Plates Grade 1	
1	Daily *	5.0	5.0	5.0	2.5	205	2	One plate sl. area "clear" & ppt. centre, but clear plaques.
2	Weekend*	"	"	"	"	275	1	One sl. ppt. & "clear" area, & one plate part sl. "clear".
				TSA(S)				
3	Daily	"	"	5.0	"	222	3	One plate part "clear" area.
4	"	"	"	7.5	"	235	3	
5	"	"	"	9.0	"	233	3	Each plate had some plaques not quite clear.
		TSA(H)						
6	"	5.0	"	7.5	"	222	3	
			TSA(H)		TSA(H)			
7	"	"	5.0	"	2.5	207	3	
		LYPA(-P)						
8	"	5.0	"	"	"	223	3	On all a few plaques sl. cloudy.

Layers 2 & 4 had Ca 6000 ppm (0.15M), calculated on amount added.

(TSA 6000 ppm Ca (0.15M) → ppt. and filtered
(Ca calculated on amount added.

H = hard S = soft * key on p. 126.

Table XLVI Experiments with one layer TSA medium.

EXPT 1

Comparison of counts given by one-layer calcium-treated TSA and four-layer methods.

No.	Phage Addition	Method			Plaque count Av. of 3
1	Phage	Usual 4-layer method (1)			608
		Layer 1 TSA (10ml)	Ca ppm	$CaCl_2$ M	
2	"	"	0	0.0	1
3	"	"	2000	0.05	130
4	"	"	4000	0.1	99
5	"	"	<6000	<0.15	15
6	"	"	8000	0.2	0
7	"	"	10000	0.25	0
	Phage +3 pads	Layer 1 TSA (10ml)	Ca ppm	$CaCl_2$ M	
8	"	"	0	0.0	2
9	"	"	2000	0.05	113
10	"	"	4000	0.1	Itr
11	"	"	<6000	<0.15	0
12	"	"	8000	0.2	0
13	"	"	10000	0.25	0
		4-layer method (2)			
	Phage	7.5ml TSA in L3			
14	"	"			233
	Phage +3 pads				
15	"	"			236

Results No. 1 apparently incorrect.

Nos.	Grade plates
1,14,15	1
2(glazed),3,4,8,9	<1
5,6,7,10,11,12,13	glazed appearance

TSA + $CaCl_2$ (filtered).

(cont'd)

Table XLVI (cont'd)

EXPT 2

One-layer calcium-treated TSA (including small amounts calcium) for phage estimations.

No.	Phage Addition	Method	TSA(H) 10 ml	Ca ppm	$CaCl_2$ M	Plaque count Av.of3	Plates Grade	Plates No. in Grade
1	Phage	(1)				221	1	(3)
2	"	(2)				228	1	(3)
3		"	"	0	0.0	0	1	(3)
4		"	"	200	0.005	34	1	(3)
5		"	"	500	0.013	46	<1	(3)
6		"	"	1000	0.025	113	1	(3)
7		"	"	2000	0.05	136	1	(3)
8		"	"	<6000	<0.15	64	Poor	(3)
9		"	"	10000	0.25	0	"	(3)
10	Phage +3 pads	"	"	0	0.0	1	1	(3)
11	"	"	"	200	0.005	26	about 1	(3)
12	"	"	"	500	0.013	24	<1	(3)
13	"	"	"	1000	0.025	88	about 1	(3)
14	"	"	"	2000	0.05	98	about 1	(3)

Nos. 1,2, 0.2 ml culture. Nos. 3-14, 0.4 ml culture, in contrast to 0.2 ml in Expt 1.

Plates 3-14 were faint; they were not as good as 1 & 2.

A few plaques in addition to above Nos. showed up by hand lens.

No. 14, all plates showed strands.

EXPT 3

One-layer calcium-treated TSA (including small amounts calcium) for phage estimations. pHs adjusted.

No.	pH	Method	TSA(H) 10 ml	Ca ppm	$CaCl_2$ M	Plaque count Av.of3	Plates Grade	Plates No. in Grade
1		(1) 4-layer				194	1	(2)
2		(2) "				223	Poor	(1)
3		(3) L1 & L2 combined				237	1	(3)
4	6.9	"	"	0	0.0	0	1	(3)
5	6.9	"	"	200	0.005	15	1	(3)
6	6.7→6.9	"	"	500	0.013	28	1	(3)
7	6.9	"	"	1000	0.025	115	1	(3)
8	6.3→6.9	"	"	2000	0.05	136	1	(3)
9	5.9→6.9	"	"	6000	0.15	101	<1	(3)
10	5.6→6.9	"	"	10000	0.25	37	Poor	(3)

Nos. 1-3, 0.2 ml culture. Nos. 4-10, 0.4 ml culture.

In No. 3, method (3), layers 1 & 2 were combined, using TSA(H), 6000 ppm Ca, filtered, 10 ml.

Next layer, 7.5 ml TSA-soft.

Top layer, 2.5 ml LYPA(H), Ca added 6000 ppm, filtered.

improved counts somewhat compared with the equivalent unadjusted medium in Expt 2. Nevertheless, all counts were well below controls, and the method was considered unsatisfactory.

Conclusion:

A medium comprising only a single layer of TSA with a range of added calcium from 0-10,000 ppm, with or without pH adjustment, proved quite unsatisfactory for phage estimations.

Attempts to Replace Two Underlayers of LYPA (Layers 1 and 2) of Method (2) by One TSA Layer

A series of experiments was carried out to investigate the replacement of the two underlayers of LYPA (layers 1 and 2) of method (2) by one TSA layer.

In Expt 1 (Table XLVII) 10 ml TSA were better than 5 ml in this single underlayer. Unfiltered media gave higher counts than filtered.

In Expt 2, 5, 10 and 15 ml volumes of TSA, calcium 6,000 ppm, filtered and unfiltered, were tried in the bottom layer. Variations were also made in the top layer. Results showed that a layer of LYPA(H) plus calcium 2.5 ml was needed on top of the culture-phage layer and that 5-10 ml TSA medium was a suitable quantity for the bottom layer where there was an LYPA top layer.

Expt 3 was carried out to find the effect of calcium alginate pads on counts when using modified method (2) in which layers 1 and 2 were replaced by 10 ml TSA(H), 6,000 ppm calcium. Results showed that 1 and 2 pads reduced the count slightly, and that 3 and 4 pads definitely reduced the count. (There was an anomaly with 2 pads, 0.5 ml citrate.)

Expt 4. A top layer was needed, and LYPA (+ calcium) was better than TSA (+ calcium) for this layer. In the section using a top layer of LYPA (+ calcium) 5 ml in the bottom layer gave higher counts than 10 or 15 ml.

Expt 5. Results showed that where 1, 2 and 3 pads were included satisfactory counts were obtained with 1 ml citrate

Table XLVII Attempts to replace two underlayers of LYPA (layers 1 and 2) of method (2) by one TSA layer.

EXPT 1

One layer TSA(H) replaced layers 1 & 2 of method (2).

Effect on plaque counts of quantity of TSA.

No.	Method					Plaque count Av. of 3
1	(1)					136
2	(2)					176
	TSA(10ml)		Ca ppm	$CaCl_2$ M		
(3	"	"	6000	0.15	F	161
(4	"	"	"	"	U	174
(5	"	5	"	"	F	127
(6	"	"	"	"	U	169

EXPT 3

One layer TSA(H) replaced layers 1 & 2 of method (2).

Effect on plaque counts of alginate pads.

	Method			Plaque count Av. of 3
1	(1)			146
2	(2)			211
3	(3)			199
	Pads	Cit. (ml)	Method (3)*	
4	1	1	"	182
5	2	"	"	174
6	3	"	"	149
7	4	"	"	137
8	1	0.25	"	184
9	2	0.5	"	128
10	3	0.75	"	159

EXPT 2

One layer TSA(H) replaced layers 1 & 2 of method (2).

Effect on plaque counts of presence or absence of a top layer.

No.	Method					Plaque count Av. of 3	
1	(1)					212	
2	(2)					243	
	TSA(H) ml		Ca ppm	$CaCl_2$ M			
3	"	5	6000	0.15	F	259	
4	"	"	"	"	U	242	
5	"	10	"	"	F	250	
6	"	"	"	"	U	247	
7	"	15	"	"	F	242	
8	"	"	"	"	U	232	
9	"	5	"	"	F	187	
10	"	"	"	"	U	191	
11	"	10	"	"	F	204	
12	"	"	"	"	U	213	
13	"	15	"	"	F	205	
14	"	"	"	"	U	184	
						With top	Without top
15	"	5	"	"	F	202(1)	198(2)
16	"	"	"	"	U	236(1)	196(2)
17	"	10	"	"	F	215(1)	182(2)
18	"	"	"	"	U	200(1)	187(2)
19	"	15	"	"	F	206(1)	190(2)
20	"	"	"	"	U	183(1)	191(2)

(cont'd)

Table XLVII (cont'd)

EXPT 4

One layer TSA(H) replaced layers 1 & 2 of method (2). Effects of (a) various quantities of TSA in bottom layer; (b) presence or absence of a layer above the phage-culture layer.

No.	Method		Plaque count Av. of 3
1	(1)		157
2	(2)		199
	L1 TSA(H) Ca 6000 ppm $CaCl_2$ 0.15M	Top layer (2.5 ml) LYPA(H)+Phos. Ca 6000 ppm $CaCl_2$ 0.15M	
	ml		
3	5	"	233
4	10	"	201
5	15	"	210
6	5	Nil	157
7	10	"	182
8	15	"	189
	TSA(H) 2.5ml Ca 6000 ppm $CaCl_2$ 0.15M		
9	5	"	188
10	10	"	180
11	15	"	180

EXPT 5

One layer TSA(H) replaced layers 1 & 2 of method (2). Effect on plaque counts of alginate pads.

No.	Method			Plaque count Av. of 3
1	(1)			185
2	(2)			213
3	(3)*			239
	Pads	Cit. ml	Method (3)*	
4	1	1	"	229
5	2	"	"	205
6	3	"	"	213
7	4	"	"	147
8	1	0.25	"	216
9	2	0.5	"	219
10	3	0.75	"	161
11	4	1.0	"	170

(cont'd)

Table XLVII (cont'd)

EXPT 6

One layer TSA(H) replaced layers 1 & 2 of method (2). Effect on counts of increased amounts of citrate for dissolving pads.

No.	Method			Plaque count Av. of 3
1	(1)			166
2	(2)			198
3	(3)*			215
	Pads	Cit. ml	Method (3)*	
4	1	1.0	"	215
5	2	"	"	186
6	3	"	"	172
7	4	"	"	154
8	1	1.5	"	223
9	2	"	"	206
10	3	"	"	201
11	4	"	"	147
12	1	2.0	"	253(2)
13	2	"	"	213(2)
14	3	"	"	220(2)
15	4	"	"	112(2)

EXPT 7

One layer TSA(H) replaced layers 1 & 2 of method (2). To find optimum quantity of medium in TSA bottom layer.

No.	Method		Plaque counts Next day PM	AM	PM
1	(1)		116	152	277
2	(2)		180	180	246
3	(3)*		180	210	236
4	TSA(H)	5.0ml	145	214	176
5	"	7.5"	165	204	184
6	"	10.0"	169	203	175
7	"	12.5"	181	247	168
8	"	15.0"	147	230	164

(cont'd)

Comments on Table XLVII

Expt 1 Plates all grade 1.

Expt 2 Top layer (H)

Nos. 3-8 LYPA(+Phos.) 2.5 ml Ca 6000 ppm (0.15M)

" 9-14 Nil

" 15-20 TSA 2.5 ml Ca 6000 ppm (0.15M)

All plates grade 1 except Nos. 10,12,14,16,18,20.

These had speckled ppt.

F = filtered U = unfiltered

Expt 3 All plates grade 1 except line 1 which were grade 1-2.

* = Method (3) anticipated, as detailed on p. 147, i.e. layers 1 and 2 of method (2) replaced by 10 ml TSA(H), Ca 6000 ppm (0.15M). Each pad = 0.04 g.

Expt 4 Plates grade 1 except following <1. No. 1 (2 plates); No. 6 (2 plates); No. 7 (1 plate); No. 8 (1 plate); also No. 7 (1 plate grade 3).

Top layer = layer above phage-culture layer.

Expt 5 All plates grade 1.

Expt 6 Plates grade 1 except No. 1 (3 plates grade <1)
No. 9 (3 " " 2-3)
No.12 (1 " " 2-3).

Expt 7 Grade of plates, G = grade

PM Of controls, 4 were G1 and 5 were <G1 to part cloudy.
Nos. 4-8, many plates part cloudy, and some impossible to count. Some were G1.

AM next day All control plates G1.
Nos. 4-8, of 30 plates, 26 were G1, 1 was G2-3, and 3 were impossible to count.

PM next day All control plates G1.
Nos. 4-8, plates <G1, except 2 G1.
Culture 0.2 ml AM, 0.3 ml AM and PM of next day.
Because of poor plates there would be inaccuracies in counts recorded, especially first column PM.

present, but that 4 pads gave low counts. With proportionate amounts of citrate incorporated 1 and 2 pads were satisfactory, but 3 and 4 pads gave low counts.

Expt 6. Counts increased slightly with larger amounts of citrate for 1, 2 and 3 pads. Four pads gave low counts and the counts decreased with larger amounts of citrate. At 1 ml citrate, fairly similar counts were obtained for 1 and 2 pads, were low for 3 pads, and were lower still for 4 pads.

Expt 7. In this experiment an attempt was made to finalize the quantity of TSA required in the bottom layer to give best results. To this end, six plates were poured with each volume of medium, and experiments were done one afternoon, the next morning, and the afternoon of that same day. Control counts were done in triplicate. There would sometimes be averages of less than six plates because some were impossible to count.

There did not appear to be any particular amount of TSA medium in layer 1 which consistently gave a high count.

In this experiment method (2) was used but with varying amounts of TSA(H) in bottom layer (5-15 ml), calcium 6,000 ppm, in place of LYPA layers 1 and 2.

Conclusions:

Layers 1 (LYPA-Phos.) and 2 (LYPA+Phos., calcium) of method (2) could be replaced by a single layer of TSA (calcium). Although a specific quantity consistently giving maximum counts was not found, it was decided that a volume of 10 ml would be appropriate. For satisfactory counts with TSA (calcium) underlayer, a 2.5 ml LYPA (calcium) layer was needed on top of the culture layer.

Using 10 ml TSA (calcium) in the bottom layer, 1 and 2 calcium alginate pads could be used with reasonable accuracy.

Further Experiments Investigating the Effect on Plaque Counts of Calcium Alginate Pads and Sodium Citrate when TSA was Included in the Medium

In Expt 1 (Table XLVIII) methods (2) and (3) were tried extensively with 1, 3 and 4 pads, and 1, 2 and 3 ml citrate, in A.M. and P.M. Results showed that with 1 pad present the count was approximately the same as control, 3 pads somewhat depressed the count, and 4 pads very much reduced the plaque count. The amount of citrate did not have much effect on the count with 1 and 3 pads present, but with 4 pads 2 and 3 ml citrate brought the counts down greatly in some cases.

In Expt 2 using method (2), 1 and 2 pads were satisfactory with 1, 2 and 3 ml citrate. Counts with 3 pads were slightly low with 1 ml citrate and were unsatisfactory with 2, 3 and 4 ml citrate.

Range of Counts Obtained by Method (2)

Using method (2), control and check plates were put up in the morning and in the afternoon (Table XLIX). The results showed that average plaque counts were within a reasonable range of the controls. Counts varied from 203 to 231 in the morning (control 215) and from 184 to 200 in the afternoon (control 188).

Table XLVIII Effect on plaque counts of calcium alginate pads and sodium citrate in methods (2) and (3).

EXPT 1

Effect on plaque counts of No. of pads, amount of citrate, in methods (2) and (3).

No.	Control method		A. M. Plaque count Av. of 3		P. M. Plaque count Av. of 3	
1	(1)		139		156	
2	(2)		200		214	
3	(3)		200		205	
	Pads	Cit.(ml)	Method(2)	Method(3)	Method(2)	Method(3)
4	1	1	190	213	213	213
5	3	"	180	179	162	165
6	4	"	120	149	106	181
7	1	2	221	221	230	236
8	3	"	183	178*	185	174
9	4	"	97	50	138	104
10	1	3	213	206*	204	218
11	3	"	188	-	172	183
12	4	"	98	62*	174	140

Culture 0.3 ml. A.M. = daily culture, broths were inoculated daily.
P.M. = weekend culture, broths were inoculated daily except at the weekend.

Grade of plates = 1,
except line 8* grade <1 (3)
" 10* " 2-3(1)
12* " 1(1),3(2)

Method(1) four layers LYPA
" (2) two layers LYPA, one layer TSA, one layer LYPA
" (3) two layers TSA, one layer LYPA.

EXPT 2

Effect on plaque counts of No. of pads, amount of citrate, in method (2).

Control method		A.M. Plaque count Av.of3	P.M. Plaque count Av.of3
(1)		119	97
(2)		184	163
(3)		210	137
Pads	Cit.(ml)	Method(2)	Method(2)
1	1	186	211
2	"	175	166
3	"	156	154
1	2	208	191
2	"	160	188
3	"	72	42
1	3	187	185(2)
2	"	196	201(2)
3	"	128	94(2)
1	4	237	199(2)
2	"	206	189
3	"	58	35

Culture = 0.3 ml.
All plates grade 1.

Table XLIX The range of counts obtained by method (2).

	Method		AM Plaque count Av.of3	PM Plaque count Av.of3	Grade of plates			
1.	(1)	Control	156	153(2)	1.	1 (1)	2^a(2)	<1 (2)
2.	(2)	"	215	188(2)	2.	1 (2)	2^b(1)	3^s(2)
3.	(3)	"	211	207	3.	1 (1)	2^b(2)	1 (2) <1 (1)
4.	(2)		219	192	4.	2^b(3)		1 (3)
5.	"		203	186	5.	2^b(3)		2^s(3)
6.	"		215	200	6.	2^b(3)		2^s(3)
7.	"		231	184	7.	2^b(3)		1 (3)

• Culture = 0.3 ml

2^a = some small plaques

2^b = slight cloudiness of some plaques

2^s = slip first layer; not left long enough before next added.

No. of plates graded in parentheses.

Effect on Plaque Counts of Heat Treatment of TSA

In the following experiment (Table L) the TSA media were: the ordinary laboratory stock; TSA heated to the minimum; and TSA subjected to extensive heating. Combinations of media were as shown in the Table. No relationship could be found between extent of heating of media and plaque counts. Apparently excessive heating did not harm the TSA medium for plaque-counting purposes.

Table L Effect on plaque counts of heat treatment of TSA [methods (2) and (3)].

Method	Medium				Plaque count Av. of 3
(1)					147
(2)	TSA(S)	LS			156
"	"	Min.			167
"	"	Ext.			169
(3)	"	LS	(Ca TSA)	LS	161
"	"	Min.	"	"	189
"	"	Ext.	"	"	174
"	"	LS	"	Min.	176
"	"	Min.	"	"	168
"	"	Ext.	"	"	182
"	"	LS	"	Ext.	190
"	"	Min.	"	"	184
"	"	Ext.	"	"	173

Culture = 0.3 ml

Grade of plates = 1 (but method (1) = small plaques)

LS = laboratory stock

Ext. = extensive heating

	To dissolve	Autoclave (opened)	Water bath
Min.	Boiling water	15 lb 15 min (soon as possible)	Min. time
Max.	Steamer up to 1 h	15 lb 20 min (after ½ h)	Long as possible before use

Bottom layers for method (3) are given after phage-culture layers.

Summary for Sub-section

Investigations were made to find whether Trypticase Soy Agar medium could replace LYPA in part or whole in the four-layer method used for estimating phage quantitatively.

A medium with one layer only of TSA and with calcium addition 0-10,000 ppm, with or without pH adjustment, was quite unsatisfactory.

TSA in quantities of 5-9 ml gave satisfactory results when used in layer 3 of method (1), and the plaque counts were higher than when using LYPA in this layer.

A layer of LYPA plus calcium was needed on top of the culture-phage layer, and LYPA was better than TSA when method(3) was used.

The two LYPA layers below the culture-phage layer of method(2) could be satisfactorily replaced by one layer TSA containing added calcium. The volume of TSA for consistent results was not accurately determined. A quantity of 10 ml was decided upon for method(3).

One and 2 calcium alginate pads could be used with reasonable accuracy both in methods (2) and (3).

(vii) COMPARISON AND REVIEW OF VARIOUS METHODS FOR PHAGE ESTIMATION

Types of Media and Comparison of Counts in Methods (1), (2) and (3)

During the course of this study many variations in details of the methods were investigated. The materials finally adopted for plating in the three methods are summarized below.

Method (1)	ml		Ca ppm		Culture(ml)	Phage(ml)
LYPA(H)+P	2.5		6000	F		
" (S)+P	5.0				0.2	0.1
" (H)+P	5.0		6000	F		
" (H)--P	5.0					
Method (2)						
LYPA(H)+P	2.5		6000	F		
TSA(S)	7.5				0.2	0.1
LYPA(H)+P	5.0		6000	F		
" (H)-P	5.0					
Method (3)						
LYPA(H)+P	2.5		6000	F		
TSA(S)	7.5				0.2	0.1
" (H)	10.0		6000	F		

P = phosphate F = filtered

Method (1) had four layers of lactose yeast agar, three of these with added phosphate. Calcium was included in layers 2 and 4 so as to supply calcium to layer 3 by diffusion.

Method (2) used TSA in the third layer in place of LYPA.

Method (3) had one thick layer of TSA plus calcium below the TSA phage--culture layer, and on the top was a layer of LYPA medium plus calcium.

Some of the phage counts obtained by methods (1), (2) and (3) are shown graphically in Fig 7. Methods (2) and (3) gave higher counts than method (1). Method (2) was chosen for routine work. (A few of the plates in method (1) were slightly under grade 1 in appearance.)

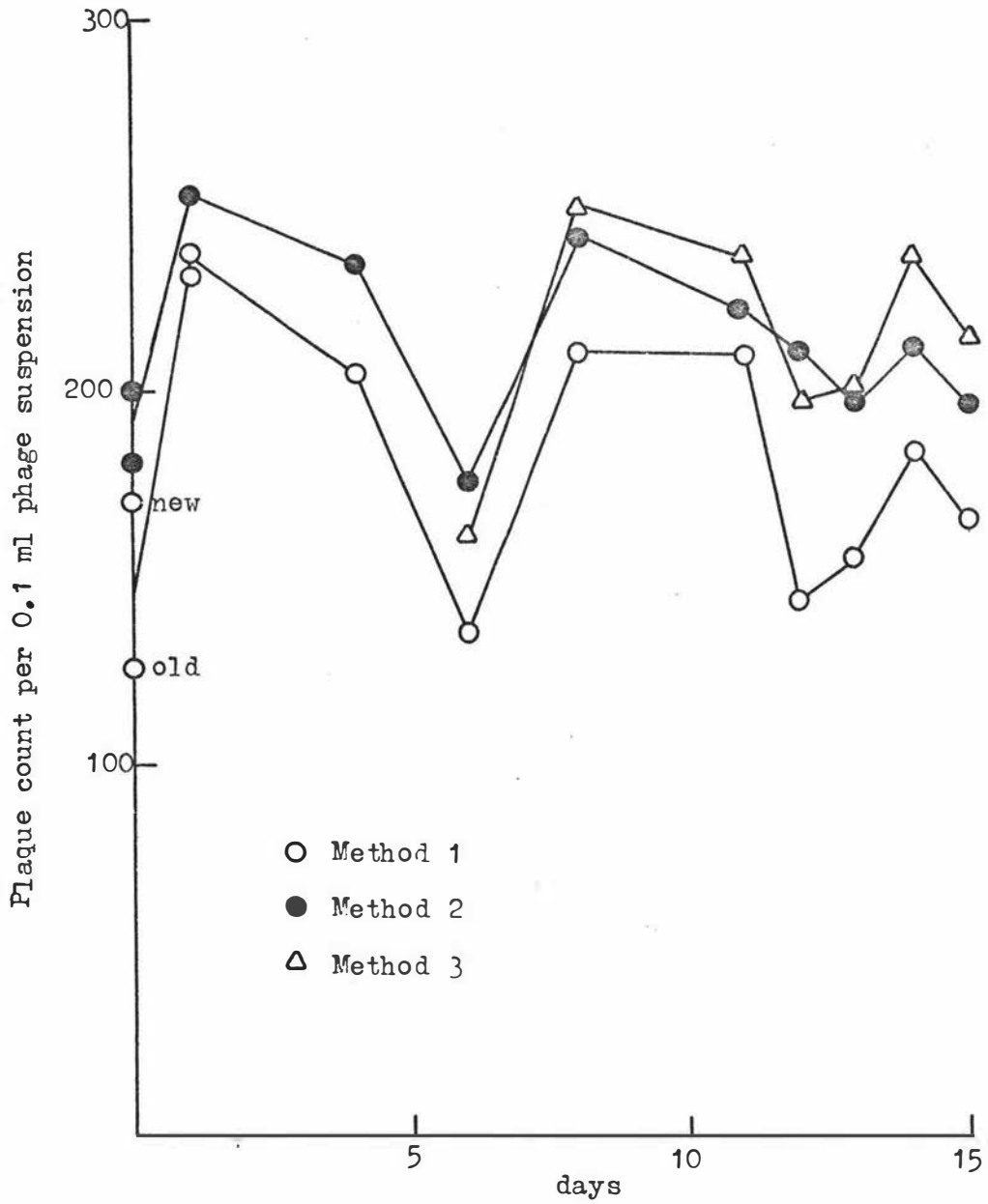

Fig 7. Comparison of plaque counts obtained by three methods for phage estimation.

Survey of Plaque Counts Obtained by Various Methods Using Media with and without Added Calcium

At this stage it was considered advisable to survey different methods for phage estimation, comparing methods with and without calcium addition. Results are shown in Table LI.

Table LI Comparison of plaque counts obtained by various methods using media with and without added calcium

Method	Amount per layer ml	Medium	P	Agar hard or soft	Ca ppm	Plaque counts 0,1ml	Av.	Appearance of plates G = good	
(1)	2.5	LYPA	+	H	6000	152,134	143	G	G
	5.0	"	+	S					
	"	"	+	H	6000				
	"	"	-	H					
(1)No Ca	2.5	"	+	H		9,8	9	G	G
	5.0	"	+	S					
	"	"	+	H					
	"	"	-	H					
(1) *	2.5	"	+	H	6000	129,156	143	G	G
	5.0	"	+	S					
	"	"	+	H	6000				
	"	"	-	H					
Normal**	2.5	"	+	S		7,4	6	opaque ppt.	<G
	10.0	"	+	H					
Normal***	2.5	"	-	S		0,0	0	G	G
	10.0	"	-	H					
(2)	2.5	"	+	H	6000	198,208	203	G	G
	7.5	TSA		S					
	5.0	LYPA	+	H	6000				
	"	"	-	H					
(1)	2.5	"	+	H	6000	177,122	150	G	opaque <G
	5.0	"	+	S					
	"	"	+	H	6000			ppt.	probably not interfere
	"	"	-	H					
Normal**	2.5	"	+	S		3,1	2	opaque <G ppt.	opaque <G ppt.
	10.0	"	+	H					

P = phosphate
* = Ca medium pH adjusted 4.9→7.1
** = two-layer soft agar method
*** = " " " " (no P soft, no P hard)
First six methods - culture 1-day-old
Last two " - culture log phase

An experiment was carried out comparing the plaque counts obtained by method (2) (four-layer) and the normal two-layer method, using small laboratory and main laboratory cultures. Counts were made by two workers. The results are shown in Table LII. Both cultures gave approximately the same counts by method (2), but counts were higher with the main laboratory culture than with the small laboratory culture when the two-layer method was used. Medium containing added calcium gave a much higher count than medium to which calcium was not added.

Table LII Comparison of plaque counts obtained by two-layer and four-layer methods.

Culture	Method	L1(LYPA) ml	Plaque counts A Av.of3	B Av.of3
SL	Normal 2-layer*	10-12	1	1
"	(2)	5	175	151
ML	Normal 2-layer*	10-12	20	15
"	(2)	5	168	149

Culture = 0.2-0.3 ml
SL = small laboratory
ML = main laboratory
A,B = two different workers
Plates all grade 1
* = top layer 5 ml soft LYPA(+P) instead of 2.5 ml.

The results shown in Tables LI and LII reveal a marked difference in counts between the various methods tested. The four-layer method (1) gave counts of 143 and 150 with calcium, and a count of only 9 when calcium was not added. The pH adjustment of medium plus calcium, method (1), did not alter the count. The four-layer method (2), with TSA in layer 3, gave a count of 203.

The two-layer method without calcium addition gave very low counts, viz. 6 and 2. The two-layer method, without phosphate in either layer, gave a count of 0.

In Table LII are shown results of the same type as those in

Table LI. They confirm the need for calcium addition, and show that the addition of calcium to the medium raises the plaque count considerably.

Conclusion:

A complicating factor that presented itself during the course of this study was that trouble could arise if calcium alginate dissolved in hexametaphosphate or citrate were added to medium containing added calcium. A method, therefore, had to be devised which overcame this difficulty. As can be seen from the foregoing results and from earlier work, a four-layer method is satisfactory, and was adopted for general use.

(viii) THE EFFECT OF CALCIUM BOROGLUCONATE ON PLAQUE PRODUCTION

Trials with Various Concentrations of Calcium Borogluconate in Media

Calcium borogluconate (CBG)readily liberates calcium ions. In the present investigation its usefulness as a source of the calcium needed in phage estimations was examined.

Experiments were carried out in which a range of concentrations of CBG in media was tried, the range including amounts that would normally be expected to affect phage production. Two-layer and four-layer plates were used, respectively, in two experiments, the results of which are shown in Table LIII.

In Expt 1 a two-layer method was used, *viz.* 10-12 ml TSA in the bottom layer, and in the top layer 2.5 ml TSA mixed with phage, culture and varying quantities of calcium borogluconate. Equal amounts of phage were added in each case. Controls were done by methods (1) and (2), i.e. using $CaCl_2$ in layers 2 and 4. Without CBG, the two-layer method did not give any plaques. There was an increase in plaque production with increasing amounts of CBG up to 0.1 molarity, but even with this concentration of CBG counts were greatly below those of the controls. Plates were not

Table LIII Experiments showing effects of various amounts of calcium borogluconate on plaque counts.

	EXPT 1 Two layers of TSA, with CBG in top layer			EXPT 2 Four-layer method Variations in quantities of CBG and $CaCl_2$			
					L2 and L4		
Method	Molarity of CBG in top layer	Plaque count Av.of3	Plaque* count Av.of3	Molarity of CBG in L_3 (TSA)	Ca ppm	$CaCl_2$ Molarity	Plaque count Av.of3
(1)		151			6000	0.15	146
(2)		166		0.0025	"	"	124
2-layer	0.0	0	1	0.005	"	"	129
"	0.0005	1	1	0.025	"	"	127
"	0.001	<1	0	0.0025	4000	0.10	112
"	0.0025	<1	2	0.005	"	"	112
"	0.005	3	6	0.025	"	"	115
"	0.01	12	8	0.0025	2000	0.05	68
"	0.05	26	16	0.005	"	"	58
"	0.1	38	38	0.025	"	"	56
	All plates grade 1.			All plates grade 1.			

* Phage with Ca alginate + citrate 2 x 0.04 g Ca alginate per 10 ml phage.

2-layer method of RJL (YE omitted)

noticeably affected by alginate. The pH of the CBG had been adjusted to approximately 7.

In Expt 2, using method (2) (with variations in $CaCl_2$ content), CBG was added to layer 3 to secure a range of molarities 0.0025-0.025, with 6,000, 4,000 or 2,000 ppm calcium in layers 2 and 4. These amounts of CBG did not raise average counts to the control values when the calcium levels were 4,000 and 2,000 in layers 2 and 4.

Under the conditions prevailing in these experiments the amounts of CBG added were inadequate to raise the average counts to those of the control plates.

Methods Used by RJL*

A colleague, (RJL), working in a nearby laboratory, developed methods for the use of calcium borogluconate (CBG) in media. They were as follows:

0.5 molar CBG stock solution 24.1 g of CBG were dissolved in distilled water (50 ml) by steaming. The solution was made up to 80 ml, cooled, the pH was adjusted to approximately 7, and the volume was made up to 100 ml with water.

Two-layer method TSA containing YE (1 g/l) was used in both layers with CBG in the top layer (0.004M).

Three-layer method Agar medium contained 1 g YE/l. The bottom layer (15 ml) was hard TSA, and the second and third layers were both 2.5 ml of soft TSA. CBG stock solution was included in each layer to give a final concentration of 0.005M. Phage, and culture (5-6 h, 30°C), were mixed with the medium in layer 2.

Broth for culture growth contained 1 g YE/l, and CBG at a concentration of 0.005M.

A count by RJL on phage suspension supplied by the author gave the following results:

0.1 ml of 10^{-6} x 0.7 phage suspension = 154 plaques.

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* Research Officer, NZDRI

(Throughout this investigation stock phage diluted to the same strength has frequently been used and similar, but variable, counts obtained.)

Comparison of Counts of Airborne Phage Carried Out by the Author and by RJL

Comparisons were made of plaque counts done by the methods of the author and RJL, respectively. For this purpose, phage suspension was sprayed into the atmosphere, plates were exposed to this atmosphere for specific periods of time, the plates were incubated, and the plaques were subsequently counted. Plates for exposure had been routinely spread with culture, but RJL introduced the method of mixing the culture with the agar medium.

Two initial experiments were unsuccessful owing to substantially lysed plates or low counts being obtained. Results from a successful experiment are shown in Table LIV.

The experiment showed that higher counts were obtained when the culture was mixed with the medium than when the culture was spread on the plate. In the RJL method, CBG appeared to be a satisfactory source of calcium. Method (2) (author) gave similar results to the pour method of RJL.

Consideration of the foregoing results suggests that possibly the calcium added to the broth may have contributed considerably to raising the plaque count.

It was considered advisable to continue the use of the four-layer method and not change to CBG because:

(1) CBG under the conditions used by the author did not give the expected increase in counts;
(2) the four-layer method had been extensively tried under varied conditions and had been checked for use with phage in the presence of calcium alginate and citrate;
(3) further work would have been necessary to find the effect of the culture-broth with and without added calcium when used in method (2).

Table LIV Comparison of author's and RJL's methods for phage estimation.

(Plates containing media used by the author and by RJL were exposed to a phage atmosphere, and comparisons of counts were made.)

		Plaque counts			
		1¼ h from spraying		2 h from spraying	
	Plates exposed to phage	Plates open for 3 min	1 min	Plates open for 10 min	2 min
Author	LYPA spread with milk culture	21	2	10	1
"	LYPA spread with 0.2 ml broth containing 0.005 M $CaCl_2$		240		32
"	Method (2) omit L4 0.2 ml broth culture mixed with TSA*		768		437
RJL	TSA(H) spread with 0.2 ml broth culture	Lysed	302	198	107
"	Top layer: TSA(S) mixed with broth culture Bottom layer: TSA(H)		691		271
"	ML(H) spread with 0.2 ml broth culture		378		200
"	Top layer: ML(S) mixed with broth culture Bottom layer: ML(H)		806		296

RJL series

ML = LYPA modified medium
H = hard
S = soft
CBG was included in all H and S agars and in broth to give a final concentration of 0.005 M.

* Doubt 0.005 M $CaCl_2$ in broth

(ix) PREPARATION OF $CaCl_2$ SOLUTION AND ADDITION TO AGAR MEDIUM

Calcium chloride solutions were prepared at intervals by dissolving $CaCl_2, 6H_2O$ in distilled water, and these solutions were then standardized. Calculated amounts of $CaCl_2$ solution were added to hot agar media, and the precipitate formed was filtered off. (Initially centrifugation was used instead of filtration.)

The method for standardizing the $CaCl_2$ solutions was as follows: a measured amount of the diluted solution was mixed with 15 ml of 0.02 M EDTA in a flask. Water, NaOH solution and Calcon* indicator were added. The remaining EDTA in the solution was titrated with 0.02 M $CaCl_2$ and the quantity of calcium in the original solution was calculated as ppm (1 ml 0.02 M EDTA = 0.8 mg calcium).

With a view to finding out by chemical analysis the amount of calcium in the agar medium, the following experiment was carried out:

Known quantities of $CaCl_2$ solution were added to agar media, the media were filtered, and the calcium remaining in the filtrate was estimated by the above method. Results are shown in Table LV.

Table LV The calcium content of agar after the addition of different quantities of $CaCl_2$, and the effect of subsequent filtration.

Hard agar + phosphate ml	Amount of 2M $CaCl_2$ added ml	Calculated Ca content of agar medium after $CaCl_2$ addition ppm	Molar	Ca content of agar medium after filtration (by analysis) ppm	Molar	Ca removed by filtration (by difference) ppm	Molar
97.5	2.5	2000	0.05	800	0.02	1200	0.03
95.0	5.0	4000	0.1	2720	0.068	1280	0.032
92.5	7.5	6000	0.15	4800	0.12	1200	0.03
90.0	10.0	8000	0.2	6720	0.168	1280	0.032

* Solochrome dark blue dissolved in anhydrous methanol.

It appeared that a constant amount of calcium was removed by precipitation and filtration regardless of the quantity of $CaCl_2$ initially present. The calcium was apparently removed by reacting with phosphate and possibly other constituents in the agar medium.

While the quantity of calcium used in experiments may appear large, it is probable that only a small proportion of the calcium reaches the phage-culture layer by diffusion.

(x) VARIATION WITHIN BATCHES OF PHAGE AND EFFECT ON PLAQUE COUNTS OF DILUTION OF PHAGE SUSPENSION

Comparison of Plaque Counts from Separately-stored Portions of the Same Batch of Phage Suspension

Separate portions of the same batch of phage suspension were compared by plaque count after storage for some months. Phage suspension from a freshly-opened bottle was somewhat higher in plaque count than the phage suspension residue from another bottle (Table LVI).

Table LVI Comparison of phage suspension from a freshly-opened bottle with residue from another bottle.

	Plaque count (Av. of 3)	
	Agar medium (prepared some days beforehand)	Agar medium (freshly prepared)
Phage suspension (freshly opened)	164	179
" " (residue)	136	121(2)

Effect on Phage Counts of Dilution of Phage Suspension

Various tests were undertaken to determine the effect of dilution of the phage suspension on plaque counts.

In Expt 1 (Table LVII) one objective was to find whether preparation of phage dilutions contributed appreciably to experimental

error. (The other objective, whether agar medium is affected by standing with $CaCl_2$, is discussed elsewhere.) From a sample of phage suspension three separate lots of dilutions (a), (b) and (c) were prepared. These were examined at intervals throughout the day. The results establish that dilution errors were not great assuming that other factors also probably affected the counts.

In Expt 2 two $<10^{-6}$ dilutions of a stock whey suspension of phage were prepared. These contained 1 calcium alginate pad (0.04 g) and 2 pads, respectively. From each of these suspensions 10^{-1} and 10^{-2} dilutions were prepared. Similarly, two $<10^{-7}$ dilutions containing 1 and 2 alginate pads, respectively, were prepared. From each of these suspensions a 10^{-1} dilution was prepared. Plaque counts were determined as shown in Table LVII. The results showed that the counts were proportionate to the extent of dilution.

Table LVII Effect on plaque counts of dilution of phage suspension.

EXPT 1

Comparison of counts on three sets of dilutions prepared from same neat phage

Phage suspension dilution	Plaque counts (Av. of 3) 10 am *	10 am †	2 pm *	2 pm †	4.30 pm *	4.30 pm †	Av. day
(a)	194	184	191	181	172	191	186
(b)	176	180	166	175	181	186	177
(c)	160	161	160	150	167	176	162

* = $CaCl_2$ added to agar medium 1 day earlier.

† = " " " " " at time of each analysis.

EXPT 2

Highly diluted phage suspensions - plaque counts on these and on decimal dilutions from them

Two suspensions $<10^{-6}$ contained 1 & 2 pads alginate, respectively, similarly $<10^{-7}$.

Phage suspension	Plaque count (Av. of 3) 1 pad	2 pads	Phage suspension	Plaque count (Av. of 3) 1 pad	2 pads
$<10^{-6}$ (as is)	185	148(2)			
10^{-1} dil.	19	19	$<10^{-7}$ (as is)	22	21
10^{-2} dil.	2(2)	3	(10^{-1} dil.)	2	0

Note: In preparing serial decimal dilutions of stock phage, the strength was reduced to 70% at 10^{-5}.

Another experiment was carried out in which phage dilutions of the approximate strength likely to be found when evaluating airborne phage were prepared using 0, 1 and 4 calcium alginate pads (0.04 g each pad). The object was to make dilutions from these suspensions, to find whether counts were proportionate to extent of dilution. The results are presented in Fig 8. Linear relationships were obtained, counts being proportional to dilution. Counts were distinctly lower when 4 pads were used, possibly due in part to the relatively large amount of calcium alginate affecting the amount of calcium available for phage proliferation.

Conclusion:

The above results demonstrated that phage dilution could be carried out without introducing major errors, and that counts were characteristically proportionate to the dilution.

(xi) SUMMARY OF BASIC METHODS ADOPTED

Four-layer Plate Method

Phage was estimated by the four-layer plate method. The diagram below illustrates the composition of each layer:

Layer 4	LYPA(H)+P	2.5 ml	Ca 6000 ppm (filtered)
" 3	TSA(S)	7.5 ml	0.2 ml culture, 0.1 ml phage
" 2	LYPA(H)+P	5.0 ml	Ca 6000 ppm (filtered)
" 1	LYA(H)-P	5.0 ml	

To a sterile Petri dish 5 ml lactose yeast agar medium were added (so-called LYPA, but with the phosphate omitted). The agar was allowed to harden and the plates were often kept in the refrigerator until required.

When used for analyses 5 ml LYPA containing added $CaCl_2$ were poured on layer 1, and the plates were allowed to set. ($CaCl_2$ had been added to this medium so as to give 0.15 M $CaCl_2$ (6,000 ppm calcium). The medium was filtered hot.) The material for the

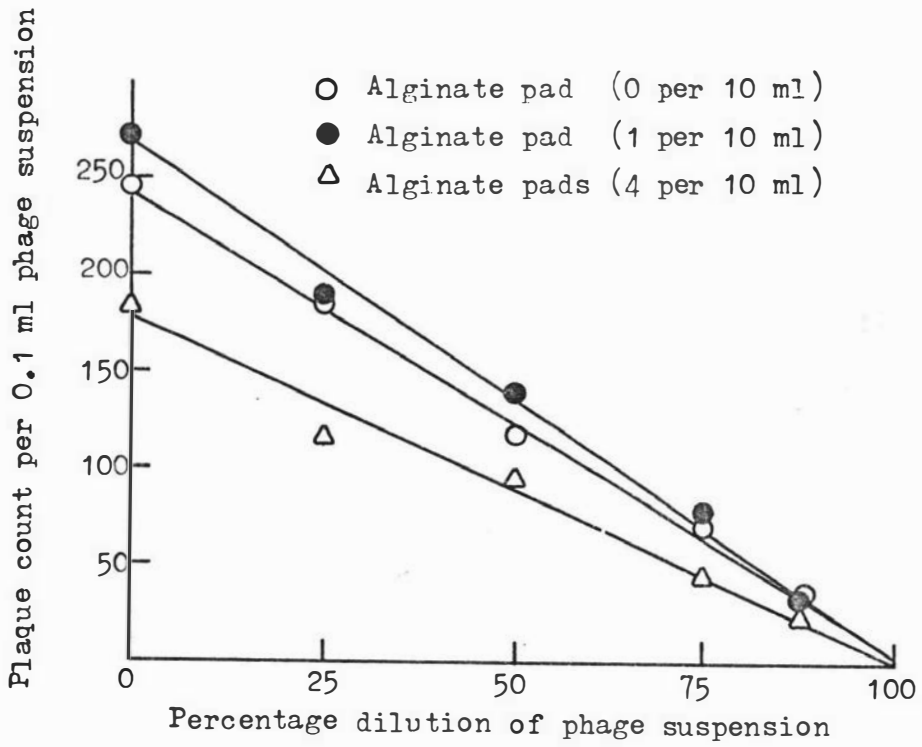


Fig 8. Relationship between plaque count and percentage dilution of phage suspension.
(Phage suspensions contained 0, 1 and 4 alginate pads per 10 ml, and dilutions of various strengths were prepared from the respective suspensions. Plaque counts were determined on 0.1 ml portions.)

third layer consisted of 7.5 ml soft trypticase soy agar (at approximately 45°C) mixed with 0.2 ml culture plus 0.1 ml phage suspension (added last). After mixing, this material was poured on top of layer 2. The fourth layer consisted of 2.5 ml of LYPA containing $CaCl_2$, similar to layer 2. The plates were incubated at 30°C overnight and the plaques were subsequently counted.

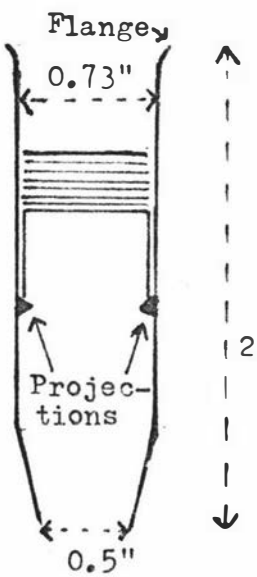
Notes:

(1) Culture. The requisite culture of lactic streptococci for phage detection was maintained by daily subculture in LYP broth, using a 0.3 ml inoculum and incubation at 22°C. When a fresh broth culture was prepared from a milk culture, broth was subcultured to broth at least once before use for analytical work. The culture used for phage counting was generally 24-30 h of age.

(2) LYPA(Hard)+$CaCl_2$. A steamed solution of $CaCl_2$ was added to hot LYPA, and the precipitate was filtered off using a hot Buchner filter funnel containing a layer of blotting paper. Throughout this study the stated calcium content of the agar medium (molarity and/or ppm) is the quantity added to the medium, but the effective amount of available calcium is, of course, lower, as is shown in Table LV.

(3) Trypticase soy agar. Soft medium contained 3% trypticase soy medium and 0.5% agar.

Estimation of Phage by Calcium Alginate Wool Pads



Seven alginate wool pads, each 0.04 g, were weighed, pressed flat in a metal mortar with a pestle, and placed on top of the grid across the surface of the cylindrical metal support held in place in a glass tube by projections from the glass wall. The pads were compacted by a weight. The apparatus was wrapped in parchment paper and autoclaved at 15 psi for 20 min.

Prior to a determination of airborne phage particles, the flowmeter was levelled. The glass tube was attached to one end of the plastic inlet tube of the flowmeter, and air was drawn through the apparatus for a definite time, e.g. 10 min. At the start of air sampling the pads were 'tightened' by lightly tapping them about 40 times with the flattened end of a sterile glass rod. The tamping of the pads, combined with the suction of air, resulted in a compact filter of alginate wool.

When air sampling was complete the pads were individually transferred, with the aid of forceps, to McCartney bottles containing 9 ml of peptone-salt solution. The bottles and contents were kept cool in an ice box containing frozen Slikka pads.

For phage estimation 1 ml of 10% (w/v) sodium citrate solution was added to the McCartney bottle and the contents were well mixed. If necessary, serial decimal dilutions were prepared by transferring 1 ml of the mixture to 9 ml of peptone-salt solution. These suspensions were called 'neat', 10^{-1}, 10^{-2}, etc. For estimation of phage 0.1 ml of suspension was plated. The number of phage particles per pad

= count x 10 (as 0.1 ml used)
x 10 (as 10 ml per bottle)
x dilution.

The counts from the pads were added to establish the total count per specific volume of air.

Notes:

(1) Peptone-salt solution contained 0.1% peptone and 0.88% NaCl. The solution was autoclaved at 15 psi (1.05 bar) for 15 min.

(2) Sodium citrate solution (10% w/v) was prepared by dissolving sodium citrate in water and steaming for 15 min.

(3) Volume of air. Generally 20 cu. ft ($0.566 m^3$) of air was sampled by drawing it through the pads and flowmeter for 10 min at the rate of 2 cu. ft per min.

Estimation of Phage Falling into Liquid

To a sterile Petri dish 10 ml peptone-salt solution were added from a McCartney bottle. The plate was exposed (lid off) to the atmosphere for 10 min. The liquid was then transferred back to the McCartney bottle, placed in an ice box and cooled with Slikka pad(s). The liquid was kept cool until analysis, when 0.1 ml portions were examined by the plating method.

Total phage particles falling into Petri dish
= plate count x 10 (as 0.1 ml used)
x 10 (as 10 ml per bottle).

Estimation of Phage Particles Falling onto Solid Medium

To a sterile Petri dish were added 5 ml LYA (without phosphate), then 5 ml LYPA ($CaCl_2$ 0.15M, filtered), then 7.5 ml soft TSA mixed with 0.2 ml host culture, giving a three-layer plate. Each layer was allowed to set after pouring. The plates were kept cool until used.

Plates were exposed at different places in the dairy factory with the lids off for 10 min. The lids were then replaced and the plates were kept cool prior to overnight incubation at 30°C. Plaques were counted subsequently.

Analyses of Whey Samples for Phage

Whey samples were taken in Bijou bottles and placed in ice in a thermos flask. On return to the laboratory the samples were kept in the cold room until analysed.

For analysis, serial decimal dilutions were prepared in peptone-salt solution and a 0.1 ml portion from each of several different dilutions was examined by the plating method. Plates were incubated overnight at 30°C and the plaques were counted subsequently.

Phage count per ml whey = plaque count x 10 (as 0.1 ml used)
x dilution.

Estimation of Phage in Milk

Milk samples were taken in 100 ml bottles and stored as described for whey samples. For examination 10 ml of the sample were placed in one tube and a further 1 ml of the sample was added to approximately 9 ml sterile skim milk in another tube. From the latter approximate serial decimal dilutions were made in tubes of skim milk so that eventually a series of tubes containing a total of about 9 or 10 ml was obtained. Two drops of a coagulated milk culture of the appropriate starter strain were added to each tube and, after incubation at 30°C for 5 h and subsequent centrifugation, the supernatant wheys were spotted on three-layer plates, which were further incubated. Results were recorded as + or - for the presence of phage in approximate specific quantities of the original sample of milk.

Standard Phage Suspension

A whey suspension of phage was prepared early (November 1968) in the present study. This whey was used over a long period as a standard suspension.

Dilutions of the suspension were prepared in peptone-salt solution, 10^{-1} to 10^{-6}, but 10^{-5} dilution was made by using 0.7 ml of 10^{-4} and 9.3 ml diluent; 10^{-6} was made by using 1 ml of approximately 10^{-5} and 9 ml diluent. This meant that so-called dilutions 10^{-5} and 10^{-6} were slightly under this strength. The reason for using 0.7 ml instead of 1 ml was in order to have a reasonable number of plaques when 0.1 ml was plated from the highest dilution; 0.1 ml of the so-called 10^{-6} dilution gave about 150-200 plaques generally. In a great many of the experiments conducted in investigating the phage methods it was convenient to have a phage suspension of known strength and this particular whey suspension was kept in the refrigerator and used over a long period. There was no appreciable decline in titre over a considerable period of time.

PART II

PHAGE IN THE ATMOSPHERE -

EXPERIMENTS WITH ARTIFICIALLY PREPARED AEROSOLS,

AND FACTORY INVESTIGATIONS

A. EXPERIMENTS WITH ARTIFICIALLY PREPARED AEROSOLS

A. EXPERIMENTS WITH ARTIFICIALLY PREPARED AEROSOLS

(i) ARTIFICIAL PRODUCTION OF PHAGE AEROSOLS AND THEIR EVALUATION

Introduction

It had been decided to estimate phage in the air by filtration through calcium alginate wool, and in view of this there was needed a phage-laden atmosphere which could be used for evaluating the method. The atmosphere of cheese factories varies widely in phage content, the number of particles per unit volume varying from nil to very high levels. For developing and testing a method of sampling it was necessary to produce artificially an aerosol which would contain a reasonably high level of phage. During the trials various points arose which needed clarification. These were examined by suitable experiments. This section deals with:

Production of aerosols;
The time of survival of an aerosol;
Factors bearing on the estimation of phage in aerosols by filtration through calcium alginate wool.

Method for Preparation of Aerosols

A simple method for aerosol production was by passing compressed air through a rubber tube into which a phage suspension was injected slowly. The turbulence in the air being forced through the tube resulted in an atomizing action on the phage suspension, and an aerosol was produced which could be discharged into a container or room.

Details of the method are as follows: a rubber tube about 3 ft (91 cm) in length and of 3/8 in. (0.95 cm) internal diameter was connected at one end to an electrically driven air pump*. The tube was pierced by the needle of a hypodermic syringe which contained a small amount of whey of high phage content, and this whey was gradually introduced while air was being forced through

* Of the two pumps used, one was a 1/3 h.p. "Tuffy" (Arnold de Vilbiss) diaphragm pump.

the tube. Initially the needle was placed in the tube near the pump, but in later experiments the position of the needle was changed to near the exit end of the tube. The open end of the rubber tube was moved about continually in order to distribute phage round the room. In this way an aerosol of high phage content could be prepared, and some of the finer particles remained suspended for several hours.

Preliminary Trials of Aerosol Production

Preliminary trials were made with the apparatus described, and results of experiments are shown in Table LVIII. At this stage of the work sedimentation plate counts were used for estimating phages. Phage suspension (0.7-2 ml) in whey was introduced from a hypodermic syringe into the rubber tube at the end near the pump, and the phage was blown into the room for 5-10 min. Plates spread with the appropriate host strain were exposed at intervals, before and after phage distribution. Plates were exposed several feet from the aerosol-generating equipment.

The two rooms used for these experiments were 11 ft x 11 ft x 10 ft (1,210 cu. ft) [3.35 m x 3.35 m x 3.05 m = 34.2 cu. m] and 8 ft x 20 ft x 10 ft (1,600 cu. ft) [2.44 m x 6.1 m x 3.05 m = 45.4 cu. m], respectively; they contained little furniture.

The counts obtained showed that the method of aerosol production resulted in quite an appreciable amount of phage being dispersed into the atmosphere. Hundreds of plaques could be obtained by sedimentation from the aerosol in a period of 10 min. The counts fell off quite rapidly in Expts 2 and 3. (Times stated in Table LVIII were counted from zero time and, therefore, included a period before phage was distributed into the air during which control plates were exposed.)

These preliminary trials indicated that an aerosol could be satisfactorily produced by the method described.

Table LVIII Sedimentation plate counts in preliminary aerosol production trials.

EXPT 1			EXPT 2				EXPT 3			EXPT 3 (cont'd)		
Min from zero time at which plates opened	Distance plates from pump (aerosol unit) ft	PLATES Plaque count	Min from zero time at which plates opened	Plates open for min	Distance plates from pump (aerosol unit) ft	PLATES Plaque count	Min from zero time at which plates opened	Distance plates from pump (aerosol unit) ft	PLATES Plaque count	Min from zero time at which plates opened	Distance plates from pump (aerosol unit) ft	PLATES Plaque count
0	3	0	0	10	3	-	0	3	0	81	3	0
"	7	0	"	"	7	0	"	7	0	"	7	0
10	3	738	10	5	3	320	"	6(D)	0	"	6(D)	2
"	7	965	"	"	7	250	15	3	87	105	3	0
15	3	928	10	5	6(D)	21	"	7	65	"	7	0
"	7	950	15	"	6(D)	91	"	6(D)	92	"	6(D)	0
25	3	495	15	5	3	124	30	3	21	135	3	0
"	7	455	"	"	7	150	"	7	25	"	7	0
35	3	316	171	10	3	0	"	6(D)	23	"	6(D)	0
"	7	324	"	"	7	0	45	3	5	165	3	0
							"	7	2	"	7	0
							"	6(D)	2	"	6(D)	0

Plates open for 10 min.
Door shut most of the time.

Phage distributed during 10–15 min period after zero time.
Door shut most of the time.

Phage distributed during 15–20 min period after zero time. Plates open for 10 min. Door open all the time.

(D) by door – opposite direction to other plates.

In Table LVIII zero time is defined as the time at which control plates were first exposed.

In Expt 1
At zero time, plates were exposed for 10 min.
At 10 min after zero time, phage distribution commenced (pump ran for 10 min, including 5 min after phage suspension had been dispensed).
At 10,15,25 and 35 min, respectively, after zero time, plates were exposed for 10 min.

The Effect on Aerosol Production of Size of Exit from Rubber Tube

The above method was examined further to find the effect on aerosol production of different sized exits from the rubber tube. Production of an aerosol by the method described depended on turbulence produced in the rubber tube. This turbulence could possibly be affected by the size of the exit from the tube, so experiments were carried out to check this point. Three different sized exits were compared, viz. end of rubber tube unrestricted, a hypodermic needle fitted in the end of the rubber tube so as to cause air passage through the narrow bore, and a glass tube connected with the rubber tube at the broad end and tapered to about 0.1 in. (2.54 mm) at the discharge end.

The hypodermic needle fitted at the discharge end in the majority of the experiments had an internal bore of approximately 0.075 in. (1.9 mm).

Phage was distributed into the air by the three methods, and plaque counts were determined by sedimentation using exposed agar plates spread with the appropriate host strain.

The results of the above experiments are shown in Table LIX. Times stated in this Table were counted from zero time and included a period before phage was distributed into the air. Phage was distributed into the air from 10-13 min after zero time, for a period of 8-10 min, part of which time included the air pump running after the hypodermic syringe was empty. Zero time was the time at which control plates were first exposed.

Highest counts were obtained with the tapered glass tube. The open rubber tube apparently did not cause enough turbulence, and the needle probably hindered production of turbulence, to some extent, by causing too much obstruction of the air flow.

As these experiments dealt only with counts obtained by sedimentation it was possible that the count of a very fine aerosol might have been underestimated.

Table LIX Comparison of different sized exit tubes in aerosol preparation.

All expts	First 2 expts	EXPT 1			EXPT 2			EXPT 3			
Distance plates from pump ft	Min from zero time at which plates opened	Plaque count (10 min) Open rubber tube	Needle	Tapered glass tube	Plaque count (10 min) Open rubber tube	Needle	Tapered glass tube	Min from zero time at which plates opened	Plaque count (10 min) Open rubber tube	Needle	Tapered glass tube
3	0	3	0	1	0	2	1	0	0	1	0
7	"	2	"	0	"	1	1	"	"	2	5
6(D)	"	2	"	1	"	0	0	"	"	0	0
3	10	16	367†	710	1	*25	**253+	10	5	11	254
7	"	0	20†	200	2	*16	**165	"	4	8	808
6(D)	"	5	50†	94	0	*21	** 59	"	2	13	93
3	30	4	6	19	0	3	13	20	1	7	76
7	"	1	2	24	"	5	2	"	1	6	66
6(D)	"	2	3	24	1	1	5	"	0	4	72
3	50	2	0	1	1	3	4	30	3	2	21
7	"	0	0	4	1	2	5	"	0	1	29
6(D)	"	1	0	3	1	1	2	"	0	2	16
Position hypodermic syringe:		Near pump			Near pump			Near exit end			

D= by door - opposite direction to other plates.

†= phage started 12 min after zero time.

*= 12 min exposure.

**= phage and plates started 13 min after zero.

Zero time is defined as the time at which control plates were first exposed.

Expt 3 Phage (end tube open) started 13 min after zero.

Effects of Quantity of Calcium Alginate Wool and Size of Holder-tube when Estimating Phage

Experiments were carried out to investigate the phage content of an aerosol by filtration through calcium alginate wool.

In an initial experiment a comparison was made between the alginate wool filtration method and the sedimentation method for examining an aerosol. At this stage of the development of test methods and media a portion (0.25 ml) of the alginate wool/phage suspension was spread on a lactose yeast phosphate agar plate on which the homologous organism had already been spread. After incubation of the plates, plaques were counted. Results of the experiment are shown in Table LX.

Table LX Plaque counts by the calcium alginate wool filtration method and by the sedimentation method.

Min after zero time at which counts started	0	20	40
* Alginate count per 20 cu. ft in 10 min	1,204,000	6,640	160
Plaque count (sedimentation) 10 "	>1,943	111	3

* Pads kept at room temperature overnight

Note on above experiment: Phage was injected into the tube for 5 min after zero time, and the pump was left on for a further 5 min.

Comparative counts by the two methods showed that plaque sedimentation counts, while not strictly proportional to alginate counts, did show some relationship.

After this particular experiment the spreading of plates for estimating phage in alginate wool was replaced by the use of soft agar. A series of further experiments for estimating phage in aerosols was carried out, and records were kept of the amount of alginate wool, the diameter of the glass tube holding the alginate wool, the voltage of the transformer, the temperature of the motor, and the phage counts per layer. These results have been recorded in tabular form (pps. 34,35 of text). They were reported in that particular section of this study because various aspects of the method were discussed there. It will be recalled that the 0.73 in.

diameter tube was selected as the most suitable size of tube as a holder for alginate wool pads. Several 0.04 g pads of alginate wool were needed to collect the majority of phage particles.

Changes in Phage Counts of Aerosols Over Several Hours

Following the previous section of the work, experiments were carried out to find how long phage remained suspended in the air and to what extent alterations occurred. The results of these experiments are presented in Tables LXI, LXII and LXIII.

In these Tables the results of five experiments are shown. Phage suspension was dispersed into a room and the amount of airborne phage in 20 cu. ft of air was examined at intervals by the alginate filtration method. At this stage of the work the alginate method was carried out by adding a mixture of phage suspension and bacteria in soft agar to a solidified plate of LYP agar. At the same time agar plates were exposed to find the counts by sedimentation.

In Table LXI (Expts 1 and 2) detailed counts for the first two experiments are given. These experiments show that the majority of the phage particles were removed in the op 0.12-0.16 g of alginate wool. In Tables LXI [Expts 1 (consolidated) and 2 (consolidated)], LXII and LXIII the results for the five experiments are given in consolidated form. For the first three experiments the sedimentation plate counts were calculated as the amount of phage falling from a volume of 20 cu. ft. In the last two experiments the sedimentation plate counts were calculated as the amount of phage falling onto plates in 5 min. These Tables show that there is a broad relationship between the alginate counts and the sedimentation plate counts; both show a somewhat similar decline in count with time.

In the experiments shown in Table LXII a fan was used. Phage was distributed into the atmosphere for 10 min from zero time. During this time the door of the room was closed. Then 4 min later the fan was turned on in a corner of the room diagonally opposite the door, and the door was opened for the remainder of the experiment. Phage counts in the room dropped very quickly (compare Expts 1 (consolidated) and 2 (consolidated), Table LXI). It was apparent that much phage was blown out of the room by the fan.

Table LXI Experiments to measure decrease in airborne phage after aerosol generation.

EXPT 1

	Time from start of phage distribution h/min	ALGINATE METHOD Ca alg. pads g	Count per layer Plaques	SEDIMENTATION PLAQUE COUNTS (A) Near pump Min	Count	Calc. av. per 5 min	(B) Near alginate apparatus Min	Count	Calc. av. per 5 min
1	.00	0.12	4.9×10^6	1	∞)	∞	1	∞)	∞
2	"	0.04	1.0×10^5	2	∞)		2	∞)	
3	"	"	2.0×10^3						
4	.20	0.12	1.2×10^6	1	427)	2135(1)	1	513)	2565(1)
5	"	0.04	2.5×10^3	2	∞)		2	∞)	
6	"	"	1.0×10^2						
7	.40	0.12	3.1×10^5	1	236)	996	1	136)	835
8	"	0.04	6.0×10^3	2	325)		2	396)	
9	"	"	2.0×10^2						
10	1.10	0.12	2.1×10^5	1	98)	447	1	85)	432
11	"	0.04	3.0×10^2	5	403)		5	439)	
12	"	"	2.0×10^2						
13	1.50	0.12	3.3×10^4	5	84)	74	5	99)	82
14	"	0.04	5.2×10^3	10	126)		10	129)	
15	"	"	$< 1.0\times10^2$						
16	2.40	0.12	7.8×10^3	5	14)	15	5	24)	20
17	"	0.04	2.0×10^2	10	33)		10	31)	
18	"	"	$< 1.0\times10^2$						
19	5.00	0.12	$< 1.0\times10^2$	5	0)	0	5	0)	0
20	"	0.04	$< 1.0\times10^2$	10	0)		10	1)	
21	"	"	1.0×10^2						
22	6.25	0.12	1.0×10^2	5	1)	1	5	0)	0
23	"	0.04	$< 1.0\times10^2$	10	1)		10	0)	
24	"	"	$< 1.0\times10^2$						
25	7.20	0.12	$< 1.0\times10^2$	5	0)	0	5	0)	0
26	"	0.04	$< 1.0\times10^2$	10	0)		10	0)	
27	"	"	$< 1.0\times10^2$						

Notes: Alginate pads kept in refrigerator 1 day. ∞ = uncountable

Phage suspension was distributed into the atmosphere over a period of 10 min, commencing at zero time. For analysis, air was drawn through alginate wool at the rate of 2 cu. ft min.

(cont'd)

Table LXI (cont'd)

EXPT 2

	Time from start of phage distribution h/min	ALGINATE METHOD Ca alg. pads g	Count per layer Plaques	SEDIMENTATION PLAQUE COUNTS Calculated No. per 5 min Av. of 2 or 3 plates Near pump	Near alginate apparatus
1	.00	0.12	5.4×10^6	6800	5220
2	"	0.04	5.2×10^5		
3	"	"	3.8×10^4		
4	.20	0.12	5.2×10^6	1864	2072
5	"	0.04	1.2×10^4		
6	"	"	5.0×10^3		
7	.50	0.12	8.7×10^5	376	376
8	"	0.04	7.1×10^3		
9	"	"	6.4×10^3		
10	1.30	0.12	3.0×10^5	186	227
11	"	0.04	2.4×10^3		
12	"	"	1.2×10^3		
13	2.10	0.12	1.3×10^5	39	36
14	"	0.04	1.1×10^4		
15	"	"	2.0×10^2		
16	2.50	0.12	5.6×10^4	23	14
17	"	0.04	9.0×10^2		
18	"	"	1.0×10^2		
19	4.50	0.12	3.0×10^3	1	3
20	"	0.04	2.0×10^2		
21	"	"	$< 1.0 \times 10^2$		
22	5.40	0.12	7.0×10^2	0	1
23	"	0.04	1.0×10^2		
24	"	"	1.0×10^2		

Details at foot of first section of Table LXI.

(cont'd)

Table LXI (cont'd)

EXPT 1 (Consol.)		ALGINATE WOOL	SEDIMEN-TATION	EXPT 2 (Consol.)	ALGINATE WOOL	SEDIMEN-TATION
	Time from zero h/min	Plaque counts per 20 cu. ft (10 min)	Plaque counts calc. for 20 cu. ft (10 min)	Time from zero h/min	Plaque counts per 20 cu. ft (10 min)	Plaque counts calc. for 20 cu. ft (10 min)
1	.00	4,986,000	>430,000	.00	5,952,600	†516,787
2	.20	1,202,600	202,100	.20	5,187,400	169,248
3	.40	319,200	78,744	.50	883,500	32,358
4	1.10	214,500	37,776	1.30	305,600	17,716
5	1.50	38,200	6,676	2.10	140,500	3,193
6	2.40	8,000	1,505	2.50	57,100	1,559
7	5.00	<300	<32	4.50	3,200	151
8	6.25	<300	<32	5.40	800	54
9	7.20	<300	<22			

Notes: Lysed plates were reckoned as having counts > 1000.
Time from zero was calculated from half-way through phage distribution to half-way through alginate filtration or (approx.) plate exposure (sedimentation). Alginate wool pads were 0.12, 0.04, 0.04 g for each filtration.
Alginate wool pads were mostly kept cool overnight.

† = 5 min late.

Method for calculating sedimentation volume count.

Area Petri dish = 9.1 sq. in. Height bench to ceiling = 7 ft 4 in. = 88 in. ∴ volume over Petri dish = 801 cu. in.
20 cu. ft = 34,560 cu. in.
The average sediment plaque count for 10 min was calculated, and this figure multiplied by $\frac{34,560}{801} = 43$
gave the sediment plaque count for 20 cu. ft over 10 min.
The factor for floor plates was $\frac{34,560}{(88+30)\times 9.1} = 32$

Comparison 20 cu. ft count by alginate method with calculated 20 cu. ft count by sedimentation method.

In the sedimentation method:

(1) all droplets of whey may not fall;

(2) plaques may be formed from whey droplets containing several phage particles. (In the calcium alginate method, the clusters of phage particles are dispersed by mixing in fluid, and plaques would thus be formed from individual particles.)

Sedimentation counts are obviously lower than calcium alginate counts.

As calcium was not included in the media at this stage, all estimation could be low.

Table LXII The effect of a fan on dispersion of airborne phage.

	Time from zero h/min	ALGINATE WOOL Plaque counts per 20 cu. ft (10 min)	SEDIMENTATION Plaque counts calculated for 20 cu. ft (10 min) In room	Outside room Table	Floor	Bench
1	0	2,714,800	†761,100	59		
2	0.25	250,400	34,400	53,320		
3	1.10	16,000	978	602	1,152	
4	1.55	< 1,000	< 86	151	288	
5	2.20	100	43	< 43	464	
6	4.00	100	< 22	43	96	< 11

Notes: See notes following Expts 1(consol.) and 2(consol.)of Table LXI.

Table LXIII Further experiments to measure decrease in airborne phage after aerosol generation.

	EXPT 1 Time from zero h/min	Rate cu. ft/ min	Alg. wool Plaque counts per 20 cu. ft*	Sedimentation Plaques calc. for 5 min In room	Cut room	EXPT 2 Time from zero h/min	Alg. wool Plaque counts per 20 cu. ft in 10 min	Sedimentation Plaques calc. for 5 min In room
1	10	2	1,926,600	4298(2)	37(1)	00	109,500	< 87(6)
2	18	4	1,721,300	1485(1)	75(2)	20	60,500	< 18(4)
3	1.20	2	189,700	176(3)	-	35	34,100	< 4(3)
4	1.28	4	299,500	77(1)	1(1)	2.10	2,300	< 2(4)
5	2.15	2	104,100	40(1)	-	3.00	600	2(4)
6	2.23	4	63,600	10(1)	-	3.45	< 300	4(4)
7	4.30	2	9,000	3(1)	0(2)	4.30	< 300	< 1(5)
8	4.38	4	4,400	-	0(2)	10 min filtrations. Rate not recorded, but probably 2 cu. ft min		

Notes: Alginate wool pads were 0.12, 0.04, 0.04, 0.04 g for each filtration in Expt 1, and 0.12, 0.04, 0.04 g for each filtration in Expt 2.

Time from zero was calculated from half-way through phage distribution to approximately half-way through alginate filtration.

* { 20 cu. ft collected in:
10 min where rate 2 cu. ft/min
5 " " " 4 " "

In relation to cheese factories these results show that it is possible for some of the phage particles distributed into the atmosphere by the atomizing action of, say, the whey separator, to remain suspended in the air for a considerable length of time, and thus to provide a possible source of contamination for starter bacteria. The simple trial with a fan suggests that airborne phage can be fairly readily displaced from the interior of a cheese factory.

Experiments to Check the Effectiveness of Filtration by Calcium Alginate Wool

Experiments were carried out to further check the effectiveness of the filtration process using calcium alginate wool. This was done to test whether any significant proportion of fine particles could be carried through several layers of alginate wool and escape detection.

In Table LXIV the results of analyses made using a two-layer method are recorded. In Table LXV the results were obtained by the four-layer method (2) with calcium included in the media. The filters used to trap airborne phage comprised 7 pads of alginate wool, each pad weighing 0.04 g. In one experiment 10 pads were used. Phage estimations were made at the beginning of the experiment (in most cases) and approximately 3-4½ h later. Twelve to 30 cu. ft of air were drawn through the apparatus at the initial determination, and 50-100 cu. ft at the second. In two experiments three sets of filtrations were made.

Dorman (1966) listed the factors which affect filtration through fibrous filters as follows: Brownian movement, inertia, the direct interception effect, electric charges upon particles and fibres and, for particles a few microns in diameter, gravitational settling. He stated that as particle size decreases down to about 0.3 μ diameter the penetration increases, and that it is predicted that at this size, or smaller, penetration decreases because of increasing diffusion rates. With increasing velocity penetration increases as diffusion becomes less important, reaches a maximum, and then decreases, the velocity for peak penetration being greater the smaller the particle.

Probably all the factors listed would apply in the experiments

Table LXIV Experiments to check efficiency of filtration. Comparison of many layers of alginate pads exposed shortly after aerosol generation and several hours later. Two-layer plate method used for phage detection.

	Plaque counts per pad								
Time filtration (min)	10	10	10	10	20	10	10	6	10
Vol. cu. ft	20	20	20	20	20	30	20	12	20
Time from zero (min)	10	10	10	10	20	10	10	8	10
Pad 1 First	9.6×10^5	4.9×10^5	1.2×10^5	3.8×10^6	3.3×10^6	4.4×10^6	2.1×10^6	1.2×10^6	6.1×10^6
2	3.7×10^5	7.6×10^4	1.1×10^6	2.0×10^6	1.9×10^6	1.3×10^6	3.5×10^5	9.2×10^5	2.6×10^6
3	3.2×10^4	2.1×10^4	1.7×10^5	2.7×10^5	5.1×10^5	4.1×10^5	4.0×10^5	3.7×10^5	5.0×10^5
4	4.2×10^3	4.6×10^3	1.6×10^5	6.4×10^4	2.1×10^5	1.9×10^5	1.3×10^5	2.0×10^5	3.9×10^5
5	9.0×10^2	3.4×10^3	1.7×10^4	1.3×10^5	3.5×10^4	2.0×10^4	4.7×10^4	4.1×10^4	5.2×10^3
6	1.0×10^2	4.0×10^2	2.4×10^3	1.8×10^4	4.2×10^3	1.0×10^4	1.7×10^4	7.4×10^4	8.5×10^3
7	2.0×10^2	6.0×10^2	1.6×10^3	4.4×10^4	1.4×10^3	2.5×10^3	1.3×10^4	1.0×10^4	1.4×10^3
8				4.1×10^3					
9				1.1×10^3					
10 Last				1.1×10^3					

(cont'd)

Table LXIV (cont'd)

	Plaque counts per pad								
Time filtration (min)	50	50	50	50	50	30	50	50	20
Vol. cu. ft	100	100	100	100	50	90	100	100	40
Time from zero	3 h/ 20 min	3 h/ 20 min	3 h/ 20 min	3 h/ 20 min	3 h/ 30 min	3 h/ 10 min	3 h/ 20 min	3 h/ 20 min	3 h/ 15 min
Pad 1 First	1.1×10^5	2.3×10^4	5.4×10^5	1.9×10^5	5.0×10^4	9.5×10^3	6.1×10^4	6.0×10^2	2.5×10^5
2	9.4×10^3	1.6×10^3	7.9×10^4	1.8×10^5	1.2×10^4	4.3×10^3	1.7×10^4	1.0×10^3	9.3×10^4
3	1.6×10^3	3.0×10^2	1.6×10^4	4.1×10^4	1.7×10^3	2.6×10^3	4.8×10^3	3.0×10^3	1.6×10^4
4	2.0×10^2	1.0×10^3	3.1×10^3	3.0×10^4	9.0×10^2	3.2×10^3	1.4×10^3	2.0×10^2	3.3×10^3
5	1.0×10^2	1.0×10^2	1.0×10^3	7.9×10^3	$<1.0\times10^2$	1.2×10^3	4.0×10^2	$<1.0\times10^2$	2.0×10^2
6	1.4×10^3	$<1.0\times10^2$	5.0×10^2	3.0×10^3	$<1.0\times10^2$	5.0×10^2	2.0×10^2	1.0×10^2	$<1.0\times10^2$
7	$<1.0\times10^2$	$<1.0\times10^2$	5.0×10^2	1.0×10^3	$<1.0\times10^2$	2.0×10^2	$<1.0\times10^2$	$<1.0\times10^2$	$<1.0\times10^2$
8				1.0×10^2	Slow filtration	Fast filtration	†	*$<1.0\times10^2$	**3.7×10^4
9				$<1.0\times10^2$				"	3.5×10^4
10 Last				$<1.0\times10^2$				"	1.1×10^4
								"	4.6×10^3
								2.0×10^2	5.0×10^2
								$<1.0\times10^2$	1.0×10^2
								"	$<1.0\times10^2$

† Moistened top pad before both filtrations (20 drops water each time).

Time from zero = half-way through phage distribution (zero) to half-way through alginate estimation.

All pads = 0.04 g

	*	**
Time filtration (min)	10	75
Vol. cu. ft	20	150
Time from zero	5 h/20 min	5 h/58 min

Table LXV Experiments to check efficiency of filtration. Comparison of many layers of alginate pads exposed shortly after aerosol generation and several hours later. Four-layer plate method (2) used for phage detection.

	Plaque counts per pad			
Time filtration (min)			10	10
Vol. cu. ft			25*	20
Time from zero (min)			12.5	11
			Av. of 3 or 6	Av. of 2 or 4
Pad 1 First			$>5.0\times10^{6}$	5.3×10^{7}
" 2			8.7×10^{5}	1.5×10^{6}
" 3			9.4×10^{4}	3.8×10^{5}
" 4			3.8×10^{4}	3.8×10^{4}
" 5			2.6×10^{4}	1.0×10^{4}
" 6			1.8×10^{4}	3.1×10^{3}
" 7 Last			1.2×10^{4}	9.0×10^{2}
Time filtration (min)	45	45	45	45
Vol. cu. ft	90	90*	90*	90
Time from zero	3h/ 23 min	3h/ 30 min	4h/ 20 min	4h/ 33 min
	Av. of3	Av. of3		
Pad 1 First	4.7×10^{5}	6.0×10^{5}	3.6×10^{4}	9.5×10^{4}
" 2	1.6×10^{5}	1.1×10^{5}	4.8×10^{3}	1.5×10^{4}
" 3	6.5×10^{4}	2.3×10^{4}	1.0×10^{3}	4.9×10^{3}
" 4	1.7×10^{4}	3.4×10^{3}	3.0×10^{2}	2.9×10^{3}
" 5	8.9×10^{3}	1.2×10^{3}	3.0×10^{2}	9.0×10^{2}
" 6	6.2×10^{3}	5.0×10^{2}	$<1.0\times10^{2}$	2.0×10^{3}
" 7 Last	3.6×10^{3}	1.0×10^{2}	$<1.0\times10^{2}$	1.1×10^{3}

Time from zero = half-way through phage distribution (zero) to half-way through alginate estimation.

All pads = 0.04 g

* assumed 2 cu. ft min

undertaken in this study. In these experiments there should be initially a great number of various sized droplets containing phage, but several h after aerosol generation ceases the heavy droplets should have settled and only a fine mist of phage should be left.

If both large and fine particles were being trapped by the fibres of the alginate wool, the fall-off in the collection of particles from first to last pads should be somewhat similar for the initial and the final analyses several hours later. Consideration of the figures showed that there was a decrease in counts from the first to the last pads at times several h from zero, and this therefore indicated that fine particles were being collected.

Triplicate Analyses

It was suggested that triplicate analyses be made of an aerosol sampled several h after phage distribution.

This was carried out in three experiments, and figures from two of these experiments are given on p. 53.

Certain anomalous results were obtained, the count per pad being often lower the greater the equivalent amount of the neat phage suspension that was present per analysis. It seemed that the amount of sodium hexametaphosphate present might have affected the results. This led to an investigation of the role of sodium hexametaphosphate in phage work, and results have been presented and discussed in the appropriate section in the text.

(ii) SUMMARY AND CONCLUSIONS FOR THIS SECTION

Work described in this section dealt with:

The preparation of an aerosol.
The time of survival of an aerosol.
Factors bearing on the estimation of phage in aerosols by filtration through calcium alginate wool.

Aerosols were successfully prepared by forcing air through a rubber tube while phage suspension was injected into the rubber tube by means of a hypodermic syringe. The discharge end of the rubber tube was partially closed by a tapered glass tube or needle.

Phage particles in the air gradually settled out, but some remained suspended in the air for several h.

Some of the phage in the air could be displaced from the room by a fan.

A tube of 0.73 in. diameter was suitable for holding calcium alginate pads. Several 0.04 g calcium alginate pads were needed for a reliable collection of most of the phage particles in a given volume of air. The pads appeared to collect even the tiny phage particles.

B. FACTORY INVESTIGATIONS

B. FACTORY INVESTIGATIONS

(i) PHAGE IN AIR IN TYPICAL NEW ZEALAND FACTORIES

Initial investigations of airborne phage distribution were made at Mangawhata Dairy Co. and at the processing plant of the New Zealand Dairy Research Institute. The main investigations were carried out at three factories, viz. Tui (formerly Ruahine) Co-op. Dairy Co. (Woodville Branch), a conventional cheese factory; Tararua Co-op. Dairy Co., a mechanized factory (Cheddarmaster); and Wairarapa Co-op. Dairy Co. (Belvedere Factory), a mechanized factory (Cheddarmaster). The results obtained at each of these three factories are first described in detail and then the significance of the observations is discussed.

Outline of Procedure

The methods used for the study of airborne phage in dairy factories have been described earlier, but some of the work described in this section was carried out before certain improvements were developed.

Apparatus was prepared and sterilized (where necessary) at the New Zealand Dairy Research Institute laboratory, and was transported by car to the cheese factory. The air filtration apparatus was set up on its table at defined locations within the factory, and air sampling by filtration was carried out at specific times during the morning and afternoon. At intervals throughout the day, plates of agar medium and plates containing 10 ml of liquid were exposed for definite times at strategic positions in the factory. Whey samples were taken on occasions, mostly at running and salting, and an occasional milk sample was also taken.

On return to the laboratory, analyses were carried out as soon as practicable. Where necessary, samples were kept in a refrigerated room until analysed.

The data accumulated from the analyses provided information about the distribution of phage in the factory throughout the day, and some about the relationship between stage of the cheesemaking

process and the phage content of the air. However, a clear-cut picture of variations in airborne phage at any particular stage of the cheesemaking process was marred at times by the drift of phage from other parts of the making room. The multiple-batch nature of the cheesemaking process and associated setting of vats at different times, the distribution into the air of phage from curd and whey by the vigorous but intermittent operations of the process, and phage distribution from the whey separator, all tended to complicate the picture of phage level at any particular spot.

(ii) TUI CO-OP. DAIRY CO. (WOODVILLE BRANCH)

Description of Factory

This factory was located on the outskirts of Woodville.

Seven cheese vats were situated in a long cheesemaking room. (Fig 9.) On one side of this room there was an alcove in which were placed two separators, which ran for 2-3 h from approximately 11.00 a.m.

Two extractor fans situated at the apex of the gable roof removed air from the factory making room. A large inlet fan, also placed at the apex of the roof, was fitted with a filter to remove as much extraneous matter as possible from the air entering the making room.

Comments on Results Presented in the Tables

Investigations at this factory were carried out as detailed under "Outline of Procedure", and results are presented in Tables LXVI-LXIX.

The phage counts at this factory were characteristically low. Whey titres were not particularly high; a count of 10^5 or 10^6 per ml is not specially high for whey sampled late in the cheesemaking process, e.g. at salting.

In Table LXVI, agar sedimentation counts near the separator

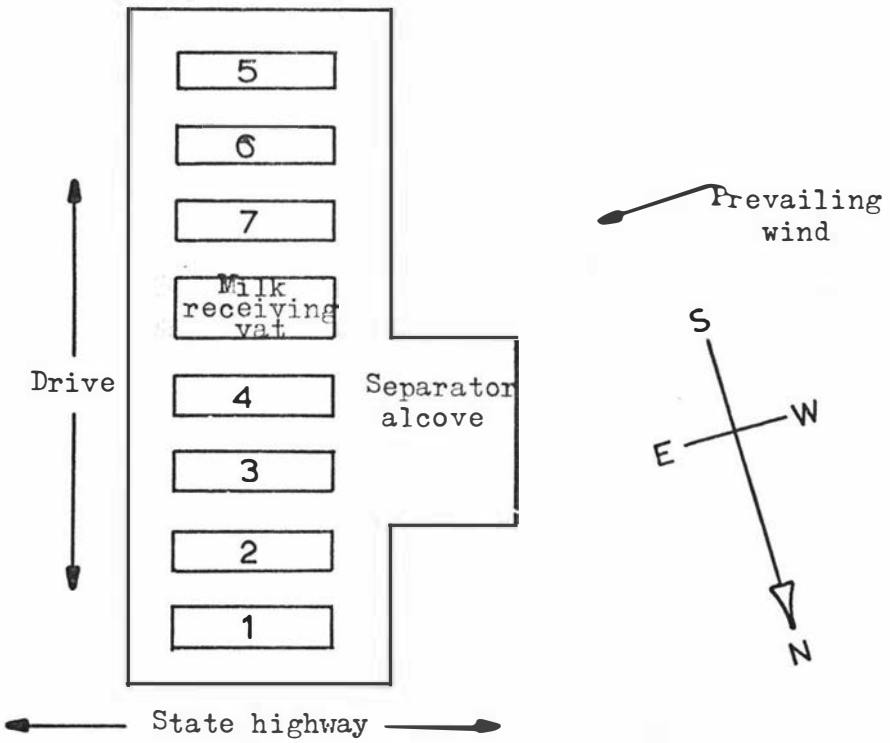


Fig 9. Layout of equipment in the cheesemaking room, Woodville Dairy Factory, Tui Co-op. Dairy Co.

were fairly high and counts by vats 1 and 5 rose distinctly about 4 h from setting. Air filtration sampling was not done at this stage of the investigation.

In Table LXVII, counts in the separator alcove and by the vats were nil or very low. The air filtration method showed a rise to 600 per 20 cu. ft 25 min after salting vat 2, but sedimentation plates exposed 55 min later did not detect phage.

In Table LXVIII, Expt 1, counts by all methods were nil or very low. In Expt 2 there was a marked rise in phage count about salting time in the separator alcove (plate and liquid counts), and near vat 2 (air filtration and agar plate counts). Wheys at approximately this time gave titres $2.7\text{-}5.0 \times 10^5$.

Table LXVI Woodville: phage counts on LYPA plates exposed at intervals throughout the day in the cheese factory.

Starter cultures: HP, E_8 and BR_4 grown together in bulk starter vessel. Counts are for hp phage.

Time	Separator room in	Separator room near	Receiving vat	Vat 1 Set: 8.15am Dry: 10.55am Salt: 1.25pm	Vat 5 Set: 9.00am Dry: 11.40am Salt: 2.15pm
a.m.					
10.30	152		5	3	2
11.00	67		1	0	1
11.30	>127		1	1	0
12.00	>104		10	0	1
p.m.					
12.37	100		13	19	3
1.00		148	24	13	15
1.30		14	39	53	163
2.00		>50	12	4	25
2.30		139	58	19	47
3.00		11	12	2	50
3.30		>170	14	2	14
3.33	>1000*				
4.00		167	56	29	24

Notes: * = 11 plates.

Plates were LYPA medium spread with HP starter. They were exposed to the atmosphere for 10 min. Ca was not included in the medium. Plates near vats 1 and 5 were exposed on boards on agitator beams, 6-7 ft above floor level. Vat 1 plates were exposed on window sill at 1.00 p.m. and 1.30 p.m.

Plates on receiving vat were on a board near the corner of the vat. Most plate counts were the averages of two plates.

Plates were exposed in the starter room for 1 h (1.05-2.05 p.m.). They gave counts of 3, 4 (bench), 0, 4 (box) per plate.

Titres were determined by spotting serial decimal dilutions of whey on LYPA plates spread with HP starter.

(cont'd)

Table LXVI (cont'd)

Titres of wheys

Vat	Time	Stage	Time from set h/min	Titre
1	10.50am	Running	2.35	10^{-2}
1	1.30pm	Salting	5.15	10^{-5}
4	11.10am	Just before running	2.25	10^{-1}
5	11.25am	" " "	2.25	10^{-2}
5	2.30pm	Salting	5.30	10^{-5}

Table LXVII Woodville: phage counts by air filtration, on exposed LYPA plates, and in exposed dishes of salt-peptone water, at intervals throughout the day in the cheese factory.

Starter cultures: E_8 (2/3 inoc.) and H_2 (1/3 inoc.) grown together in bulk starter vessel. Counts are for e8 phage.

				Vat 2				Vat 4(Colby)		Vat 6	
				Set: 8.10am				Set: 8.30am		Set: 9.00am	
				Dry: 10.55am				Dry: 11.45am		Dry: 11.40am	
				Salt: 1.05pm				Salt: 1.15pm		Salt: 1.50pm	
	Separator		Air count 20 cu.ft	Plate		Liq. 10ml		Plate	Liq. 10ml	Plate	Liq. 10ml
Time	Plate	Liq. 10ml	End	End	Side	End	Side	Side	Side	Side	Side
a.m.											
10.00	0	<100	50	0	0	<100	<100	0	<100	0	<100
10.30			<50								
11.00	0	"	"	0	0	"	"	0	"	0	"
11.30			"								
12.00	0	"	"	0	0	"	"	0	"	0	"
p.m.											
12.30			100								
1.00	8	"	150	0	1	"	"	0	"	0	"
1.30			600								
2.00	0	"	-	0	0	"	"	0	"	0	"
2.30			<50								
3.00	0	"	100	0	0	"	"	2	"	0	"
3.30			<50								
4.00	0	"	100	0	0	"	"	1	"	0	"

Notes:

LYPA plates were spread with E_8 starter on the day before the experiment, and were kept cool until used. (Ca not added.)

Liquids were examined by method which included use of Ca.

Vat 5: Set: 8.45am, Dry: 11.25am, Salt: 1.35pm
" 7: " 9.15am, " 12.20pm, " 1.50pm.

Separators: Both on 10.30am. One off 12.45pm, other off 12.55pm.

Whey counts

Vat	Time	Stage	Time from set h/min	Count/ml
2	11.00am	Drying	2.50	1.2×10^5
2	1.05pm	Just before salting	4.55	6.4×10^6
5	11.25am	Running	2.40	3.0×10^2
6	2.40pm	Hooping	5.40	1.7×10^4
7 (Colby)	3.10pm	Hooping (last bucket)	5.55	2.2×10^5

Table LXVIII Woodville: phage counts by air filtration, on exposed plates, and in exposed dishes of liquid, at intervals throughout each of two days in the cheese factory.

Starter cultures: HP (1/3 inoc.) and AM_2 (2/3 inoc.) grown together in bulk starter vessel on both days. Counts are for hp phage on each day.

	EXPT 1 Vat 2 Set: 8.05am Dry: 10.50am Salt: 1.21pm							EXPT 2 Vat 2 Set: 8.35am Dry: 11.20am Salt: 1.50pm						
	Separator room		Air count 20 cu. ft	Plate		Liq.		Separator room		Air count 20 cu. ft	Plate		Liq.	
Time	Plate	Liq.	End	End	Side	End	Side	Plate	Liq.	End	Vat End	Press Side	Vat End	Press Side
a.m.														
7.30	0	<50	<50	0	0	<50	<50							
8.00	"	"	50	"	"	"	50							
9.00	"	"	<50	"	"	"	<50	0	<100	<50	0	0	<100	<100
10.00	"	"	"	"	"	"	"	"	"	"	"	"	"	"
11.00	-	50	"	"	"	"	"	"	"	"	2	"	"	"
12.00	0	<50	50	"	-	"	"	"	"	"	0	"	"	"
p.m.														
1.00	"	"	<50	"	1	"	"	1	"	50	"	3	"	"
2.00	-	-	"	"	0	"	"	1,200	109,000	700	16	25	"	100
3.00	1	<50	"	"	"	"	"	22	<100	<50	0	0	"	<100
4.00	"	"	"	"	"	"	"	0	"	"	"	"	"	"

Notes:
Milk entering Vat 3. Phage absent in 10 ml.
Vats 2-7: Set: 8.05-9.10 a.m.
Separator: ran 10.38 a.m.-approx. 12.15 p.m.
Times for commencing plate exposures not precise.

Notes:
Air filtration 6 ft away at 1.00 and 2.00 p.m.
Milk entering Vat 2. Phage absent in 10 ml (HP)
Vats 2-7: Set: 8.35-9.30 a.m.
Separator: ran 10.55 a.m.-1.50 p.m.
Times: as for Expt 1.

(cont'd)

Table LXVIII (cont'd)

EXPT 1 Whey counts					EXPT 2 Whey counts				
Vat	Time	Stage	Time from set h/min	Count/ml	Vat	Time	Stage	Time from set h/min	Count/ml
2	9.13am	Stirring	1.8	<10	2	11.09am	Whey half run	2.34	<10
2	10.13am	"	2.8	10	2	12.14pm	Stagnant	3.39	7.9×10^3
2	10.43am	Near end running	2.38	<10	2	1.15pm	After raking	4.40	5.0×10^5
2	1.21pm	Salting	5.16	3.9×10^4	2	1.55pm	Just salted	5.20	2.7×10^5

Experiment to Find Average Number of Phage Particles in Each Droplet of Whey

Experiments were carried out at Woodville to find the number of phage particles in whey droplets. The method used was to expose agar plates spread with culture alongside broth or Ringer's solution in a Petri dish in a phage atmosphere. After incubation and counting of agar plates, and analysis of broth (by spreading a known volume on a plate already spread with culture, incubating, and counting) the counts were compared.

The results are shown in Table LXIX. As this experiment was carried out before the role of calcium in phage estimations was investigated, accurate counts could not be obtained. Nevertheless, results showed that the average whey droplet contained a considerable number of phage particles, and that this number could vary considerably.

Table LXIX Woodville: investigation of number of phage particles in individual whey droplets.

			Agar plates		Broth or Ringer's		No. particles
Time(pm)	Place		No. of plates	Av. count	No.	Count*	per droplet
12.55-1.05	Separator alcove	†	5	>217	1	57,100	<263
1.28-1.38	"	††	6	31	1	600	19
1.50-2.00	"	††	6	15	1	100	7
2.10-2.20	"	††	14	10	1	9,000	900

* This is count for total exposed volume, calculated from fraction of this volume used for analysis. Counts are recorded to the nearest hundred.

†= corner ††= diagonally opposite

Notes:

The separator was stopped at 1.15 p.m. No reason can be advanced for the high broth count in the 2.10-2.20 p.m. determination except perhaps that the airborne droplets may have been larger than usual at this time and hence contained more phage particles.

Some plates were spread with coagulated milk culture, and some with broth culture. Counts were generally lower on broth culture plates than on milk culture plates.

Conclusions:

Experiments at Woodville showed a characteristically low phage level in the air and in the whey.

Droplets of whey contained from a few to a high number of phage particles.

(iii) TARARUA CO-OP. DAIRY CO.

Description of Factory

The Tararua Dairy Factory is a mechanized cheese factory with Cheddarmaster equipment and Large Hoops. There were four large vats situated as shown in the plan of the factory (Fig 10) and, of these, three vats were generally used.

Two different starter strains were used each day, on a four-day rotational basis. The two starters were grown in one bulk starter vessel and the appropriate volume of the mixture was pumped into the vat. The starter was added to the vat 10 min before setting.

The vats were set at 9.05, 9.30, and 9.50 a.m., respectively.

The Cheddarmaster unit was started at 11.30 a.m. Curd and whey were pumped to the Cheddarmaster draining conveyor. The curd was drained and was eventually deposited in cheddar boxes. About 2 h later the curd was milled and salted on the same Cheddarmaster unit. From here the curd went to the presses (Large Hoops and some conventional 40-lb hoops). Washing of the Cheddarmaster unit was finished by about 3.30 p.m. Fans were turned on or off to maintain pleasant working conditions throughout the day; they were usually run for about 6-8 h per day.

A separator was situated in a corner of the cheese room. The separator ran from approximately 11.30 a.m. to 1.45 p.m. The whey was piped to a tank outside the factory and the cream was discharged into a container inside the making room.

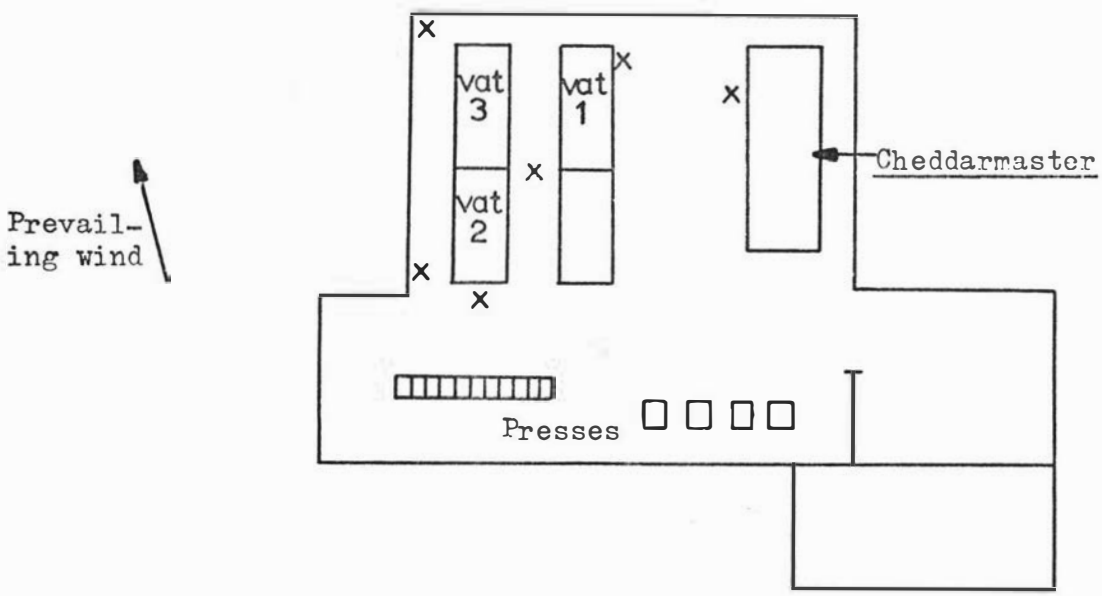


Fig 10. Layout of equipment in the cheesemaking room at the Tararua Dairy Factory. A cross identifies each sampling point used.

Comments on Results Presented in the Tables

Investigations at this factory were carried out on four occasions by the procedure detailed under "Outline of Procedure". The results are presented in Tables LXX-LXXIII.

Several points of interest were apparent from this first experiment (Table LXX). The alginate filtration method revealed a regular increase in airborne phage particles to a maximum at 1.30-2.30 p.m. and then a slight decrease. Large and fine droplets were collected in this method, and the figures served as a standard against which the sedimentation counts could be compared. Sedimentation counts started to rise appreciably at noon, and high counts were obtained by sedimentation and filtration methods from 1.30-3.30 p.m. Sedimentation counts fell rapidly by 4.00 p.m.

Meantime, counts near the separator were high at noon, 1.00 p.m. and 2.00 p.m. Whey counts were high by noon (vat 2, 1.3×10^8 at 11.55 a.m.).

A high phage content of the air was associated with dispersal of whey of high phage titre at running, and then by further vigorous agitation of product at milling and salting at which time the phage content of the whey was very high.

In the early stages of the process some of the airborne phage detected could result from splashing of whey during operation of the vats.

Highest sedimentation counts were obtained near the Cheddarmaster and the separator.

In the second experiment (Table LXXI) a series of results similar to those in Table LXX was obtained, but both whey counts and atmospheric counts were lower. It was noted that there was a very rapid fall in counts from 3.00 p.m. to about nil at 4.00 p.m.

In the third trial (Table LXXII) the results followed a pattern similar to those obtained earlier but counts were higher. Whey counts of up to 2.8×10^9 were obtained, and some sedimentation and air filtration counts were very high. At 4.00 p.m., counts were

still appreciable. From notes it will be seen that the incoming milk contained hp phage. It is relevant to note that at this factory four suppliers took whey home for pig-feeding and this may have led to contamination of the milk with phage.

The results obtained in the fourth, and last, trial at Tararua (Table LXIII) showed whey counts which were exceptionally high. Some of the sedimentation and air filtration counts were also very high. The pattern of the results was similar to that for earlier trials at Tararua. In spite of the very high counts obtained at 3.00 p.m., counts at 4.00 p.m. were low, and practically nil at 4.45 p.m.

Table LXX Phage counts on exposed agar-medium plates, in exposed dishes of liquid, and by air filtration, at intervals throughout one day at the Tararua mechanized cheese factory.

Starter cultures: HP and AM_1 grown together in bulk starter vessel. Plates and liquid exposed for 10 min. Counts are for hp phage.

	By Cheddar-master		Side vat 1 (towards Cheddar-master)		By separator		Wall oppos. vat 2		Vat 2 end		Vat 2 end 20 cu.ft air
Time	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	
a.m.											
7.03	0	<50	0	<50	3	<50	466	<50			
7.40											<50
8.00	0	"	4	50	0	"	16	"			
8.30											"
9.00	0	"	3	1000	0	"	0	"			
9.45											50
10.00	9	"	7	1800	6	"	200	"			
10.30											<50
11.00	2	"	5	<50	3	"	0	"			
11.30											150
12.00	16	"	19	"	596	300	23	"			
p.m.											
12.30											300
1.00	44	"	63	50	1452	800	111	"			
1.30									15	<50	5750
2.00	275	126000	169	350	151	2250	37	250			
2.30									17	50	5050
3.00	118	200	69	"	19	<50	35	<50			
3.30											1850
3.36									41	50	
									16		
4.00	0	<50	0	<50	0	<50	0	<50			
Next	3	<22	1	-	30	5244	4	<22	1	22	333
day	3	<22	3				17		2		

(cont'd)

Table LXX (cont'd)

Whey from vat 2

Time	Stage	Time from set	Count/ml
a.m.		h/min	
10.13	Commenced stirring	.43	1.0×10
10.40	Stirring	1.10	2.3×10^{3}
11.55	Started to run	2.25	1.3×10^{8}
p.m.			
12.38	Cheddaring box	3.08	5.8×10^{6}
2.35	Milling	5.05	1.5×10^{9}
2.43	Salting	5.13	1.1×10^{8}
2.54	Over end	5.24	4.0×10^{9}

Notes:

Plates for exposure, 3 layers (third layer TSA with culture mixed in).

Vat times

Vat	Set	Dry	Salt
	a.m.	a.m.	p.m.
1	9.05	11.40	1.58
		p.m.	
2	9.30	12.05	2.43
3	9.50	12.26	3.04

Examinations made the following day were undertaken in the afternoon, using 1 h air filtration and $\frac{3}{4}$ h plate exposures. Results are calculated to the usual 10 min. HP starter was not used this day and the counts reflect residual airborne material plus phage liberated by removal of cheese from the presses in the morning and from cold curd (trimmings from the pressed cheese) returned to the vats.

Fourteen agar-medium plates were exposed at 4.00 p.m. (for 10 min) on the ground outside the factory:

12 plates = 0

2 " = 1 plaque each.

Table LXXI Phage counts on exposed agar-medium plates, in exposed dishes of liquid, and by air filtration, at intervals throughout a second day in the Tararua mechanized cheese factory.

Starter cultures: HP and AM_1 grown together in bulk starter vessel. Plates and liquid exposed for 10 min. Counts are for hp phage.

Time	By Cheddar-master Plate	Liq.		Side vat 1 (towards Cheddar-master) Plate	Liq.	By separator Plate	Liq.	Wall oppos. vat 2 Plate	Liq.	Vat 2 end 20 cu.ft air
a.m.										
8.35	1	<50		2	<50	0	<50	0	<50	<50
10.00	0	"		126	17850	0	50	1	"	"
11.00	9	"		-	-	1	<50	1	"	"
12.00	16	"		70	50	232	"	32	"	300
p.m.										
1.00	55	"		46	<50	960	700	93	"	"
2.00	75	50		121	"	150	250	175	100	2550
Ca*	85			131		60		84		
2.40			Alcove 109 †							
2.40			Storage room 3 ††							
3.00	205	150		233	400	80	<50	333	100	450
Ca*	208			188		87		252		
4.00	0	<50		0	100	0	50	0	<50	<50
Ca*	2			6		0		0		
Next a.m. approx.										
8.30	<1	<17		1	<17	1	<17	1	<14	<10

Counts on whey from vat 2

Time	Stage	Time from set h/min	Count/ml
11.12am	Mixing in vat	1.42	3.0×10
12.03pm	Towards end running	2.33	6.1×10^4
1.58pm	Milling - just before salting	4.28	1.1×10^7

Notes:

* = $CaCl_2$ in culture mixed with TSA.

† = Near Cheddarmaster and in alcove off make room (count is av. of 4).

†† = Door of storage room mostly shut (count is av. of 8).

Plates were 3-layered, culture mixed with TSA in 3rd layer.

(cont'd)

Table LXXI (cont'd)

Vat times

Vat	Set	Dry	Salt
	a.m.	a.m.	p.m.
1	9.05	11.45 p.m.	1.50
2	9.30	12.05	2.10
3	9.50	12.30	2.35

Tests for hp phage in milk

Sample day of experiment. From vat before starter.		Sample taken next day from vat. Starter in other end.	
	Phage		Phage
10ml milk	+	10ml milk	+
1ml "	+	1ml "	-
0.1ml "	-	0.1ml "	-

Examinations made the following day were undertaken in the morning, using 50 min air filtration and 30 min plate exposures. Results are calculated to the usual 10 min. Phage could have been present in the atmosphere as explained in the notes on the previous experiment, but apparently there was very little when the tests were carried out.

Table LXXII Phage counts on exposed agar-medium plates, in exposed dishes of liquid, and by air filtration, at intervals throughout the third day at the Tararua mechanized cheese factory.

Starter cultures: HP and AM_1 grown together in bulk starter vessel. Plates and liquid exposed for 10 min. Counts are for hp phage.

Time	By Cheddarmaster		Side vat 1 towards Cheddarmaster		By separator		Wall oppos. vat 2		End between vats 1 & 3		End between vats 1 & 3
	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	20 cu. ft air
a.m.											
9.15	0	<100	3	<100	2	200	0	<100	2	<100	50
10.00	3	<100	37	-	2	<100	0	<100	3	<100	<50
11.00	0	<100	0	900	13	100	0	<100	1	<100	100
12.00	21	<100	9	-	174	<100	10	<100	4	-	<50
p.m.											
1.10	91	200	209	1600	772	2000	325	100	162	300	10200
2.00	7	600	46	2400	10	1100	2	100	4	100	6200
3.05	707	5600	712	5800	137	52400	362	16300	640	8200	467100
4.00	436	1900	36	-	6	300	4	2900	28	100	1900

Counts on whey from vat 1

Time	Stage	Time from set h/min	Count/ml
a.m.			
10.30	Stirring curd and whey	1.10	1.2×10^3
p.m.			
12.10	Started pump curd and whey to tower	2.50	5.8×10^5
2.32	Cheddar tower before milling	5.12	2.8×10^9
2.47	Cheddarmaster milling	5.27	1.2×10^9
2.55	After salting	5.35	2.7×10^9

(cont'd)

Table LXXII (cont'd)

Vat times

Vat	Set	Dry	Salt
	a.m.	p.m.	p.m.
1	9.20	12.17	2.50
3	9.45	12.43	

Tests for hp phage in milk*

Sample of milk from vat 1 (9.15 a.m.)

	Phage
10ml milk	+
1ml "	+
0.1ml "	+
0.01ml "	-

* Sample may have contained starter.

Cheddaring tower, not cheddaring boxes, was used for cheddaring on this occasion. There was milk in vats 1 and 3 only.

Air filtration apparatus was stationed in the passageway between vats 1 and 2, and near the junction of vats 2 and 3.

Agar-medium plates exposed in factory several yards from vats. 3.55-4.05 p.m.

Plaque counts per plate	
23	63
18	30
23	27
32	39
23	28

Separator running 12.05-1.15 p.m. (approx.)

Table LXXIII Phage counts on exposed agar-medium plates, in exposed dishes of liquid, and by air filtration, at intervals throughout the fourth day at the Tararua mechanized cheese factory.

Starter cultures: HP and AM_1 grown together in bulk starter vessel. Plates and liquid exposed for 10 min. Counts are for hp phage.

Time	By Cheddarmaster		Side vat 1 towards Cheddarmaster		By separator		Wall oppos. vat 2		Vat 2 end			By Cheddarmaster	
	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	20 cu. ft air	20 cu. ft air	Plate
a.m.													
9.35	2	<100	15	<100	7	<100	25	<100	1	<100		800	
10.15	0	"	304	1000*	23	"	1	"	0	100		100	
11.00	"	"	1	<100	1	"	1	100	7	<100		50	
12.00	"	"	0	"	0	"	0	<100	2	800		100	
p.m.													
1.00	143	100	169	300	700 approx.	5400	201	100	133	400		5050	
2.00	87	200	32	400	29	100	49	300	80	600		11100	
3.00	473	17600	>500	703000	440	16300	427	11200	541	39400		838500	448†
4.00	11	<100	6	<100	4	<100	2	200	0	<100		550	
4.45	0	"	0	"	1	"	0	100	0	"		<50	
Next day a.m.													
6.30	3	300	26	<100	1	<100	1	<100	0	100	200		
7.33	0	<100	2	"	1	"	0	"	0	<100	<50		
8.25	28	300	20	100	13	"	2	"	19	600	4050		

* Splashed?

† Average of 4.

(cont'd)

Table LXXIII (cont'd)

Counts on whey from vat 2

Time	Stage	Time from set	Count/ml
a.m.		h/min	
11.25	Stirring curd and whey	2.05	1.9×10^5
p.m.			
12.14	Started pumping	2.54	2.8×10^6
2.58	Start Cheddarmaster (vat 2)	5.38	6.3×10^{10}
3.09	Salting	5.49	7.0×10^{10}

Notes:

Vat times

Vat	Gal	Set	Dry	Salt
		a.m.	p.m.	p.m.
2	1875	9.20	12.20	2.35
3	1475			

Vat 2 slightly slow this day.

Tests for phage in milk

Vat 2 ml milk*	Next day		
		Past. from bag*	Vat*
	Phage	Phage	Phage
10	+	+	+
1	+	+	+
0.1	+	-	+
0.01	-	-	+
0.001			-

* No starter in milk sample as taken.

Separator ran 12.24-1.15 p.m.

Determinations next day. During the period from 6.50 a.m. until 8.35 a.m. the 40-lb cheeses from the conventional presses were unwrapped, bandages were scraped, and cheese was cut out from the Large Hoops. The 2nd and 3rd "next day" determinations of airborne phage (below the dotted line) were made while these operations were taking place.

(iv) WAIRARAPA CO-OP. DAIRY CO. (BELVEDERE FACTORY)

Plant Layout at Belvedere Cheese Factory

Belvedere Cheese Factory has closed down since the following results were obtained. It was located a short distance from Carterton. The layout of the making room is shown in Fig 11. The factory had six 2,600-gal vats which were filled up to three times a day and thus 46,800 gal of milk could be dealt with daily. There were two Cheddarmaster conveyors, one for draining, and one for mellowing and salting. Conventional presses were located in the centre of the factory.

The whey separator was located in a room adjacent to the cheese vats. A door from this room opened into the factory opposite the cheese vats, and this door was opened on occasions. The separator ran from between 11.00 and 11.30 a.m. to about 4.30 p.m., with short shut-downs.

A large ventilation unit with a relatively low efficiency filter was located at the end of the making room farthest from the vats, i.e. near the Cheddarmaster equipment. This unit was installed between first and second surveys of airborne phage.

Investigations at this factory were carried out as detailed under "Outline of Procedure", and results are presented in Tables LXXIV-LXXXI.

(The factory plan and details are from a brochure prepared by the New Zealand Dairy Research Institute, to inform and guide visitors to the Belvedere Factory.)

In the early stages of the study ML_8 was used as test organism but later HP was adopted because this strain was used in the other factories studied.

Comments on Results Presented in the Tables

In the first survey (Table LXXIV) an increase in phage count was detected about the time of running of the first vat. Atmospheric phage counts were much higher by the Cheddarmaster than near the vats.

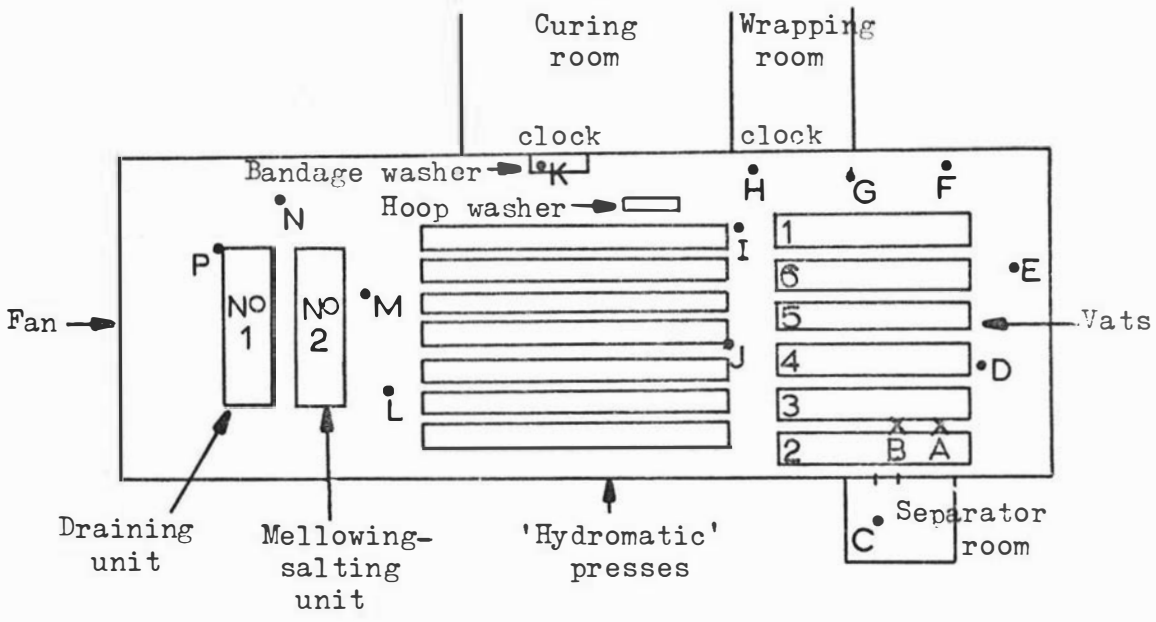


Fig 11. Layout of equipment in the cheesemaking room at the Belvedere Dairy Factory. A letter identifies each sampling point used.

When the door to the separator room was opened at 2.05 p.m. the sedimentation count (which had been 167 at 1.55 p.m.) rose to >1,375.

In the second survey (Table LXXV) counts were moderately low throughout the day, and were no higher in the vicinity of the Cheddarmaster equipment than near the vats.

Counts obtained throughout the third survey (Table LXXVI) were very low, except in the separator room at 3.00 p.m. and 3.30 p.m.

During the fourth survey (Table LXXVII) the phage counts in the vicinity of the Cheddarmaster equipment started to rise soon after running commenced. Near the vats, counts did not rise much until about 2 h later. High counts were obtained in both areas about 3.30 p.m.

In the fifth survey (Table LXXVIII) most counts remained very low until 3.00-3.30 p.m.

Between the fifth and sixth surveys the analytical method for estimating phage in whey was changed and calcium was also added to the medium (see *).

The host organism was ML_8 for surveys one to five and HP for the sixth and seventh surveys.

In the sixth survey (Table LXXIX) whey counts, sedimentation counts, and air filtration counts were all very high. Phage p_2 grows on HP, and as both P_2 and HP were used as starters this day the high initial counts were no doubt due to detection of p_2 phage on the HP test organism.

In the seventh survey (Tables LXXX, LXXXI) counts were fairly high about $\frac{3}{4}$-1 h after the first vat was run. About the time milling commenced, the air filtration method of phage detection showed a very marked increase in count. Whey counts were fairly high this day.

This was the first Belvedere experiment in which calcium was incorporated in the exposed plates.

Unwrapping, weighing and wrapping of cheeses.

As is usual the day following manufacture, the cheeses were taken from the presses and the bandages were taken off and flung into a heap as a prelude to gathering up for washing. The cheeses were then partially scraped. The cheeses were next taken into a wrapping room (Fig 12) where they were weighed, cut to adjust weights, and then wrapped. Some of these movements could result in phage from the cheeses and bandages being dispersed into the atmosphere. Plate exposures were made with the results shown in Table LXXXI.

* Relationship between plaque count of whey expressed as "titre" and as "count per ml"

In early experiments (up to fifth survey inclusive) phage in whey was estimated by spotting loopsful of serial decimal dilutions of phage in Ringer's solution on a mat of the host organism spread on LYPA. Calcium was not added. The highest dilution giving plaques was called the "titre".

In later experiments (sixth and seventh surveys) phage was estimated by plating 0.1 ml quantities of phage suspension with medium containing added calcium. The serial decimal dilutions of phage were prepared in peptone-salt solution.

Obviously the second method gave higher counts than the first.

A rough relationship between counts by the two methods is illustrated by the following hypothetical case:

Count by first method: if two plaques were obtained at 10^{-2} dilution (titre 10^{-2}), then count per ml =
$2 \times 250 \times 100 = 5 \times 10^4$ per ml
(a loopful is approximately 0.004 ml).

Count by second method: would be approximately 10 times greater (or more) than by first method = 5×10^5 (or more) per ml.

Results in Table LI show that the addition of calcium to the medium can cause more than a ten-fold increase in plaque count.

Table LXXIV Making details and plaque counts obtained with a slit sampler and by exposing agar-medium plates at intervals throughout the day at the Belvedere mechanized cheese factory.

Time	Vat numbers and details 1	2	3	4	5	6
a.m.						
10.50		Stir				
11.27	C.Run					
11.45	N.F. Run	Stir	Stir	Stir	C.S.	
12.00		C.Run	"	"	Stir	
p.m.		N.F.				
12.15	Wash	Run	"	"	"	
12.45			N.F. Run	"	"	Stir
1.00			Wash	"	"	"
1.15				N.F. Run	"	"
1.30					"	"
2.00					Empty	"

C.S. = commenced stirring; Stir = stirring
C.Run = commenced running; Run = running
N.F.Run = nearly finished running; Wash = washing vat

Time	Slit sampler ½ cu. ft (A)	Sedimentation counts (10 min) Near vats 2, 3 (A)	Time	Cheddarmaster area Near wall (N)	Near rope conveyor (L)
a.m.			a.m.		
10.50	0				
11.00	0	0			
11.15		1	11.15	0	0
11.20	1				
		First vat run			
11.40	0	22	11.45	105	110
12.00	18	34			
p.m.			p.m.		
12.20	19	95	12.15	895	100
12.35	18	22	12.45	277	127
12.50	28	88	1.15	478	142
1.25	127 (B)	83 (B)	1.45	641	268
1.55	144	167			
2.05		>1375			
2.10	∞				
2.15		1589	2.15	728	370
			2.45	688	239
3.20	97	380	3.20	445	70
3.40	296	79	3.45	1171	229
3.50		126			
3.52	712				
4.00	909	410			

∞ = probably > 500

(cont'd)

Table LXXIV (cont'd)

	Cheddarmaster operations
a.m.	
	Stirrers rotating - water rinse.
11.30	No curd on No. 1 conveyor until approx. 11.30 a.m.
11.45	No. 1 being washed, draining curd
p.m.	
12.15	Some boxes turning (cheddaring)
12.45	" " "
2.15	Draining terminated about this time

Notes:

For location of sampling points A,B, N and L see Fig 11.

1.55 p.m. Sampling (plates, slit sampler) moved from "A" to "B".

2.05 p.m. Separator door opened (opposite "B" location).

3.50 p.m. Separator door shut (separator has been off approx. ½ h).

Plates spread at Belvedere with ML_8 and R_1 clotted cultures.
Results for R_1 are not shown, as most plates had no plaques.

Starters: 1 bulk vessel ML_8
1 " " ML_8 and R_1
1 " " BK_5 and P_1

Starters from bulk starter machines were mixed before use in the vats as required, to give specific combinations.

Table LXXV Making details and plaque counts using agar-medium plates exposed at intervals throughout the second day at the Belvedere mechanized cheese factory.

Vat details			Cheddarmaster area	
Vat 1	12.20–1.20pm	Ran between these times	11.00am	Draining curd in Cheddarmaster conveyor
2	10.54am	Started to run		
3	11.10am	" " "	1.15pm	Milling and salting started
4	11.50am	1/3 run		
5	12.20pm	½ run		
6	12.20–1.20pm	Ran between these times		

Sedimentation counts (10 min)

Time	Near vats 2 & 3 (A) Av. 2	Separator room (C) Av. 2	Time	Cheddarmaster area Near wall (N)	Near rope conveyor (L)
a.m.			a.m.		
10.50	2		10.45	0	0
11.10	0		11.15	3	1
12.00	0		11.45	16	1
p.m.			p.m.		
12.20	2		12.15	18	2
			12.45	14	2
1.20	11		1.15	18	13
1.50	28		1.45	51	39
2.00	25				
2.10	46		2.15		20
2.20	29				
2.30	11				
3.20	16	282			
3.30	10	1			
3.40	24	1			
4.00	5 min.	Two plates each end cheese presses: 151, 25, 14, 21 plaques.			

For location of sampling points A, C, N and L see Fig 11.

Notes:

Separator ran approx. 10.45 a.m.–2.05 p.m.

Door to separator room was open to factory 1.50–2.45 p.m.

Fan had been installed between the first survey (Table LXXIV) and this trial.

The slit sampler was used at times to attempt to find the effect of a filter fitted to it but, because of low counts without a filter, the experiment was unsatisfactory.

Plates LYPA (no Ca added) some spread before, some on day of, experiment, with ML_8.

Starters used this day: ML_8, Z_8, SK_3.

Table LXXVI Making details, whey titres and plaque counts obtained with agar-medium plates exposed at intervals throughout the third day at the Belvedere mechanized cheese factory.

Vat details		
Vat 1	1.00–1.30pm	Run
2		
3	11.30am	½ run
4	12.00pm	½ run
5	12.30pm	2/3 run
6	12.30–1.00pm	Run

Cheddarmaster	
11.10–11.30am	Started draining
2.00pm	Started milling

Whey titres

11.45am	Vat 4	Shortly before running	Neat	1	plaque
12.45pm	" 6	" " "	"	0	"
2.35pm	" 5	Milling	10^{-2}	2	"

Sedimentation counts (10 min) (Av. 2)

Time	Near vat 3 (A)	Separator room (C)	Cheddarmaster area: Near wall (N)	Cheddarmaster area: Near rope conveyor (L)
a.m.				
10.30			0	0
11.00	0	0	0	0
11.30	0	0	0	0
12.00	0	0	1	0
p.m.				
12.30	0	0	1	3
1.00	1	0	1	3
1.30	1	0	1	1
2.00	1	0	1	3
2.30	1	1	2	1
3.00	2	90	1	0
3.30	1	381	1	0
3.55	0–1(8)*		0	0
3.55	Stairway to receiving platform		10 min	1(1), 0(3)
	Edge of press		"	1(1), 0(3)
	Receiving platform		"	1(2), 0(2)
4.00	Packing room		"	0(4)
	Test room		"	1(1), 0(2)

* No. cf plates in parentheses

For location of sampling points A,C,N and L see Fig 11.

Notes:

Separators 11.30 a.m.–3.00 p.m.

Door opened at times to separator room.

Plates LYPA (no Ca added) spread on day prior to this experiment with ML_8.

Starters used in factory: ML_8, SK_3.

Table LXXVII Making details, whey titres and plaque counts obtained with a slit sampler and by exposing agar-medium plates at intervals throughout the fourth survey at the Belvedere mechanized cheese factory.

Vat times			
a.m.	Vat		
11.05	6	Start	run
11.20	1	"	"
11.50	2	"	"
p.m.			
12.10	3	"	"
12.30	4	"	"
12.50	5	"	"
2.40	6	"	"
2.55	1	"	"

Cheddarmaster	
	a.m.
Curd draining	11.05
Estimate to cheddar box	20 min
Estimate cheddaring	1 h 45 min
	p.m.
Estimate milling	1.10
Recorded salting and milling	1.30

Whey titres			
a.m.	Vat		
11.05	6	10^{-2}	Start run
11.35	1	10^{-2}	Half run
p.m.			
12.40	4	10^{-1}	" "
1.15	6	10^{-5}	Milling
1.40	1	10^{-5}	"
2.00	2	10^{-4}	"
2.20	3	10^{-5}	"

(cont'd)

Table LXXVII (cont'd)

Tests started between or at times below	Slit sampler near vats 2 & 3 (A) ½ min Av.2 or 3	Sedimentation counts (10 min) Near vats 2 & 3 (A) Av.2 or 3	Time	Cheddarmaster area Near wall (N) 1 or Av.2 or 3	Near rope conveyor (L) 1 or Av.2 or 3	Press near vats (J) 1 or Av.2 or 3
a.m.			a.m.			
			10.00	0	0	
10.50-58	0	0	11.00	0	0	
11.30-37	1	4	11.30	2	20	2
12.00-04	1	4	12.00	10	15	8
p.m.			p.m.			
12.30-32	2	4	12.30	28	15	15
1.00-06	2	6	1.00	34	25	11
1.30-39	40	54	1.30	40	121	87
2.00-10	57	69	2.00	75	102	174
2.35-44	30	24	2.30	45	85	100
2.45-54	17	19				
3.00-08	25	22	3.00	149	52	67
3.30-39	177	>750* >800(6)**	3.30	103	509	121
3.40		424(8)			2pm moved to beneath cheese mill	

* Of 3 plates, 1 estimated and other 2 almost completely lysed.
** No. of plates in parentheses.

For location of sampling points A,N,L and J see Fig 11.

Notes:

Separator door closed during analysis, as far as was known.

Method whey titres = Serial decimal dilutions in ¼-Ringer's, and spotted on plates.

Plates: LYPA medium (no Ca added) spread day before experiment with ML_8.

Starters: Vats 6,1,2,3,4,5 ML_8 R_1
" 6,1 (refill) AM_2 ML_1

Table LXXVIII Making details, whey titres and plaque counts obtained with a slit sampler and by exposing agar-medium plates at intervals throughout the fifth survey at the Belvedere mechanized cheese factory.

Vat times

a.m.	Vat	
10.35	5	Started to run
11.00	6	"
11.20	1	"
11.40	2	"
12.00 p.m.	3	"
12.20	4	"
1.55	5	"
2.20	6	"
2.35	1	"

Cheddarmaster

	a.m.
Started to run	10.35
Estimate start cheddar boxes	10.55
Estimate start milling	10.55 + 1 h 45 min = 12.40 p.m.

Whey titres

a.m.	Vat		
11.45	2	Neat	Half run
p.m.			
1.50	6	10^{-3}	Milling
3.50	4	10^{-3}	Salting

(cont'd)

Table LXXVIII (cont'd)

Tests started between or at times below	Slit sampler near vats 2 & 3 (A) ½ min Av.2 or 3	Sedimentation counts (10 min)			
		Near vats 2 & 3 (A) Av.2 or 3	Cheddarmaster area Near wall (N) Av.2 or 3	Near rope conveyor (L) Av.2 or 3	Press near vats (J) Av.2 or 3
a.m.					
10.40			1	0	0
10.45-54	0	0			
11.00-05	0	0	0	0	0
11.30-37	1	1	0	0	0
12.00-06	0	1	0	2	1
p.m.					
12.30-37	0	1	0	2	1
1.00-05	3	0	2	4	3
1.30-38	1	1	2	2	1
2.00-06	0	1	2	1	2
2.30-35	0	2	3	4	2
3.00-02		4	4	12	4
3.30		9	34	122	10
4.00		12	>90	36	12
4.30			13	17	16

For location of sampling points A,N,L and J see Fig 11.

Notes:

Separator door opened several times after commencement of experiment.

Plates: LYPA medium (no Ca added) spread with ML_8.

Starters: ML_8 R_1 ML_1 AM_2 in each bulk vessel, respectively.

Vats 5,6,1,2,3,4 = $1\frac{3}{4}\%$ (ML_8 + R_1)

5,6,1 (second fill) = 2% (ML_1 + AM_2)

Table LXXIX Making details, phage counts in whey and plaque counts obtained by air filtration and by exposing agar-medium plates and dishes of liquid at intervals throughout the sixth survey at the Belvedere mechanized cheese factory.

Vat details

Vat	Dry	Mill
	a.m.	a.m.
1	8.15	10.40
2	8.35	11.00
3	8.55	11.10
4	9.15	11.30
5	9.35	11.50
		p.m.
6	11.25	1.25
7	11.45	1.45
	p.m.	
8	12.05	2.05
9	12.25	2.35
10	12.45	3.00

Phage counts in whey

Time	Vat	Stage	Count/ml
a.m.			
11.07	9	Stirring	4.4×10^6
11.40	7	Running	4.5×10^4
p.m.			
12.36	10	Stirring	2.2×10^7
1.05	9	40 min after drying	1.5×10^8
2.25	10	1 h 40 min " "	4.4×10^9
2.58	10	5 min before milling	1.2×10^{10}
3.00	9	Salting	1.9×10^8

(cont'd)

Table LXXIX (cont'd)

Time	At end vats (D) (Alginate method) 20 cu. ft air	Sedimentation counts (10 min) End vats (D) Plate	Liq.	Wall side vats (F) Plate	Liq.	Near clock (H) Plate	Liq.	Cheddarmaster area Near clock (K) Plate	Liq.	Cheddarmaster (No. 1 (P) Plate	Liq.
a.m.											
10.00	5200	12+	850	3	200	0	50	12	1200	12	89500
10.30	15950	17	750	9	3200	41	700	48	8650	235	280500
11.00	38725	36+	3050	40+	2400	50	10200	77	7850	217	269000
11.30	38775	57	3200	33	3550	57	4400	72	11000	32	3300
12.00	12900	37	900	26	400	43	450	31	1400	46	4750
p.m.											
12.30	26200	85	1350	19	1100	59	2450	35	6500	82	21050
1.00	16350	37	750	28	950	47	1000	103	2950	220	910000
1.30	35050	47	2100	27	1300	23	1600	43	1150	59	9500
2.00	58400	54	2150	88	4550	71	6700	175	20250	362	100000
2.30	149750	125	5450	36+	6050	60+	12650	99+	8850	285	585000

For location of sampling points D,F,H,K and P see Fig 11.

+ count could be slightly higher.

Notes: Fan was shut off when vats 1-5 started to run until last of vats (6-10) had clotted, to prevent blowing phage over vats during this period.

On this day 10 vats were used, 5 in the first fill and 5 in the second. The vats were numbered 1-5 and 6-10, but as no record was kept of which actual vats were used, the numbers do not correspond with vat numbers in the plan of the factory. The numbers merely refer to the sequence in which the vats were used; the details given correspond with those specific vat numbers.

Liquids estimated by medium with Ca addition.

Plates: LYPA medium (no added Ca) spread with HP.

Starters: in 1st fill P_2 AM_2 } Phage p_2 grows on HP. This could account for high
in 2nd fill HP R_1 } counts early in the trial.

Table LXXX Making details, phage counts in whey and plaque counts obtained by exposing agar-medium plates and dishes of liquid and by air filtration at intervals throughout the seventh survey at the Belvedere mechanized cheese factory.

Vat details				
No.	Set	Dry	Mill †	Salt †
	a.m.	p.m.	p.m.	p.m.
1	9.10	12.15	2.25	2.30
2	9.40	12.35	2.45	2.50
3	10.05	1.00	3.15	3.20
4	10.25	1.15	3.40	3.45
5	10.40	1.35	3.55	4.00
6	10.50	1.50	4.15	4.20

† calculated

Phage counts in whey			
Time	Vat	Process	Count/ml
p.m.			
12.30	2	Started run	1.7×10^6
12.34	2	Draining	1.9×10^6
3.00	2	Milling	4.0×10^9
3.07	2	Salting	2.7×10^8

(cont'd)

Table LXXX (cont'd)

	Sedimentation counts (10 min)								
	End vats (E)		Wall (against wrapping room) (G)		Near presses approx. (I)		Near Cheddarmaster salt unit (M)		Near Cheddarmaster salt unit (M) Alginate method 20 cu. ft air
Time	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	
a.m.									
10.05	5	<100	9		0		0	<100	50
11.00	0	<100	0		0		0	<100	<50
12.00	4	<100	3		0		1	<100	50
p.m.									
1.00	58	100	118		145		203	1800	16400
2.00	238	800	161		134		175	3200	64100
2.30	269	1900	297		281		339	22300	336700
3.00	141	8000	202		154		212	16200	261750
*4.25	327	2273	256		408		245	2455	45520
5.00	226	3400	196		261		347	19000	2750
5.45	57	100	54		1		5	<100	700

* Exposure times varied, so calculated to 10 min.
For location of sampling points E,G,I and M see Fig 11.

Notes:

The alginate air filtration apparatus was placed about 6 ft from the Cheddarmaster (towards the presses).

Methods: (Sedimentation: 3 layer (Ca added) media. HP starter in top layer.
(Whey counts, liquid counts: 4 layer (Ca added) media.
(Air filtration. Counts on 4 layer (Ca added) media.

Vat numbers refer to the order of filling used this particular day, not to those shown in Fig 11.

(cont'd)

Table LXXX (cont'd)

Examination of milk for hp phage			Phage present or absent in quantities of milk noted below				
Time	Vat	Sample	10 ml	1.0 ml	0.1 ml	0.01 ml	0.001 ml
a.m.							
9.35	2	Milk entering vat	-	-	-	-	-
10.55	6	" " "	-	-	-	-	-

Starters on day of manufacture:

HP (1 vessel), R_1 (1 vessel), HP + R_1 (1 vessel).

Starters were mixed before being used in the vats.

Treatment of cheeses the day after manufacture.

An account of the removal of cheese bandages and the subsequent handling of the cheeses has been given in the text. These operations were carried out from 8.00 a.m. to 11.00 or 11.30 a.m., with a break from 10.00 to 10.20 a.m. A diagram showing the layout of areas for removal of cheese bandages, weighing, and wrapping of cheeses is given in Fig 12. See also Table LXXXI.

(v) EXPERIMENT COMPARING THE "hp" PHAGES FROM THE THREE FACTORIES

An experiment was carried out to compare "hp" phages from the three factories. To each of three vats of milk was added a small number of phage particles isolated from the respective factory. A fourth vat served as control. Phage levels were measured in whey samples taken from each vat at intervals throughout the making process. Results showed that the three vats with added phage gave approximately the same phage counts at the corresponding times throughout the experiment, showing that the phages from the three districts had similar characteristics and were probably one and the same 'strain'. This indicated that the differences in phage levels in factories were, therefore, due to factors such as procedure, equipment, etc. in the specific factory rather than to a difference in strains of "hp" phage.

(vi) DISCUSSION OF RESULTS, AND ACCOUNT OF CONCLUSIONS DRAWN, FROM SURVEYS OF PHAGE DISTRIBUTION IN FACTORIES AT WOODVILLE, TARARUA AND BELVEDERE

On a number of occasions detailed surveys were made of the phage distribution in factories at Woodville, Tararua and Belvedere. The object of the investigation was to obtain a broad but quantitative understanding of the distribution of phage in the air during the cheesemaking process. The surveys were made collaterally with the investigation of the role of calcium in phage estimations. Hence

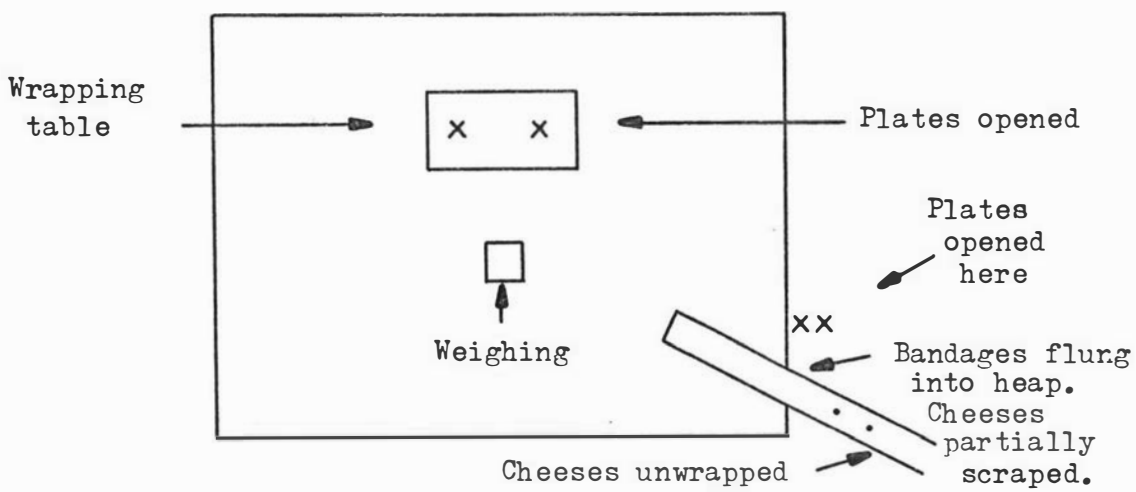


Fig 12. Diagram showing layout of areas for removal of cheese bandages, weighing, and wrapping of cheese (Belvedere Dairy Factory).

Table LXXXI Counts of airborne phage the day following cheese manufacture at the Belvedere mechanized cheese factory.

	Sedimentation counts (10 min)								Near Cheddarmaster salt unit (M) Alginate method 20 cu. ft air
	End vats (E)		Wall (against wrapping room) (G)		Near presses approx. (I)		Near Cheddarmaster salt unit (M)		
Time	Plate	Liq.	Plate	Liq.	Plate	Liq.	Plate	Liq.	
a.m.									
7.40	0	<100	11		14		16	<100	600
9.32	36	100	126		7		1	<100	750
10.25	19	200					18	<100	7100
10.55	11	300					40	100	<50

Sedimentation counts (plates)

Time	Place	Exposure time	Plaque count
a.m.		min	
10.28	Wrapping room	5	5
10.28	" "	10	36
10.57	Unwrapping area	5	30
10.57	" "	10	100

For location of sampling points E,G,I and M see Fig 11.
For location of sampling points, wrapping room, unwrapping area see Fig 12.

Notes:

Examination of milk for hp phage

Time	Vat	Sample	Phage present or absent in quantities of milk noted below: 10 ml	1.0 ml	0.1 ml	0.01 ml	0.001 ml
a.m.							
9.22	2	Start filling vat	+	+	-	-	-
10.38	6	Milk entering vat	+	+	+	+	-

Starters: H_2 (1 vessel), SK_4 (1 vessel), $H_2 + SK_4$ (1 part vessel) } mixed.

the methods used varied somewhat on occasions. In Table LXXXII is set out the presence or absence of calcium in the medium used in each phage detection method employed on specific factory visits.

Table LXXXII Presence or absence of added calcium in media used in phage estimation methods employed on specific factory visits.

		Slit sampler	Whey		Sedimentation			Air filtration
			Titre	Count	Plates		Liq.	Alginate
Factory	Table	No Ca	No Ca	+ Ca	No Ca	+ Ca	+ Ca	+ Ca
Woodville	LXVI		"		"			
"	LXVII			"	"		"	"
"	LXVIII(1)			"		"	"	"
"	LXVIII(2)			"		"	"	"
Tararua	LXX			"		"	"	"
"	LXXI			"		"	"	"
"	LXXII			"		"	"	"
"	LXXIII			"		"	"	"
Belvedere	LXXIV	"			"			
"	LXXV	"			"			
"	LXXVI		"		"			
"	LXXVII	"	"		"			
"	LXXVIII	"	"		"			
"	LXXIX			"	"		"	"
"	LXXX			"		"	"	"

Whereas certain facets of information could be gathered from surveys which used media lacking added calcium, it was obvious that for strictly comparative purposes only the results obtained with calcium in all media were of value. In Table LXXXII the experiments marked in rectangles were, therefore, of special value.

In assessing numbers of airborne phage particles throughout the day at factories, the samples were generally taken at hourly intervals. Such a routine is reasonable and practicable for sampling when dealing with a system where vats of milk were set at different times, and where several different stages of the manufacturing process might be going on at any particular time. The contribution to airborne phage

of the making operations associated with any one vat could still be followed to some extent, although there was the possibility of phage levels at any specific location in the making room being influenced by phage from some other area at that particular time. Nevertheless, main trends in phage levels could be established reasonably well.

In general it was found that phage developed in the vats to a varying extent, and that atmospheric counts (unless influenced by phage from some other area) were comparatively low until running off of whey commenced or the curd was drained on the curd-conveyor of the Cheddarmaster equipment. At this stage there was often a distinct increase in phage in the air. The higher levels of atmospheric phage were associated with processes applied to the curd after cheddaring, i.e. milling, salting, and other vigorous operations to which the curd was subjected in the vats or on the curd-conveyor.

The amount of phage transferred to the atmosphere at any stage clearly depended on the extent to which phage had developed in the curd and whey, and the subsequent amount of movement to which the product was subjected, the basic factor, of course, being the numbers of phage particles in the product. Low or high counts of phage in whey were to a great extent reflected in low or high phage levels in the atmosphere. The separator made a small or large contribution to the atmospheric phage depending on:

(i) whether it was situated in the making room or in a separate closed room;
(ii) the length of time for which it was run;
(iii) the phage content of the whey; and
(iv) movements of the air.

In summary, the factors which determined the amount of phage in the air were:

Quantity of phage in whey (and curd) at different times.
Distribution of phage from whey and/or curd (Running/draining conveyor.
(Milling.
(Agitation in vat or on curd-conveyor.
Distribution of phage by separator.
Air movements by fan, etc.
Removal of bandages next day.

There was thus a rise in phage content of the air to a maximum during the latter part of the cheesemaking process on each day of manufacture, followed by a quick fall as making operations ceased. The following morning some phage was distributed into the atmosphere as the bandages were removed and the cheese prepared for packaging.

Comparison of the results obtained at the three factories showed that the non-mechanized Woodville factory had a characteristically low level of phage in the atmosphere.

It may be concluded that the higher levels of phage sometimes obtained at the other two factories were partly due to the Cheddarmaster equipment. The ratio of liquid to spread plate counts in this area was at times high and this was presumably due (in part at least) to large sized droplets. It would appear that in factories with Cheddarmaster equipment air currents should be avoided between such areas and the vicinity of vats which are being filled while processes, such as draining, milling and salting, are proceeding. Similarly, air current from the region of separators should be avoided. Extractor fans should be provided in the areas where phage is being generated.

In Table LXXXIII are shown the maximum atmospheric phage counts and whey counts (and titres) for various days.

Perusal of the Table confirms that low and high phage counts in whey were associated with low and high phage counts, respectively, in the atmosphere. "Low whey counts" were approximately 5×10^4 to 5×10^6 phage/ml, and "high whey counts" were approximately 5×10^8 to 5×10^{10} phage/ml.

The alginate filtration method proved of great value and served as a reliable standard for phage estimation in the atmosphere. Counts obtained by plate sedimentation methods ran somewhat parallel with it. This, therefore, indicated that the widely used method of airborne phage estimation by sedimentation using agar-medium plates

Table LXXXIII Maximum phage in whey and in air. A relationship between these can be seen in an overall survey of counts for hp phage. The interpretation of results is somewhat complicated by the fact that both mechanized and non-mechanized equipment had been used.

	Phage content of whey		Maximum phage content of atmosphere							
			Plaque counts (sedimentation, plates exposed 10 min)							
Factory	Titre† or Phage/ml		Near vat	Near vat	Near vat	Near Cheddar-master	Near separator	Air filtration 20 cu. ft	Phage under investigation	Ca addition to medium
Woodville	1.0×10^{-5}	Salting	53	163	58*		>170		hp	–
	6.4×10^{6}	Before salting	0	2			8	600	e_8	–+
	3.9×10^{4}	Salting	1				1	50	hp	+
	5.0×10^{5}	After raking	25				1200	700	"	+
Tararua	4.0×10^{9}	Over end conveyor	169	111**		275	1452	5750	hp	+
	1.1×10^{7}	Before salting	233	333		208	960	2550	"	+
	2.8×10^{9}	Cheddar tower; before milling	712	362	640	707	772	467100	"	+
	7.0×10^{10}	Salting	>500	427	541	473	700	840000	"	+
Belvedere	1.0×10^{-2}	Milling	2			2 3	381		ml_8	–
	1.0×10^{-5}	Milling	>800	174		149 509			"	–
	1.0×10^{-3}	Salting	12	16		>90 122			"	–
	1.2×10^{10}	5 min before milling	125	88	71	175 362		149750	hp	–+
	4.0×10^{9}	Milling	327	297	408	347		336700	"	+

†Highest dilution giving plaque(s) see p. 210. * = receiving vat

** = there were counts of 466 & 200 at 7.00 & 10.00 a.m., respectively.

can give a very approximate but useful indication as to whether there is comparatively much or little phage in the atmosphere. Clearly, however, spread plates give lower estimates than liquid plates particularly in locations where large, or coarse, droplets are encountered which may contain numerous phage particles.

The slit sampler method was not considered satisfactory for phage estimation.

An account was given (p. 224) of an experiment comparing the "hp" phages from the three factories. It was concluded that the differences in phage levels in the factories were due to factors, such as procedure, equipment, etc., in the specific factory rather than to a difference in strains of "hp" phage.

Whey droplets were shown to carry an average of less than one to several hundred phage particles in each droplet. This was established (Tables LXIX, LXXXIV) by dividing the liquid count by the plate plaque count. Differences in phage content of whey droplets might depend to some extent on the location in the factory, and Table LXXXIV shows results for samples taken near, and far from, the Cheddarmaster draining conveyor.

Table LXXXIV Liquid count divided by plate count (i.e. average number of phage particles in each droplet) in various locations in factories with Cheddarmaster equipment.

	Belvedere		Tararua							
Time	End of vats	Near Cm*	Opp. vat	Near Cm	Opp. vat	Near Cm	Opp. vat	Near Cm	Opp. vat	Near Cm
p.m.										
1.00	2	9					<1	2	<1	<1
2.00	3	18	7	458	<1	<1	50	86	6	2
2.30	7	66								
3.00	57	76					45	8	26	37
							4pm		4pm	
4.25	7	10					725	4	100	<9
5.00	15	55								

* Cm = Cheddarmaster equipment

It would be expected that heavy droplets of whey near the source would contain more phage particles per droplet than those drifting to further parts of the making room. This appeared to be the case at Belvedere, though it is not known to what extent the droplets from near the Cheddarmaster equipment would have reached the other end of the making room in a given test interval of 10 min.

At the Tararua factory the results were not clear-cut, but this may be connected with the comparatively small size of the making room and the relative nearness of the sampling points.

SUMMARY OF RESULTS FROM SURVEYS OF PHAGE DISTRIBUTION IN FACTORIES AT WOODVILLE, TARARUA AND BELVEDERE

A quantitative survey of the distribution of phage in the atmosphere of the making rooms of cheese factories was carried out.

The phage content of the air varied greatly, and depended on the amount of phage developed in the milk, curd, and whey, and the extent to which the products were agitated during manufacture.

Maximum numbers of airborne phage particles were found late in the making process, after cheddaring (milling, salting, etc.).

The alginate filtration method of sampling airborne phage was of value as a standard with which other methods of estimation could be compared.

The majority of phage particles in the air normally sedimented out fairly rapidly when the cheesemaking process had concluded for the day.

Fresh phage was distributed into the air the morning following manufacture when the bandages were unwrapped from the cheeses and flung into a heap.

The whey separator dispensed large quantities of phage into the air. The separator should be kept isolated from the cheesemaking room.

At two of the three factories studied the Cheddarmaster equipment was a source of relatively large whey droplets of phage. This equipment should either be separated from the remaining equipment in the make room, or arrangements should be made for air currents to be directed towards the Cheddarmaster area.

The sedimentation method used for estimating the quantity of airborne phage in a dairy factory was shown to give an approximate idea of comparative amounts of phage in the atmosphere.

The average whey droplet contains more than one phage particle. Hence, when sedimentation agar plates and liquids are exposed for the same time and at the same place the spread plate gives lower counts than the liquid medium.

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