ABSTRACT

'FOAM SEPARATION AS A METHOD OF WATER AND EFFLUENT PURIFICATION - VIRAL-SURFACTANT INTERACTIONS'

R.A. Irwin

The factors governing virus inactivation and removal during water treatment by foam flotation were assessed using a bacteriophage (MS-2) and a quaternary ammonium surfactant (Arquad T50) as a model system. The importance of virus aggregates was recognized and comparison of various deaggregation procedures showed that dilution in distilled water effectively caused complete dispersion with no loss of infective titre. The phage was resistant to pH inactivation within the range 5.0 to 8.0 with maximum survival at pH 6.0. Using a specially developed isoelectric focussing technique, MS-2 was found to exist in one of two interchangeable states characterized by isoelectric points at pHs of 4.1 and 7.6. This was postulated to result from reversible, configurational alterations in the coat proteins.

The toxicity of cationic and anionic detergents was found to increase abruptly at the higher and lower isoelectric points respectively. This pH-dependent inactivation resulted from ionic interaction of the polar groups with the coat proteins. The inactivation rate decreased with time and this was postulated to result from interference by the adsorbed hydrocarbon groups of the surfactant molecules. This hydrophobic adsorption also caused some inactivation and activation energies for the ionic and non-ionic inactivation mechanisms were calculated to be 33.7 $kJmole^{-1}$ and 43.2 $kJmole^{-1}$ respectively.

Ions enhanced surface inactivation in aerated suspensions to an extent related to their size and charge. In laboratory flotation experiments however, they reduced both inactivation and removal by Arquad; this ionic interference again being proportional to ionic radius and charge.

These results were interpreted in terms of the effects of surfactants and ions on the hydrated layers surrounding bubbles and virus particles. Many similarities with the regulating action of reagents in mineral flotation were noted and it was concluded that in this context, the behaviour of viruses was analagous to that of mineral particles.

'FOAM SEPARATION AS A METHOD OF WATER AND EFFLUENT PURIFICATION'

A thesis submitted to the
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1. INTRODUCTION

In the field of public health engineering, the problems associated with wastewater treatment and potable water supply are becoming increasingly more complex. Many modern industrial processes produce large volumes of chemical effluents whose concentration and toxicity have adverse effects upon the biological treatment plants at the sewage works to which they are discharged. Similarly, the expanding population of towns and cities has increased the volume of sewage presented to many works beyond their capacity and with land at a premium, extensions to the biological treatment plant are frequently very costly or impossible. Consequently many works are finding great difficulty in maintaining a treated sewage discharge to watercourses which complies with the standards of the Royal Commission on Sewage Disposal of not more than 30 mgl^{-1} suspended solids and 20 mgl^{-1} Biological Oxygen Demand (BOD).

At the same time, domestic and industrial requirements for potable water are steadily increasing. Previously, increased demand was met largely by the construction of reservoirs but due to environmental and economic constraints this is no longer practicable. Thus increasing use is being made of watercourses, many of which receive a treated sewage discharge upstream of the abstraction point. This poses problems not only of clarification and sterilization but also of the removal of potentially harmful chemicals such as polyaromatic hydrocarbons and hormones, originating from a sewage discharge, and also various pesticides, nitrates, etc. from surrounding farmland.

These problems associated with modern sewage treatment and potable water supply have prompted the water industry to investigate new treatment processes which may be used in conjunction with, or as

an alternative to, existing methods. Many are high-rate, physico-chemical processes having the advantages of compact design, insensitivity to toxic chemicals and ability to handle a wide range of flow rates and contaminant concentrations. Such processes include reverse osmosis, electrodialysis, tertiary filtration, ozonation, activated carbon adsorption and flotation.

As an industrial process, flotation is over a century old, the first patent for its use in mineral ore separation being granted to William Haynes in 1860. Essentially, it involves the concentration at bubble interfaces of soluble or insoluble components in a liquid, and their subsequent removal from the bulk solution as a froth or foam. Various classification schemes have been devised for flotation processes, some of which have been reviewed by Kishimoto (83). One of the simplest and most convenient however is that proposed by Rubin (117), based on the relative gas flow rate and size of the particles removed (Table 1).

Table 1 Classification of Foam Separation Processes (after Rubin (117))

Relative Gas Flow Rate	Particle Size Range		
	Molecular	Microscopic	Macroscopic
High	Foam Fractionation	Foam Flotation	Froth Flotation
Low	Ion Flotation	Micro-Flotation	Precipitate Flotation

The low flow rate processes are impractical for water treatment due to the lengthy retention time required for the removal of particulate

material. Their use is limited to the recovery and concentration of valuable ions from aqueous solutions (117, 123, 97). The remaining three, high rate processes are all suitable for water and sewage treatment. However, due to the complex nature of the feed, which contains many organic and inorganic components both in solution and as particulates, all three processes frequently occur simultaneously. Thus soluble proteins may be removed by foam fractionation (121, 94), bacteria (49, 50) and their spores (17, 37) by foam flotation and algae (90) by froth flotation. Although the separation of viruses has been termed foam fractionation (67), they are microscopic particles and not solutes and it is therefore considered more appropriate to refer to their removal as a foam flotation process.

Since both solutes and particulates are removed by flotation during water treatment, the high flow rate processes may be more conveniently classified on the methods used for bubble generation (141). These are listed in Table 2 and described below.

Table 2 Types of Bubble Generation Systems used in Flotation

System	Method of Bubble Generation
1. Dispersed Air Flotation	a) Compressed air through spargers
	b) Mechanically-Induced Air Flotation
2. Dissolved Air Flotation	a) Pressurized feed stream
	b) Pressurized recycled effluent stream
	c) Evacuated flotation tank
	d) Hydrostatic pressure release
3. Electrolytic Flotation	a) Electrolysis of water

In the dispersed air system (1a), air is pumped into the flotation tank through porous plates or tubes called spargers. Bubble size can be varied by altering the porosity of the spargers, generally made of stainless steel or carborundum, and is usually in excess of 1000 μm (140). The bubble density per unit volume of liquid can be controlled by regulating the air flow rate.

Mechanically-induced air flotation (MIAF)(1b) units exploit the vortex created by a rotating impeller to draw air down its hollow shaft and distribute this as small bubbles via a disperser (see figure 1).

All dissolved air flotation systems rely on a pressure reduction to precipitate dissolved air as bubbles from a supersaturated solution. This can be achieved by prior pressurization of all or part of the feed stream (2a), or a recycled fraction of the effluent stream (2b), and subsequent pressure release in the flotation tank. Air is introduced either before or after the pressurizing pump and the mixture is usually then passed to a saturation tank where equilibration of the air occurs. The pressure is released upon injection into the open flotation tank where precipitation of the dissolved air occurs as bubbles of between 30 and 120 μm in diameter (46). The amount released depends upon the degree of saturation of the pressurized stream, its flow rate, and the saturation pressure. Chemicals, usually coagulants and flocculants, are either added prior to the pressurization pump, with suitable mixing and flocculation stages, or are injected separately into the flotation tank which then also acts as the flocculator. A schematic diagram of a typical dissolved air flotation system utilizing pressurized recycled effluent is shown in figure 2.

Figure 1. Schematic Diagram of a Typical Mechanically Induced Air Flotation Cell.

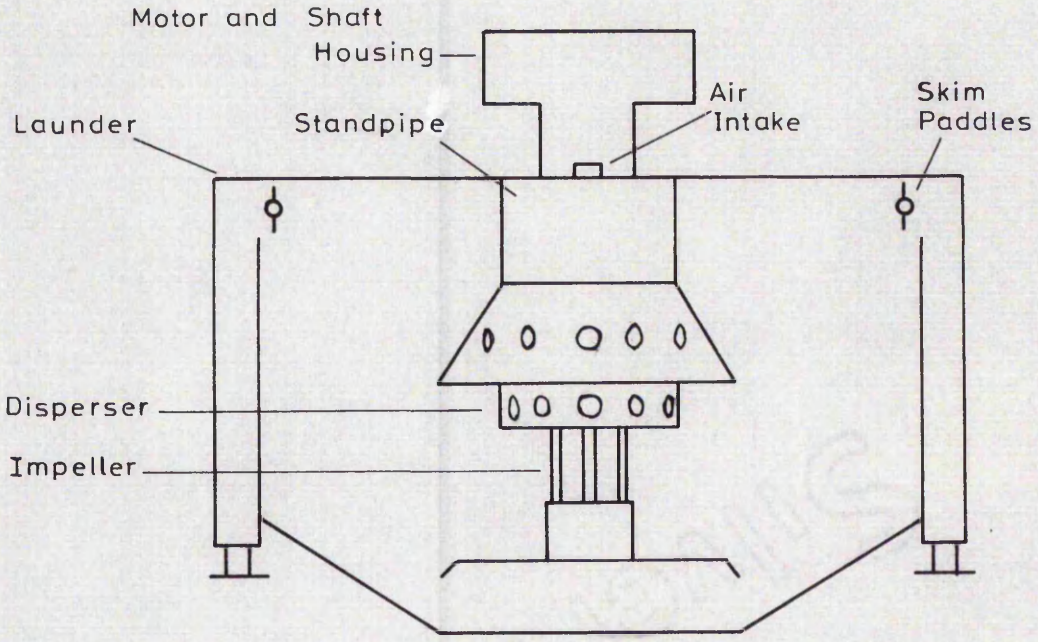
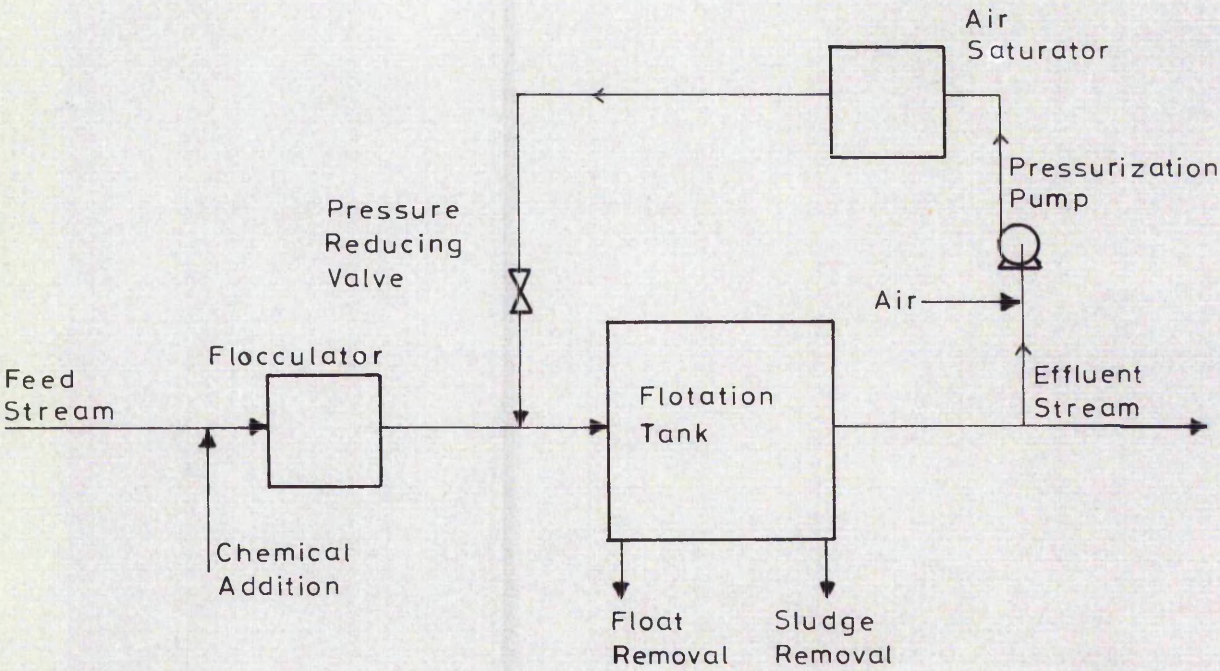


Figure 2. Schematic Diagram of a Dissolved Air Flotation System.



An alternative system, sometimes called vacuum flotation, (2c) utilizes the air dissolved at atmospheric pressure by pumping the feed stream into an enclosed flotation tank and reducing the pressure above the liquid. The relatively low volume of air released makes this process unsuitable for either wastewater treatment or potable water production.

A novel aeration method (2d) has recently been developed by the Environmental Systems Division of AB Electrolux and is known as the Micro Flotation System (116). The feed stream is pumped down a shaft approximately 10 m deep countercurrent to air rising from an aerator located at the bottom. Hydrostatic pressure ensures that the stream is 50 to 70% saturated with air at the bottom. As the stream passes to a riser column and flows upwards, bubbles are released as in conventional dissolved air systems.

Electrolytic flotation (3a) differs from the other processes not only in the method of bubble generation but also in the type of gas from which the bubbles are formed. Electrodes are placed horizontally in the flotation tank and application of a low voltage direct current across them results in electrolysis of the water to release oxygen and hydrogen as small uniform bubbles from the anode and cathode respectively. The critical factor in 'electro-flotation' development is the design of the electrodes and their materials of construction (46, 73).

Removal of impurities from an aqueous solution by flotation requires not only a large surface area in contact with the effluent but also a means of concentrating those impurities at the bubble interfaces. This may be achieved by the addition of chemicals which are surface active or which render the impurities surface active.

Alternatively dissolved air systems generally utilize chemicals which coagulate or flocculate the impurities and the resulting flocs trap the precipitated air bubbles and are buoyed to the surface to be removed. Flocculation is not practical in dispersed air systems however because the turbulence created by the larger bubbles tends to disrupt the delicate flocs . Under these circumstances therefore surface active agents such as detergents, either naturally present or added to the feed, are utilized. These adsorb to the suspended material, rendering it hydrophobic and causing it to be concentrated at the bubble interfaces. The nature of the interactions occurring when chemicals adsorb to interfaces will be discussed in greater detail later.

It will be apparent from the above remarks that the particular constituent removed during flotation will be dependent upon the type and concentration of chemical additive used. Furthermore, the operating conditions will vary depending upon the concentration of contaminating material present and the degree of purification required. Thus flotation plants vary considerably in design depending upon the type of effluent to be treated. Examples of effluents successfully treated by flotation include those from machine shops (10) and paper (68, 86) and food (110, 145, 147) processing plants, with many more examples being cited by Chase (29) and by Rovel (142). In the field of water and wastewater purification, flotation has been investigated in a wide range of applications from clarifying borehole water to thickening of surplus activated sludge.

At a recent conference organized by the Water Research Centre, UK (142), a number of the papers discussed the use of dissolved air flotation for potable water production. Data was also presented

on the performance of 5 flotation units commissioned by the W.R.C. and operated on various types of water. These each had a capacity of 2300 m^3d^{-1} at a surface loading of 260 $m^3m^{-2}d^{-1}$. This compares very favourably with conventional rapid gravity sand filtration with a maximum loading of around 150 $m^3m^{-2}d^{-1}$ (131). In a total treatment time of 20 to 25 minutes, these units removed some 60-90% of the turbidity and approximately 90% of the algae to yield a 'float' with a solids content of some 6 to 8%. The type and concentration of chemical added varied with the water type, for example ferric sulphate at 3.7 mgl^{-1}Fe was used for stored river Stour water and 50 mgl^{-1} alum ($Al_2(SO_4)_3.21H_2O$) for river Severn water, which had a higher turbidity and pH.

The air was provided by recycling 6-8% of the treated effluent after passing through an air saturator at a pressure of 50-60 psi. Data presented at the conference by Zabel and Hyde showed that, at these pressures, the saturator would provide approximately 75 to 100 mg of air for flotation per litre of recycle water. At 8% recycle this was equivalent to 6-8 g air per m^3 of water to be treated, comparing favourably with the value of 3-7 g air m^{-3} of Thames water derived from pilot studies at the W.R.C. However, this mass of air introduced as large bubbles would cause disruption of the floc and highly inefficient flotation. Clearly the important parameters for maximizing flotation efficiency are bubble size and bubble density per unit volume of feed. Thus for example the volume of air contained in a 1 mm bubble is equal to that contained in 8,000 bubbles of 50 microns diameter. Bubble size and density can readily be determined by direct observation (i.e. photography) within the flotation cell but no such data can be found in the literature relating to water

treatment by flotation. The parameters usually quoted are the recycle ratio and saturation pressure although the factors determining the number and size of bubbles released are more complex including degree of saturation achieved, water temperature, design of the pressure release valve, effectiveness of mixing, etc.

At the same conference, a paper was presented by Rosen and Morse describing a number of flotation plants operating in Sweden and Finland. These units ranged in size from 4320 m^3d^{-1} to 24000 m^3d^{-1} and were of three basic designs, one of which incorporated a conventional sand filter in the base of the tank and is marketed by Water Management Ltd., UK as a Flofilter. All these units operated at lower surface loadings than those of the W.R.C. i.e. 100-140 $m^3m^{-2}d^{-1}$, with a recycle ratio of 7-12% at 70-85 psi and an alum dose of 30-45 mgl^{-1}. Very little performance data was given but the units were quoted as reducing turbidity and colour by 90% after flotation and filtration.

Other smaller units were described by Gardner and by Stock (142) who presented data from a unit treating deep-well water at a surface loading of 511 $m^3m^{-2}d^{-1}$. This relatively high rate was achieved with a $FeCl_3$ dosage of 51 mgl^{-1}Fe and air supplied by recycling 5% of the effluent at a saturation pressure of 105 psi. The unit was designed for permanganate value reduction and the high level of 20 mgl^{-1} was successfully reduced by 54%. Although not directly comparable with the W.R.C. units due to differences in plant design and operation, the lower sludge solids content (0.5-1.0% $^w/_v$) and higher residual coagulant level (8 mgl^{-1}asFe) found may have been attributable to the considerably greater surface loading rate of this unit.

Dissolved air flotation plants for the treatment of primary sewage effluents have been described by a number of authors (47, 71, 92, 101, 144). These units had surface loadings ranging from 97 to 294 $m^3m^{-2}d^{-1}$ with one laboratory scale unit (101) operating at 360 $m^3m^{-2}d^{-1}$. Coagulant doses (aluminium sulphate or ferric chloride) were higher than those used for water treatment at around 400 mgl^{-1}, as necessitated by the higher solids load in the influent. However, one unit used no coagulant addition (71) and another utilized lime at 350 mgl^{-1} (101). Removals ranged between 65 and 95% for suspended solids and Chemical Oxygen Demand (COD) levels were reduced by between 25 and 72%.

Once again, comparison of plant efficiencies was difficult since insufficient data was provided regarding air requirements. However, from the data of Levy, White and Shea (92) it was possible to calculate an optimum air supply of between 3 and 14 gm^{-3} of sewage. This compared with the value of 3-7 gm^{-3} of Thames water obtained by the W.R.C. (142) and suggested that the air requirement was relatively independent of the solids concentration in the feed water.

Several plants for thickening primary and secondary sewage sludges by dissolved air flotation have been described by Katz and Glinopolos (82). In this application, the surface loading rates were expressed as weight of biomass added per unit area of flotation tank per unit time ($Kgm^{-2}d^{-1}$) and the efficiency measured in terms of the sludge float concentration (% $^{W}/_{V}$). The units described had surface loadings of 48-98 $Kgm^{-2}d^{-1}$ for activated sludge, 98-195 $Kgm^{-2}d^{-1}$ for mixtures of primary and activated sludges and up to 268 $Kgm^{-2}d^{-1}$ for primary sludge alone. The sludge float concentrations

ranged between 3.7% and 8.6% $^{w}/_{v}$, the lower values being obtained at the higher sludge loading rates. These results were obtained with no chemical addition but data given for plants utilizing polyelectrolytes at dosages of between 1.3 and 11.7 g/Kg dry sludge solids indicated that they could either increase the sludge float concentration at high sludge loadings or increase the solids recovery in the float, i.e. lower the effluent suspended solids content.

Data obtained from a flotation unit operated at the Aycliffe Sewage Treatment Works, UK (142) showed that a float concentration of 4% could be achieved at a solids loading of 232 $Kgm^{-2}d^{-1}$ using a polyelectrolyte dose of 8.9 gKg^{-1} dry solids. This was equivalent to an hydraulic surface loading of 78 $m^{3}m^{-2}d^{-1}$ which is lower than that achieved in water or sewage effluent reatment and reflects the considerably increased influent solids load.

Unfortunately the author does not provide sufficient information to calculate the amount of air available for flotation per unit volume of feed although the Aycliffe unit used a pressure of 70 psi and a recycle ratio of 140% by supplementing flotation effluent with works final effluent. Even allowing for differences in saturator efficiency and tank design, this unit clearly used far greater amounts of air than are typical of water or sewage effluent treatment. Maddock (142) suggested that in commercial sludge thickening practice the air to solids ratio varied between 15 and 30 $cm^{3}g^{-1}$. Assuming a density for air at 15°C and atmospheric pressure of 1.29 gl^{-1}, this represented a mass of air available per Kg of solids ranging between 19 and 38 gKg^{-1}. At a sludge solids content of 5 Kgm^{-3} the air consumption would therefore be 95-190 gm^{-3} which is considerably in excess of the amount used to treat effluents (3-14 gm^{-3}).

Assuming ideal gas behaviour and a molecular weight for air of 28.9, a value of 91.5 gm^{-3} can be calculated from the data of Katz and Glinopolos (82), in reasonable agreement with the value proposed by Maddock.

In contrast with dissolved air flotation, dispersed air flotation has received little attention as a means of water treatment. One objection to the use of the process which has often been raised is the possible health hazard resulting from the residual surfactant in the product water. However it has been pointed out by Grieves and his co-workers (60) that one quaternary ammonium surfactant (cetyldimethylbenzylammonium chloride) has approval from the U.S. Food and Drug Administration for human ingestion of up to 40 mgd^{-1} as throat lozenges.

A number of small scale feasiblity studies have been performed since Hopper first suggested the process in 1945 (74). In 1954, Moore and Bryant published the findings of a research program sponsored by the U.S. Army to investigate the effectiveness of foam flotation as a water purification process for military field installations (104). Good removals of various micro-organisms were reported from synthetic waters, but several components of natural waters such as di- and tri valent cations, turbidity and colour adversely affected the removals, necessitating higher surfactant doses and the authors reported that the process was unsuitable for water purification.

This conclusion was not endorsed by Grieves and his co-workers who in two series of investigations, also sponsored by the U.S. Army, showed that water clarification was feasible and produced a projected cost analysis (54, 59, 60, 63). They showed that turbidities of up to 150 Jackson Candle Units (JCU) could be reduced to less than 4 JCU by increasing the surfactant dose to 60 mgl^{-1} and the retention time

to 45 minutes whereas Moore and Bryant did not extend the aeration period beyond 20 minutes or the surfactant dose above 50 mgl^{-1}. Probably the most significant difference between their experiments was the air rate used. Transforming the data as before, Moore and Bryant experimented within the range 0.7-12.9 Kg air per m^3 raw water whereas Grieves and his co-workers used rates of between 57 and 237 Kgm^{-3}.

These air rates are several orders of magnitude greater than those associated with dissolved air flotation, reflecting the different removal mechanisms. In dispersed air flotation, contaminants are adsorbed to the bubble interfaces as a monolayer whereas in dissolved air flotation they are first concentrated by flocculation and subsequently trap air bubbles in the resulting floc. The former mechanism is therefore dependent upon interfacial surface area to a far greater extent than the latter, and this is reflected in the higher air consumption.

Grieves also noted the adverse effects of trivalent cations such as Fe^{+++} and Al^{+++} but found that this could be overcome by the addition of bentonite as a flotation aid (61) and the residual surfactant concentration of 1-2 mgl^{-1} could be reduced to less than 0.1 mgl^{-1} by passage through an activated carbon column (59).

Dispersed air flotation of sewage effluents, received some attention in the early 1960's as a means of detergent removal as the increased use of the relatively non-biodegradable alkyl benzene sulphonates (ABS) led to foaming problems at sewage works and in effluent receiving waters. Considerable data is available on the 2270 m^3d^{-1} flotation plant at Pomona, California (2, 21, 118) which shows that this unit removed up to 75% of the ABS from activated

sludge plant effluent. A residence time of 5 minutes was used at an air rate of 6.45 Kgm^{-3} effluent.

Using higher air rates of between 7.7 and 38.7 Kgm^{-3}, Jenkins successfully treated primary effluent by dispersed air flotation (78). Unfortunately he presented no absolute values for removal of ABS and other components but quoted results as enrichment ratios, i.e. constituent concentration in the foam divided by constituent concentration in the effluent. At ABS enrichment ratios between 50 and 100 he found suspended solids and COD enrichment ratios of 20-50. He also studied a number of plants treating secondary effluents, including the Pomona unit, and found that they removed between 22 and 36% COD and between 43 and 75% suspended solids, leaving an effluent containing less than 1 mgl^{-1} ABS. He concluded that better results were obtained treating secondary effluents. The largest unit examined, at Whittier Narrows, Los Angeles, also had the highest surface loading of any water or wastewater flotation unit at 712 $m^3m^{-2}d^{-1}$. The removals obtained with this unit were comparable with those obtained from lower rate units although insufficient data is available to explain how this greater efficiency was achieved.

All these plants were designed to remove detergents already present in the sewage but in 1943, Hansen and Gotaas had shown that dosing primary sewage effluent with 60 to 80 mgl^{-1} of a cationic surfactant prior to flotation gave excellent results (69). Up to 85% of the BOD and 97-100% of the suspended solids and bacteria were removed. However there is no evidence in the literature to suggest that surfactant addition for sewage flotation has been attempted since that date.

In addition to the practical experience gained from the operation of pilot and full-scale flotation units, considerable theoretical data is available from the complementary laboratory studies carried out on various waters and wastewaters. Foremost in this field with respect to dissolved air flotation is the W.R.C., Stevenage, U.K. (e.g. 75, 104, 142). Factors shown to be of importance during studies were as follows:

1. Air/solids ratio
2. Surface loading rate
3. Degree of air saturation
4. Method of bubble generation
5. Efficiency of bubble entrapment
6. Efficiency of flocculation stage (where added).

It should be noted that the air solids ratio and the efficiency of bubble entrapment are interrelated since theoretically activated sludge solids, for example, were expected to float at an air/solids ratio of 0.18 $cm^3 g^{-1}$ whereas in practice 15-30 $cm^3 g^{-1}$ were required (142). Their laboratory studies showed that even using highly effective needle valves for pressure reduction, bubble entrapment was only 20-50% efficient. Maddock (142) concluded that this was due to large bubbles, resulting from coalescence, which escaped to the surface and that the efficiency was even lower in full-scale units. However, he overlooked the physical and chemical factors involved during solids adsorption to an interface which are vitally important in mineral ore flotation and are probably involved during bubble entrapment by floc particles. These factors will be discussed more fully later.

Flocculation is not unique to flotation and is extensively used for water clarification. The controlling factors for effective flocculation are the type and dosage of coagulant used and the design of the flocculation tank and the retention time therein.

The most extensive studies of the numerous influential factors in dispersed air flotation have been undertaken by Grieves and his co-workers. They determined the effect of flotation column design (65), surfactant feed concentration, air and feed rates and retention time (62, 66) and of liquid depth and position of feed entry within the flotation cell (66). These studies were performed in continuous flow flotation columns using solutions of anionic (ABS or dodecyl sodium sulphate) or cationic (ethylhexadecyldimethylammonium bromide (EHDA-Br) detergents. In addition, the effects of temperature (48, 57, 66), pH (48, 56) and colloidal and particulate matter (33, 55, 58) have also been determined. These authors adopted an empirical approach to the analysis of their results but some workers have attempted to derive mathematical models by analysis in terms of a mass balance and the surface adsorption equilibrium (39, 83, 105). However, this approach is only suitable for the prediction of fractionation performance for one or two components under laboratory conditions since the analysis becomes impossible when considering the numerous interractions within a chemically complex water or effluent.

In the analysis of their results, Grieves and Wood (65) found that foam separation of surfactant was independent of liquid solution height above the diffuser. They therefore concluded that overall foam enrichment was governed by the separation occurring in the regions adjacent to the foam-solution interface. For a given frationation

column, the optimum interface position was determined by the minimum liquid height allowing uniform bubble dispersion and maximum foam column height for increased foam concentration and reduced foam flow rate.

In later experiments (66), they found that separation of surfactant was unaffected by variations in residence time. At an air rate of 25.8 Kgm^{-3}, the feed surfactant concentration of 200 mgl^{-1} was reduced to 95 mgl^{-1} throughout a range of residence times from 18 to 206 minutes. This result is apparently in contradiction to later work by Grieves where improved removal efficiences were obtained as the retention time was increased between 13 and 205 minutes (60). However these were results of pilot studies on natural water clarification using higher air rates (56-95 Kgm^{-3}) lower surfactant concentrations ($\simeq$62 mgl^{-1}) and more than 98% removal ($\leqslant$1 mgl^{-1} in the effluent) of surfactant.

They also found that the ratio of residual to feed surfactant concentrations was directly proportional to the ratio of air to feed flow rates. Thus for a given feed concentration, the residual concentration was determined by the volume of air delivered per unit volume of feed. As discussed earlier however the significant parameter was bubble surface area which was not solely governed by air volume but by other factors such as diffuser porosity. However, the relationship between residual concentration and air rate does have important implications for full-scale plant design since theoretically the same removal should be achieved with several small units in parallel as with one large column provided the air rate to feed rate ratios are the same.

It was also found that the position of the feed entry to the column affected the flotation efficiency. Feeding into the foam phase improved surfactant removal and reduced the collapsed foam volume compared with introducing the feed below the foam-solution interface. An optimum position was found at which the volume of collapsed foam was at a minimum.

The effect of temperature upon flotation may be deduced from the Gibbs equation which relates the surface excess concentration of a surfactant to its bulk liquid concentration (57) thus:

$$\tau = -\frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

where: τ = the excess concentration of solute over 'c'

c = bulk solution solute concentration

R = gas constant

T = temperature

$\frac{d\gamma}{dc}$ = rate of change of surface tension with solute concentration.

Thus the adsorption of surfactant to the bubble interfaces should be reduced as the temperature increases. As expected, the residual concentration increased with temperature, as did the residual flow rate (66). This was investigated further by Grieves and Bhattacharyya (57) who concluded that foam drainage increased and foam stability decreased with temperature as the viscosity of the liquid in the foam decreased. After fractionating EHDA-Br over a temperature range of 14^{o}-54^{o}C they found that these factors resulted in increased foam and residual concentrations and decreased foam flow rate with increased temperature.

Studies by two groups of workers have shown that high pH values increase the fractionation of anionic ABS but decrease the removal of cationic EHDA-Br. Gassett and his co-workers (48) found that the removal rate, as well as the final percentage removal of ABS from solution, was increased when the pH was raised from 4.0 to 11.0. They explained their findings in terms of the effect of pH on micelle formation, the critical concentration above which such aggregates were formed being pH-dependent.

The more extensive studies conducted by Grieves and Bhattacharyya (56) using EHDA-Br showed that removal efficiency passed through a maximum at pH values of 2.7 or 3.3 depending whether hydrochloric acid or suphuric acid respectively, was used for pH control. However, adding the same concentrations of chloride and sulphate ions as their sodium salts, they found very similar removal efficiences and concluded that most of the effects observed were attributable to the anions added rather than pH. They postulated that adsorption of surfactant to an interface resulted in the formation of an electrical double layer of surfactant ions in the surface and below it a region poorer in solute. This formed an activation barrier to further surfactant adsorption which could be overcome by an increase in solution ionic strength. In this regard $SO_4^{=}$ was more effective than Cl^- while OH^- (elevated pH) had little influence. The reduction in surfactant removal below pH 2.7 or 3.3 was explained by the effect of electrolytes on foam drainage.

At higher pH values (6.1→3.5) removal efficiency increased due to increased surfactant adsorption. Below pH 3.3, adsorption levelled off but increased foam drainage due to the raised ionic concentration caused the residual concentration to increase once more.

These workers also reported that such a relationship between foam stability and electrolyte concentration has been found for anionic surfactants. However Gassett et al did not record such an effect since their apparatus removed the foam from the surface of the solution preventing formation of a foam column.

All these laboratory experiments were conducted using pure solutions of surfactants and relate solely to the fractionation of those surfactants. However in water and wastewater treatment, flotation occurs in a multicomponent system. Thus although flotation of sewage effluents was primarily undertaken for ABS removal, the numerous organic and inorganic solids and solutes present were clearly involved in the process as some were concentrated with the surfactant in the foam phase. The organic components of such an effluent are composed largely of dissolved proteins, carbohydrates and lipids, and particulate material such as micro-organisms. Laboratory experiments have demonstrated that such material can be separated and purified from solution by foaming. London and his co-workers showed that the enzymes catalase and urease could be separated from each other without loss of activity, by fractionation of a mixed solution utilizing the inherent foamability of proteins (94). They noted that separation was independent of temperature within the range 10^{o}-30^{o}C and that addition of '2 drops' of the non-ionic detergent Tween 80 significantly increased separation which they concluded to be due to the stabilization of the foam phase. They also observed optimal fractionation within the pH range 4.8 to 5.6 coinciding with the point at which, for most proteins, the number of ionized cationic and anionic groups on each molecule is equal; this being known as the isoelectric point. A similar finding was reported

by Schnepf and Gaden using bovine serum albumin (121). They concluded that protein mixtures could be at least partially fractionated provided the individual components differed sufficiently in surface activity and/or isoelectric pH, and produced a stable foam.

The flotation of micro-organisms has been studied by a number of workers and the selective separation of bacterial spores from vegetative cells in autolysed cultures by aeration has been reported (e.g. 17, 51). Much of this work has been reviewed by Dobias and Vinter (37) who collated the results of these studies with their own data and concluded that a critical factor affecting flotation was the chemical composition of the cell surface. The hydrophobic or hydrophilic character of this surface could be modified by the addition of suitable surface active agents thereby radically affecting flotation behaviour.

Gaudin and his co-workers found that inorganic salts also affected the flotation of Escherichia coli (49, 50). They concluded that phosphates and carbonates enhanced flotation whilst the effectiveness of cations was generally greater for higher atomic weights and valencies.

The flotation of insoluble inorganic material has also been studied by Crandall and Grieves using various mineral clays (33). From their extensive investigations they proposed a mechanism whereby cationic surfactant (EHDA-Br) adsorbed to the negatively charged clay particles making them hydrophobic and causing their accumulation at gas-liquid interfaces. Excess, non-adsorbed surfactant created a stable foam enabling the particles to be removed from the bulk solution. At high pH values ($\geq$7.0) good removals were noted due to the preferential

adsorption of cationic surfactant to the negatively charged sites on the clay surface. As the pH was lowered (7.0-3.5) removal decreased due to competition for the anionic sites from excess H^+ ions. Below pH 3.5, the removal efficiency again increased which was thought to be due to the adsorption of divalent sulphate ions (from H_2SO_4 used for pH adjustment) by the now positively charged clay. This resulted in another charge reversal allowing renewed adsorption of cationic surfactant.

The mechanisms involved in the flotation of inorganic particles have been extensively studied with respect to mineral ores which are frequently separated and concentrated by flotation in the mining industry. In their excellent book on the subject, Klassen and Mokrousov analysed all the phenomena involved in terms of the role of hydrated envelopes surrounding mineral particles, air bubbles, molecules and ions in a flotation mixture (84). These phenomena may be grouped into three broad categories as follows:

1. Interactions between flotation reagents and the mineral surface.
2. Interactions between flotation reagents and the bubble surface.
3. Attachment of mineral particles to bubble surfaces.

Flotation reagents are the most influential components in a flotation system and a study of their mode of action therefore has great practical significance. By careful selection of reagents, one can enhance bubble attachment of a particular mineral and suppress that of others in a mixed ore pulp, and by creating a stable froth or scum enable that mineral to be readily separated and collected. These reagents may be classified into three groups according to their

action:

1. Those which concentrate at the mineral/water interface, i.e. all collectors and many activators.
2. Those which concentrate at the air/water interface, i.e. frothers and some inorganic electrolytes.
3. Those which remain in the bulk solution, i.e. activators, depressants and pH regulators.

Collectors and frothers are surface active agents since, by definition, they concentrate at interfaces. Such molecules consist of two groupings, i.e. a polar or hydrophilic group and a non-polar or hydrophobic group, usually comprised of a hydrocarbon chain. Their solubility in water is due to attraction between the polar group and the water molecules but Van der Waals forces tend to repel the hydrophobic group. At a mineral/water interface the polar group adsorbs to the mineral surface. This adsorption is dependent upon ionization of the polar group and its subsequent electrostatic attraction for the mineral surface, assuming them to be of opposite charge. The degree of adsorption is related to the ionic radius of the polar group. It has been found that the closer this corresponds to the crystalline lattice dimensions of the mineral, the more effective will be adsorption (84).

The hydrophobic groups of collector molecules decrease the hydration of mineral surfaces to which they are adsorbed. This hydration zone is formed by the attraction of the water dipoles to the uncompensated electrostatic forces of atoms or ions in the surface. The intercalation of hydrophobic groups reduces both the interactions between the water dipoles themselves and their attraction to the surface. In general, the greater the chain length of the hydrophobic

group, the greater its effect upon the hydration sheath up to a definite limit. Collector adsorption therefore increases the hydrophobicity of the mineral surface, reducing its 'wettability' and enhancing its flotation properties.

Frother molecules are also assymetric with hydrophilic and hydrophobic groups and in fact many surfactants can act as both frothers and collectors. At a gas-liquid interface, a frother molecule aligns with the polar group in the liquid phase and the hydrophobic group in the gaseous phase. At low concentrations, the hydrocarbon chains lie in the plane of the interface but at higher concentrations they rise from the surface and eventually form a condensed, monomolecular layer with all the chains aligned perpendicularly. The low affinity of surfactants for water molecules reduces the cohesive forces at the interface thus lowering the surface tension. The extent of this reduction is determined by the surface concentration of surfactant and the chain length of the hydrophobic group. As a result of these properties, frothers favour the formation of finer bubbles, prevent bubble coalescence and, most important, form a stable froth or foam. The reduction in bubble size is partly due to the lowering of the surface tension, which allows greater contraction of the interface, and partly to the prevention of coalescence into larger bubbles. This results from the presence of the hydrophilic groups within the surface layer which actively interact with the water molecules and stabilize the hydration zone adjacent to the surface. This increases the strength of the region surrounding the bubble, preventing its destruction upon collision with other bubbles.

A froth or foam is formed as bubbles leave the liquid surface surrounded by a relatively thick (approximately 1μ) film of liquid(84).

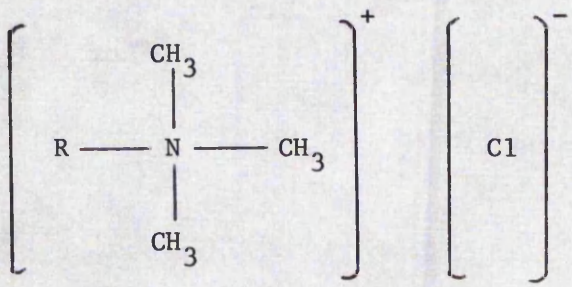
As water drains from the froth, the bubble walls thin until adjacent boundary hydrated layers come into contact with each other. Further thinning greatly reduces their stability and strength resulting in film disruption and collapse of the froth. By stabilizing the hydrated layers as already described, frothers greatly increase the resistance of these layers to thinning, imparting greater mechanical strength to the froth. Furthermore, by increasing the elasticity of the interfaces within the foam, frothers enable these films to counteract mechanical stresses arising within themselves. An expansion of a film, for example, reduces the concentration of surfactant per unit area, increasing the surface energy and strength of the film. Compression, however, increases surfactant concentration and reduces the film strength allowing contraction. These phenomenon are short-lived however, since diffusion tends to equilibrate localized concentration differences, although this may be slow for large, organic frother molecules.

The range of surface active molecules available is extensive but those of particular interest to the water industry are the detergents. These contain one or more long hydrocarbon chains per molecule which may be branched or have attached benzene ring structures. Smith has classified them according to the charge on the hydrophilic grouping as shown in Table 3 (125).

Table 3 Classification of Synthetic Detergents

Hydrophilic Group	Examples
Anionic	Carboxylate
	Sulphate
	Sulphonate
	Others
Cationic	Amine Salts
	Quaternary ammonium
	Quaternary heterocyclic
	Non-nitrogenous
Non-ionic	Polyoxyethylene
	Polyhydric alcohol
Ampholytic	Amino carboxylic
	Amino sulphonic

The detergent used throughout these studies was Arquad T-50 which was kindly donated by Armour Hess Chemicals Ltd., Leeds. It is a cationic alkyl quaternary ammonium chloride having the following general formula:



The 'R' group represents a hydrocarbon chain of varying length, derived from a fatty acid. The exact proportions of the various chain lengths present and the composition of Arquad T-50 are shown in Table 4.

Table 4 Composition of Arquad T-50

'R' Groups	Number of Carbon Atoms per Chain	Percentage Composition
Tetradecyl	14	3
Hexadecyl	16	27
Octadecyl	18	16
Octadecenyl	18	48
Octadecadienyl	18	6
Active Ingredient		50
Sodium chloride		1
Isopropanol		36
Water		13.5

The third major group of phenomena involved in flotation are those associated with the attachment of particles to air bubbles. This may be achieved in two ways: 1. By collision between particles and bubbles, as in dispersed air flotation,

2. By precipitation of bubbles on particles from a supersaturated solution, as in dissolved air flotation.

During collision the resistance to disruption of the bulk liquid layer and the hydrated layers separating the particle from the bubble must be overcome for successful attachment. When the bubble and particle surfaces are completely hydrophilic, i.e. fully hydrated,

the free energy of the liquid film continuously increases as it is thinned and no attachment is possible. When the surface hydration is lowest, i.e. completely hydrophobic, the free energy decreases as the film is thinned and spontaneous bubble-particle attachment occurs. Completely hydrophobic particles, however, have a strong tendency to coalesce into large aggregates in aqueous suspensions, making them undesirable in a flotation system. When the surfaces are partially coated with surfactant, an initial increase in film free energy occurs as the surfaces approach and water molecules are removed from the hydration layers. However, as these layers are much reduced due to the presence of adsorbed hydrophobic molecules, a point is reached when the surfaces attract each other, the free energy of the film decreases sharply and attachment occurs.

The force required to overcome the free energy barrier to attachment is provided by collision of particles and bubbles within the flotation cell. However very small particles tend to flow around bubbles due to their low momentum which is insufficient to allow them to penetrate the more ordered, and therefore more viscose, hydrated layers surrounding the interface. It can be seen therefore that attachment is also dependent upon the particle size and the degree of turbulence within the flotation cell.

Depending upon the relative sizes of the bubbles and particles one or more particles may be attached to one bubble, one or more bubbles attached to one particle or aggregates of bubbles and particles (known as aeroflocs). The latter situation is most commonly found in dissolved air flotation. The mechanism of attachment in this case is by formation of gas bubbles on the particle surface. Gas molecules, or sub-microscopic aggregates of molecules, from a

supersaturated solution diffuse through the hydrated layers to the solid surface from which they displace water molecules more readily than they separate the molecules themselves. Clearly the greater the surface hydration, the greater will be the diffusion path for the gas molecules and bubble formation will be slower and less complete. Once at the surface, the bubble nuclei expand rapidly due to inward diffusion of gases, provided the partial pressure within the bubble is less than that of the surrounding solution.

Bubble-particle attachment, induced by the use of suitable collectors and frothers, is sufficient for flotation to occur. However the process can be considerably enhanced or suppressed or made selective for specific particles in a mixture by the addition of certain flotation reagents known as regulators. These may be organic or inorganic and have various modes of action. They may adsorb to particle surfaces and either increase hydration, thus depressing flotation, or decrease hydration, causing activation. Typical depressants in mineral flotation are starches and tannins whose highly hydrated molecules adsorb to certain mineral surfaces in thick films. Certain depressants act by desorbing previously adsorbed collector from the mineral surface; for example sodium sulphide desorbs xanthates from the surface of sulphides (84). They may also act without removal of the collector, by adsorbing to those portions of the surface which are not covered as for example potassium chromate which depresses the flotation of lead sulphide with xanthate.

Some regulators can cause activation of flotation by forming 'bridges' between the particle surface and the collector. Thus heavy metal ions adsorb to the surface of quartz and subsequently react with oleate ions resulting in chemical adsorption of this ion on the

quartz.

Acids and alkalis can also affect flotation by altering the pH. The effects of pH regulation are dependent upon the particular flotation system to which it is applied. Thus the hydrogen ion concentration can affect the degree of dissociation of collector molecules into ions. Hydroxyl and hydrogen ions can also diffuse into the hydrated layers surrounding a particle and affect their electrical properties. Such alterations in these potentials and in collector dissociation can profoundly affect the adsorption of collector to the particle surface.

Hydroxyl and hydrogen ions can also depress flotation of anionic and cationic collectors respectively by competing with them for adsorption to the particle surface or the electrical double layer surrounding the interface. Thus, for example, hydrogen ions increase the degree of dissociation of a cationic collector, favouring its adsorption to a negatively-charged surface. However hydrogen ions may also compete with the collector for adsorption to that surface which suggests that adsorption of collector will be maximal at a specific pH value. As already noted, Grieves and Bhattacharyya (56) reported pH optima for the flotation of a cationic surfactant, presumably corresponding to maximum adsorption to the gas-liquid interface.

Various inorganic salts also have a regulating effect upon flotation as demonstrated by the studies of Gaudin and his co-workers (49, 50) and Crandall and Grieves (33) which have already been discussed. In addition, several workers have studied the flotation of coal in solutions of inorganic salts; much of this work being reviewed by Klassen and Mokrousov (84). The results showed that there

was considerable adsorption of ions on the coal, reducing its surface hydration, and therefore improving the attachment of particles to bubbles. No satisfactory explanation why further reduction in the hydration of an already weakly hydrated particle surface should enhance its flotation has yet been proposed.

Much of the flotation theory discussed so far has been related to mineral ore separation. In such a system, the flotation tank feed or 'pulp' contains extremely large amounts of dissolved and suspended inorganic material and requires relatively large concentrations of flotation reagents for successful separation. In water and wastewater treatment the main constituents requiring removal are organic and, except in the case of sludge thickening, are present in much lower concentrations. Futhermore, for sewage and water treatment the objective is to remove as many of the constituents as possible from the water whereas the mining industry generally requires separation and concentration of only one component. Nevertheless flotation has been successfully applied to water treatment and as already noted many of the results can be explained in terms of the theory derived from mineral separation practice.

It is the aim of this research to study the interaction of a model virus with certain flotation reagents and to determine whether their behaviour in a flotation system can be defined in terms of mineral flotation theory.

As previously stated the particular flotation reagent used throughout these studies was a quaternary ammonium detergent (Arquad T-50) which acted as both collector and frother. However many workers have noted the toxicity of similar surfactants towards microorganisms such as bacteria (70, 119) and their spores (114). Much of this work

has been reviewed by Newton (106) who proposed that surface-active compounds combine with and modify the bacterial cell wall and/or membrane in such a way as to cause disruption and leakage of cellular constituents. Inhibition of this toxicity, however, has been demonstrated by certain phospholipids added either before or simultaneously with the detergents (9). Phospholipids possess the typical polar-nonpolar structure of a surface-active compound and it was proposed that they competed with detergents for sites on the bacterial cell. This is in agreement with the mechanism of action of certain organic depressants of flotation, although phospholipids were unable to desorb previously adsorbed detergent since they were ineffective unless added before or simultaneously with the detergents.

The binding of the cationic detergent - cetyltrimethylammonium bromide (CTAB) to yeast cells has also been investigated by Fujita and Koga (44). They found that increasing the pH of the medium increased the amount of CTAB adsorbed and calculated that for 100% inactivation of a yeast cell suspension, the cells must adsorb an amount of CTAB equal to that required to form a monolayer over the entire surface area of the cells. The pH effect was unexpected since flotation theory predicted that greater adsorption of cationic detergents would occur at lower pH values. However, the proteins contained in the yeast cell membrane exposed numerous ionizable carboxyl and amino groups to the surface. At high pH values this would therefore have an overall negative charge and thus adsorb greater quantities of cationic detergent.

In similar studies using various surfactants and dyes Riemersa found no variation with pH through the range 3.2 to 7.0 (113). Below pH 3.2 and above pH 7.0 he found greatly increased sensitivity to

cationic dyes and surfactants whilst the anionic sodium dodecylsulphate only caused cell lysis below pH 3.2. He proposed that the primary sites of adsorption were the anionic phospholipids of the cell membrane. These results apparently contradict those of Fujita and Koga (44) although they measured CTAB adsorption whereas Riemersa measured the resulting cytolysis of the yeast cells. Adsorption of surfactant to the cell membrane is clearly a complex phenomenon, as is the subsequent lysis resulting from such adsorption.

At lower concentrations than those required for cytolysis, detergents inhibit yeast metabolism. Armstrong (5) showed that such inhibition by dodecyl trimethylammonium bromide could be overcome by the addition of metallic cations whose order of increasing effectivess was trivalent>divalent>monovalent. Again the proposed mechanism was competition with detergent ions for anionic sites on the cell surface.

The cell membrane of yeasts and bacteria is a very complex structure which allows numerous compounds to pass into or out of the cell by diffusion or by active transport at specific sites. Adsorption of surfactants to such a surface may therefore be expected to interfere with at least some of these processes, impeding cellular metabolism and ultimately causing cytolysis. However synthetic detergents have also been found to be toxic towards viruses, whose outer wall or capsid is a much simpler structure by comparison, and which possess no metabolic activity.

Klein and his co-workers (85) exposed Influenza A, Vaccinia virus and two bacteriophages to twenty different detergents and concluded that cationic surfactants were more toxic than anionic and non ionic detergents caused least inactivation. These findings were

verified by Stols and Veldstra (129) for Turnip Yellow Mosaic Virus (TYMV). They used quaternary ammonium compounds of the general structure $C_nH_{2n+1}N.(CH_3)_3Br$ and found no significant inactivation with a hydrocarbon chain length of less than n = 6. The inactivation mechanism which they proposed involved complexing of the quaternary ammonium groups with the ribonucleic acid (RNA) inside the virus particle, causing it to uncoil and expand thus rupturing the virus coat. However if this were correct, one would expect more rapid inward diffusion of the shorter chain homologues of the surfactant and thus greater inactivation. Clearly the hydrocarbon chain was also actively involved and the inactivation mechanism therefore more complex.

In an extensive review of the literature, Armstrong and Froelich (4) concluded that the cationic surfactant benzalkonium chloride, was effective against most virus groups except the picornaviruses which were highly resistant to most detergents including quaternary ammonium compounds. However in all these cases the viruses were suspended in media containing high concentrations of salts and/or proteins such as tissue culture fluid (26), buffers and serum solutions (4), and ground cell filtrate (85). As already explained, these substances can all act as regulating agents in flotation and as such could have profoundly affected the adsorption of surfactant to the virus capsid. The concentrations of such salts and proteins are considerably lower in natural waters and sewage effluents and thus the toxicity of surfactants towards the picornaviruses in these media may be much greater.

The resistance to inactivation of the picornaviruses is undoubtedly related to their structure. They are small, isometric

particles between 20 and 30 nm in diameter with a particle weight of 4-7 x 10^6 daltons (43). The capsid is composed exclusively of repeating protein subunits, called capsomeres, assembled into an icosahedral structure around an RNA core. This is in contrast to several other virus groups such as Myxoviruses, Paramyxoviruses, Herpesviruses, etc. whose more complex capsids also contain lipids and carbohydrates, rendering these viruses susceptible to inactivation by lipid solvents such as ether, chloroform or bile salts.

The relatively simple organization of the protein capsid of picornaviruses also confers resistance to various other inactivating agents including chlorine (88, 120), ozone (22) and formaldehyde (28, 132) as well as to extremes of heat (146, 150) and pH (7). Some of these investigations have included studies on the rate of inactivation and several workers have attempted to determine the mechanisms involved in the lethal event by detailed analysis of the 'survival curves' produced (6, 45, 72, 76). Many of the results are conflicting as for example the data on thermal inactivation. Ginoza used RNA isolated from Tobacco Mosaic Virus (TMV) and concluded that the inactivation rate followed first order kinetics (52), i.e.

$$\frac{N}{No} = e^{-kt}$$

where N = concentration of viable viruses at time t

No = initial concentration of viable viruses at time zero

However, both Kaplan (81) and Bachrach and co-workers (7), using Vaccinia virus and Foot-and-Mouth Disease virus respectively, plotted survival curves of a two-component character which they attributed to inherent heterogeneity of the virus.

More complex survival curves have been reported for formaldehyde inactivation of poliovirus showing an initially rapid rate of inactivation which decreases regularly with the time of treatment. Gard (45) postulated that this resulted from a reaction between formaldehyde and the viral coat protein which gradually decreased its permeability thus slowing penetration of formaldehyde to the sensitive RNA core. However Hiatt (72) pointed out that several other mechanisms could be used to construct survival curves which fitted the data equally well and that such a fit was not proof of the mechanism.

Undoubtedly a greater knowledge of the properties of the viral capsid surface is essential to the understanding of viral inactivation mechanisms. Like all proteinaceous material, the properties of the viral coat proteins in solution are determined by the large number of free ionizable groups of the amino acids comprising the polypeptide chains. The ionization of these amino and carboxyl groups is pH-dependent thus:

Low pH: $R - COO^- + H^+ \rightleftharpoons R - COOH$

$R - NH_2 + H^+ \rightleftharpoons R - NH_3^+$

High pH: $R - COOH \rightleftharpoons RCOO^- + H^+$

$R - NH_3^+ \rightleftharpoons RNH_2 + H^+$

At a certain pH value characteristic of the particular protein, equal numbers of carboxyl and amino groups will be ionized and the overall net charge on the protein molecule will be zero. This is known as the isoelectric point and at this pH the protein will remain stationary when placed in an electric field. At any pH value above the isoelectric point the molecules will carry a net negative charge and migrate to the anode. The magnitude of the charge increases as

the pH is raised, thus increasing the velocity of migration. Similarly, below the isoelectric pH the molecules carry a net positive charge and migrate towards the cathode.

This principle is utilized in the separation of proteins on the basis of their rates of migration in an electric field at a particular pH and is known as electrophoresis. Various electrophoretic techniques have been applied to the study of intact viruses. Early workers used the Tiselius apparatus to observe the movement of the boundary between a pure buffer and a buffered solution of the virus under an applied electric field (e.g. 102). Also popular was the Northrop-Kunitz micro-electrophoresis cell in which the movement of collodion particles coated with viruses was observed microscopically in various buffered solutions (102). These studies performed on the PR8 strain of Influenza virus, showed that it could be separated and purified from a crude cell extract and that its isoelectric point was at pH 5.3.

Both these methods have disadvantages, however, including susceptibility of the apparatus to thermal convection currents, the need for elaborate optical equipment and relatively large amounts of virus suspension. The first of these drawbacks can be overcome by electrophoresis in a porous, inert support medium, generally connected between buffer reservoirs containing the electrodes. During electrophoresis, the viruses and extraneous proteins migrate as discrete bands within the support material and may be visualized by staining or the support may be sectioned and the viruses eluted and assayed from each strip. The choice of support material for intact viruses is limited to those of sufficient porosity to allow

free migration of the particles. Two which have been used successfully are filter paper (53, 151) and agar (23) for TMV and bacteriophages, respectively. The first two studies demonstrated the feasibility of using electrophoresis to purify TMV from infected plant cell extracts whilst the third study by Burnet and McKie attempted to determine the surface charge on twenty different bacteriophages. The results showed that all of them exhibited a negative surface charge at pH values above 4.0.

More recently, gels of polyacrylamide have been used to separate and purify intact viruses (13, 30, 130) and viruses treated with sodium dodecyl sulphate to disrupt the viral coat proteins (130). The latter study, employing the bacteriophage Qβ, demonstrated the existence of only two different proteins comprising the capsid.

A technique of great potential has recently been developed which utilizes the ionization properties of complex, synthetic ampholytes to establish a pH gradient between the cathode and anode (87). Any proteinaceous material within this gradient will migrate under the influence of the applied electric field until, on reaching its isoelectric pH, its net charge becomes zero and it remains stationary within the gel. This procedure is known as isoelectric focussing and has the advantages of high resolving power, speed and simplicity. The isoelectric points of a number of plant viruses and Qβ bacteriophage have been determined by this procedure and the majority were found to lie between pH 4.0 and pH 4.3.

It was noted by Strauss and Kaesberg (130) that the number of ionizable groups on the surface of Qβ bacteriophage was relatively small. This fact and the general polypeptide structure of proteins would suggest that the phage surface may be at least partially

hydrophobic. The floatability of naturally hydrophobic coal particles has been noted earlier and it is therefore possible that virus particles may similarly be attracted to gas-liquid interfaces. This may result in virus inactivation without prior adsorption of surfactant. Unfortunately, testing this hypothesis by aeration would also cause mechanical agitation of the suspension which itself may cause inactivation.

Published data on mechanical agitation of bacteriophage (25), equine encephalitis virus (100) and Rauscher virus (91) concluded that inactivation did occur although in each case the method used did not preclude inactivation by adsorption to a gas-liquid interface. Campbell-Renton (25) and McLimans (100) used vigorously-agitated vials containing small volumes of virus suspension whilst Levy and his co-workers (91) used an Ultra-Turrax tissue grinder. All these workers reported rapid inactivation of viruses suspended in buffers or culture media, but Campbell-Renton found considerably less inactivation in distilled water or 1% $^{w}/_{v}$ peptone solution. These results are consistent with flotation theory since inorganic salt solutions enhance surface adsorption of naturally hydrophobic particles and peptone may be expected to protect the virus by competitive adsorption to the interfaces.

Similar experiments carried out by Adams (1) showed that shaking glass vials completely filled with suspension caused no loss of bacteriophage titre, presumably discounting inactivation by mechanical agitation. He confirmed that surface inactivation occurred and that this could be prevented by addition of sufficient protein to saturate the gas-liquid interfaces.

Extensive studies on the inactivation of viruses in aersols have been performed by Trouwborst and his co-workers (35, 135-139). They verified that inactivation was due to surface adsorption and that in salt solutions, this increased with the ionic strength (136, 137). Peptone, apolar amino acids (e.g. leucine, phenylalanine) and non-ionic detergent decreased inactivation (137, 139). They found that surface inactivation was accompanied by loss of infectious RNA from encephalomyocarditis virus (35) and loss of deoxyribonucleic acid (DNA) from T1 bacteriophage (138). They suggested that the increased surface tension of the interface in salt solutions may have caused nucleic acid release by physical disruption of the particle. However this increase is small compared with the effect electrolytes have on the rate of particle-interface attachment and on the tenacity of that attachment which are more likely causes of increased inactivation.

The aim of the research presented here was to investigate the factors affecting virus inactivation during dispersed air flotation and to equate these with the parameters of importance in mineral particle flotation. This was hoped to be of use in assessing virus removals during water and wastewater treatment by flotation and it was therefore considered important to choose a virus representative of those found in sewage. Prominent among these are the Enteroviruses comprising the subgroups Poliovirus, Coxsackievirus and Echovirus. These grow in the intestinal walls of infected patients and are excreted in large numbers in the faeces (27). As they are picornaviruses they are highly resistant and much evidence suggests they can survive sewage treatment processes (14, 15, 89, 96, 98, 143) and potable water purification systems (14, 15, 27, 31, 32, 42, 126)

and so become a potential health hazard. They are also extremely long-lived and can survive for up to several months in natural waters (8, 14, 31, 143). A number of outbreaks of viral diseases including poliomyelitis (11, 93) and infectious hepatitis (36) have been attributed to a sewage contaminated water supply and much of this literature has been reviewed elsewhere (15). Chang has pointed out that sub-clinical infections from contaminated water supplies may considerably outnumber clinical cases (27).

For the purpose of this study, it was decided to use the Escherichia coli bacteriophage MS-2 as a model virus. This is a picornavirus which very closely resembles the enteroviruses in both size and structure and was used for two reasons. Firstly, to obviate the health hazard ensuing from flotation experiments in the laboratory, where the aeration of suspensions would inevitably produce aerosols. The use of pathogenic viruses would have necessitated elaborate precautions for the sterilization of the potentially virus-laden exhaust air. Secondly, culturing host bacteria and the assay of their bacteriophages is simpler and quicker than the maintenance of slow-growing and expensive tissue culture cell lines required for the multiplication and enumeration of enteroviruses, thus allowing increased experimentation in a given time.

MS-2 bacteriophage is a member of the RNA-containing coliphages which are male-specific, adsorbing to the F-pilus of the host bacterium. They have icosahedral symmetry with a particle diameter of 23 to 25 mm. They contain single-stranded RNA with a molecular weight of 1.2×10^6 daltons consisting of 3 genes whose nucleotide sequence has recently been elucidated (40, 41, 103). These code for the coat protein, A-protein and the replicase enzyme. There is

one A-protein molecule per virion which has been shown to be located in the phage capsid and to be involved in the adsorption of phage to the sex pilus (34).

2. METHODOLOGY

The distilled water used throughout was produced by a Jencons Autostill Eight Plus electric still and stored in 20 ℓ glass aspirator bottles until required. All glassware was cleaned by boiling for 15 minutes in a 0.1% $^{v}/_{v}$ solution of QED contained in a 15ℓ stainless steel bucket. After cooling, the glassware was rinsed in 3 changes of tap water and 5 changes of distilled water before drying in a warm air cabinet. Pipettes were then plugged with cotton wool and placed in metal canisters and phage assay tubes were capped and stacked on metal trays prior to sterilization. This was achieved by heating to 180°C for two hours in a hot air oven.

All prepared media were dispensed into clean glassware prior to sterilization by autoclaving at a temperature of 121°C for 15 minutes. All manipulations involving sterile media, bacteria or bacteriophage were performed using standard aseptic techniques.

The addresses of suppliers of equipment and materials used throughout this study will be found in Appendix V. Unless otherwise stated, all chemical reagents were of Analar grade and obtained from BDH Chemicals Ltd. and items of laboratory equipment were obtained from A. Gallenkamp and Co. Ltd.

2.1. Foam Flotation Apparatus

2.1.1. 200 ml Capacity Units

Key to Schematic Diagram (Figure 3)

1 - Flotation columns

2 - Feed lines of 3 mm internal diameter plastic tubing

3 - Liquid feed pump (Watson-Marlow MHRE MCIO)

4 - 5ℓ bacteriophage stock feed bottle contained in expanded polystyrene ice-bucket.

5 - Magnetic stirrer

6 - 1ℓ surfactant solution feed bottles

7 - Air flow meters (GPE Meterate Tube D ST.ST. Float)

8 - Air flow regulators (Hoffmann clips)

9 - Air supply ring manifold of 3 mm ID plastic tubing

10 - Air filter (Glass fibre packed in a Pyrex 500 ml Buchner flask)

11 - Excess air bleed-off

12 - Air feed pump (Charles Austen Model MU 13/26).

The flotation columns were constructed from Perspex tubing with a wall thickness of 3 mm and an internal diameter of 32 mm. A scale diagram of one of these columns is shown in figure 4.

The spargers consisted of Jobling 32 mm x 3 mm sintered glass discs having a porosity of 8-15 microns.

The four columns were housed in a specially constructed, temperature-controlled water bath. This consisted of a Perspex tank 600 mm x 340 mm x 340 mm insulated with expanded polystyrene 30 mm in thickness. An aquarium-type 100 watt heater and thermostat maintained the temperature to within 0.5^{o}C of that selected.

Figure 3. Schematic Diagram of Small-Scale Foam Flotation Equipment.

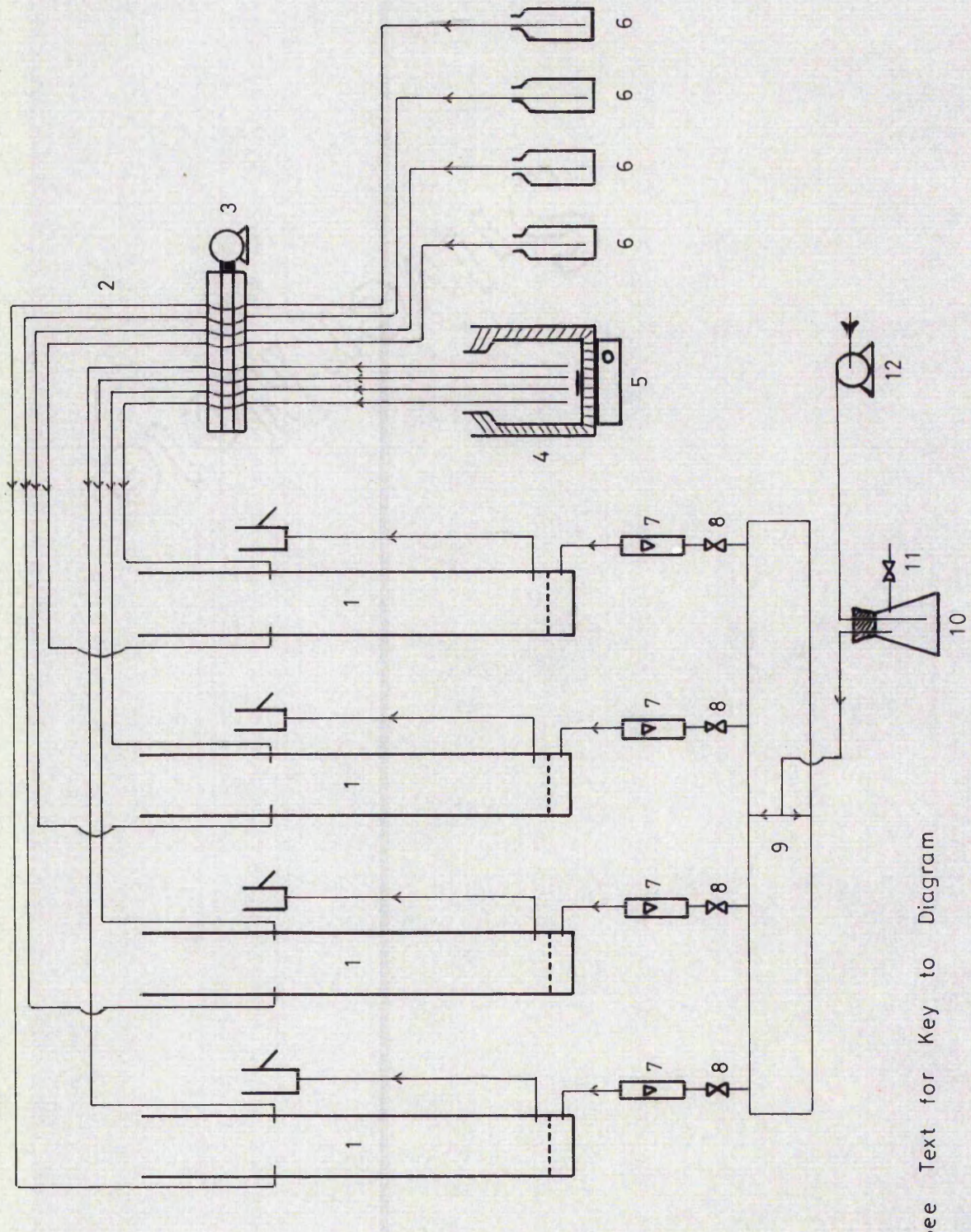
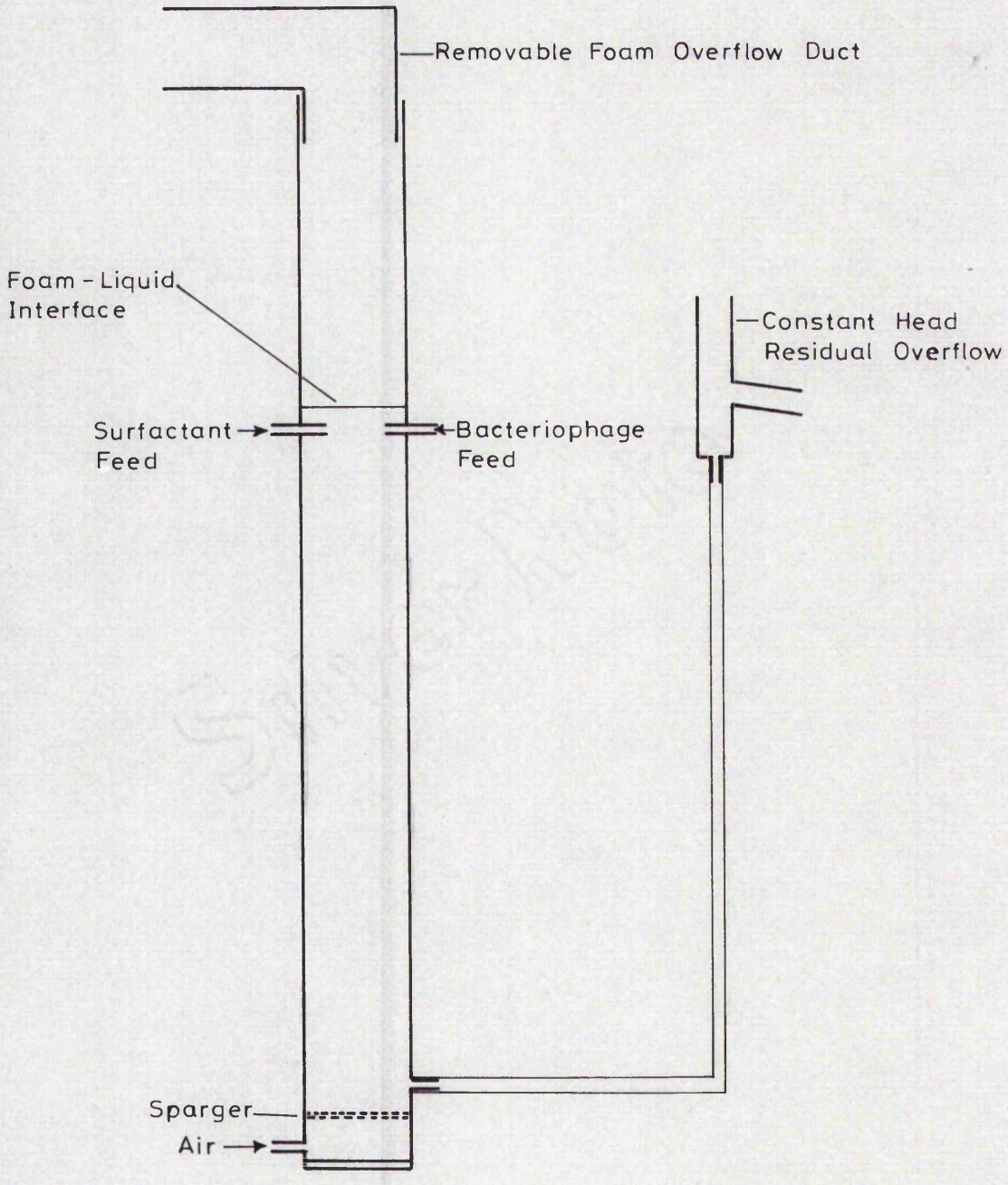


Figure 4. Schematic Diagram of 200ml Foam Flotation Column.



SCALE - 1 : 4

An LEEC glass fibre ultra filter unit was incorporated into a hood fitting over the tank and exhaust air from the columns was extracted through this filter by a suction fan and vented outside the laboratory.

Air supplied by the pump (12) was filtered through glass fibre wool (10) and delivered via the ring manifold (9) through the regulators (8) and flow meters (7) to the columns. In the majority of the experiments the air flow rate was held constant at 0.5ℓ per minute per column.

The separate bacteriophage (4) and surfactant (6) feeds were delivered to the columns by the feed pump (3). The ten channel module of this pump was fitted with 8 silicon rubber tubes of 4 mm internal diameter. For the majority of the experiments, the speed of the pump was adjusted to give a flow rate of 5 ml per minute per tube. The total flow rate per column was thus 10 ml per minute and the bacteriophage and surfactant feeds were mixed to give a dilution ratio of one to one. The volume of liquid within each column was maintained at 200 ml by adjustment of the height of the constant head overflow, giving a residence time of 20 minutes.

The bacteriophage feed suspension was prepared by addition of 0.5 ml of crude phage lysate to 5ℓ of pre-cooled ($4^{o}C$) distilled water and overnight storage at $4^{o}C$ to ensure deaggregation. This volume was sufficient for two experimental runs. The feed solutions of surfactant and specific salt under investigation were prepared at twice the experimental concentration required to allow for dilution by the bacteriophage suspension.

At the start of each run, the air pump (12) and ultra filter fan were activated and 200 ml of distilled water added to each column.

The feed tubes were connected to the appropriate stock solutions and the feed pump (3) started. After operating for 100 minutes, steady state conditions were attained and samples of treated water collected from the residual overflows. These were suitably diluted and assayed for remaining bacteriophage. The phage titre of the feed suspension was taken as the mean of assays performed at the start and finish of each run.

Between experimental runs, the feed tubes and columns were thoroughly flushed with tap water, drained and refilled with distilled water. Experiments were conducted in replicates of four, so that each experimental treatment was tested in each column and the mean of the results calculated. In this way the effect of performance differences between the columns was minimized.

2.1.2. 2400 ml Capacity Unit

Key to Schematic Diagram (Figure 5)

1 - Air supply pump (Edwards EB3A vacuum and compressor pump)

2 - Air filter (2 QVF funnels packed with glass fibre)

3 - Air bleed-off valve (Rotaflow stopcock TF2/13)

4 - Air saturator (50 mm x 600 mm QVF with sintered glass filter stick sparger, containing 800 ml water)

5 - Air flow meter (Meterate GPE type G glass float)

6 - Liquid drain valve (Rotaflow stopcock TF2/13)

7 - Foam recycle and sample control valve (3-way glass stopcock)

8 - Foam breaker (Hot air blower)

9 - LEEC Ultra filter fan unit

10 - Surfactant stock solution magnetic stirrer

11 - Surfactant stock solution reservoir (20ℓ glass aspirator)

Figure 5. Schematic Diagram of Large-Scale Foam Flotation Equipment.

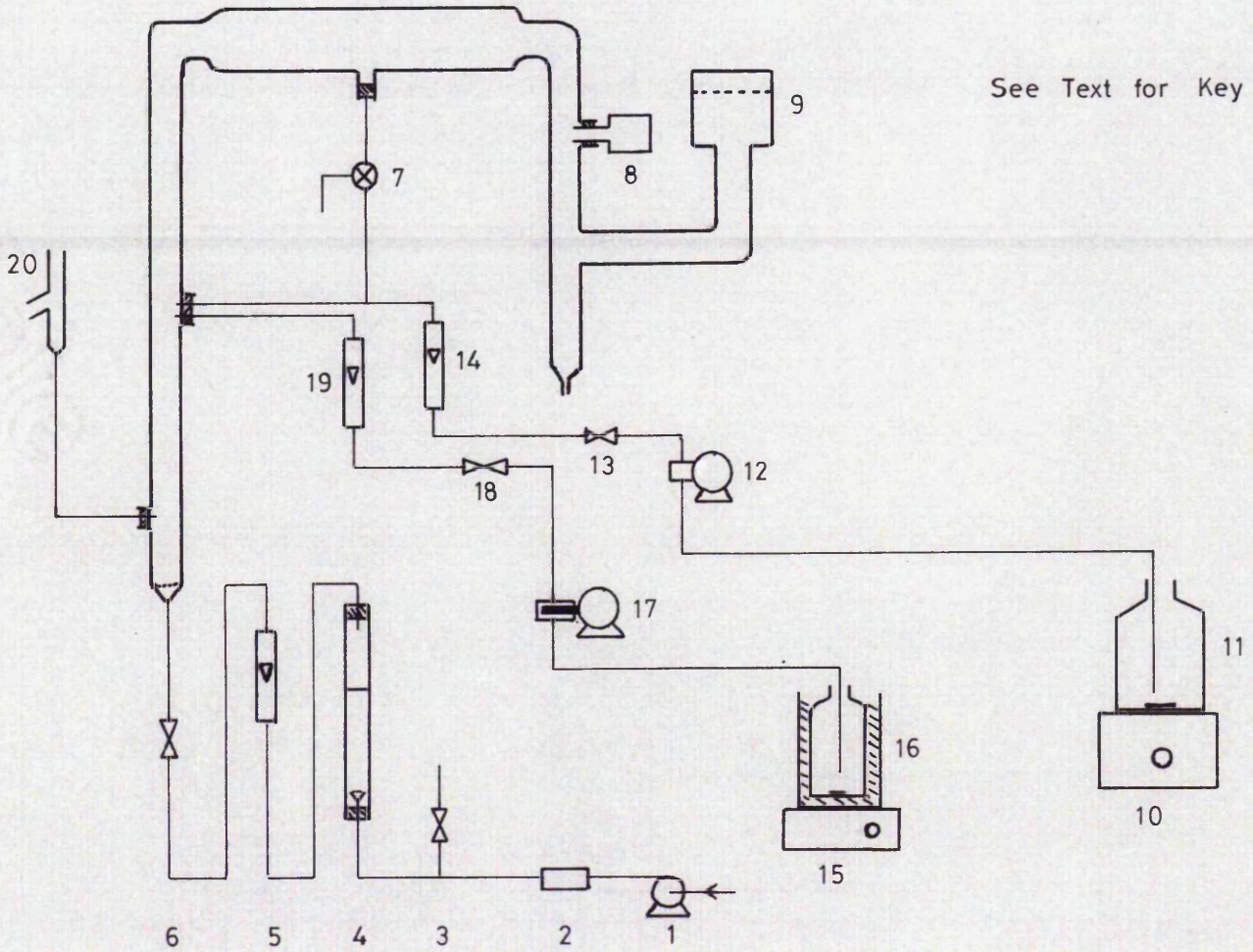
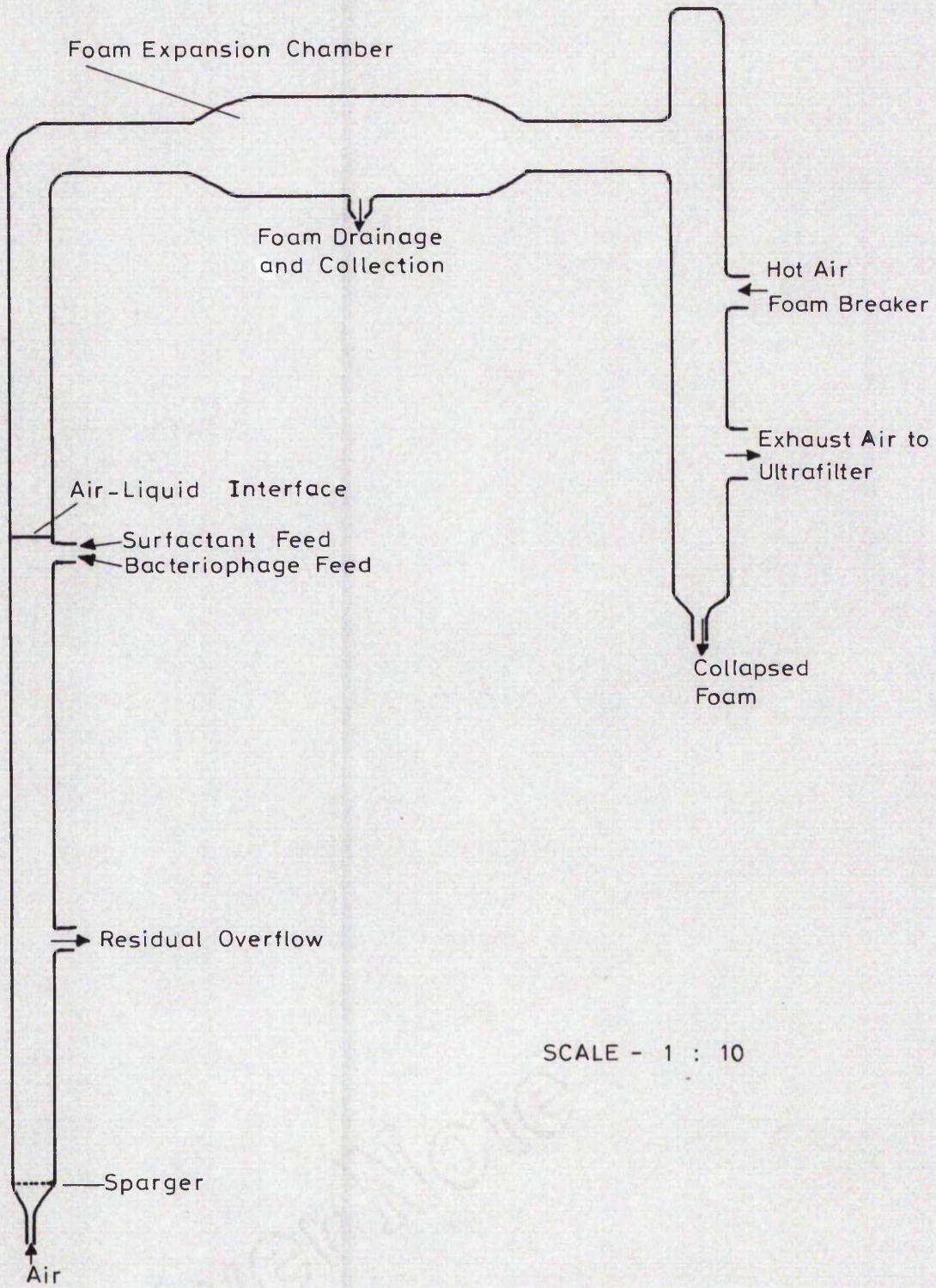


Figure 6. Schematic Diagram of 2400ml Foam Flotation Column.



12 - Surfactant solution feed pump (Watson Marlow MHRE 200)

13 - Surfactant solution flow regulator (Rotaflow stopcock TF2/18)

14 - Surfactant solution flow meter (Meterate GPE type E st st float)

15 - Bacteriophage stock suspension magnetic stirrer

16 - Bacteriophage stock suspension reservoir (1ℓ glass bottle in Thermos flask filled with crushed ice)

17 - Bacteriophage feed pump (Watson Marlow MHRE MCIO)

18 - Bacteriophage suspension flow regulator (Hoffmann clip)

19 - Bacteriophage suspension flow meter (Meterate GPE type B st st float)

20 - Specially constructed glass residual overflow.

The construction of the flotation column was based on a design by Guy (67) and is shown to scale in figure 6.

The column was assembled from 750 mm ID QVF tubing, using the appropriate gaskets and couplings. The sparger consisted of a sintered glass filter unit of 650 mm diameter, having a porosity of 5 to 15 microns, which was fixed to the end of the column with Araldite. The air and surfactant feed lines were constructed from 10 mm internal diameter plastic tubing and 3 mm tubing was used for the bacteriophage feed line.

At the start of an experiment the air supply pump (1) was activated and the drain valve (6) opened. This valve prevented liquid draining back through the sparger into the air line and causing a fluctuating reading on the flow meter (5). The flow rate was regulated by adjustment of the air bleed-off valve (3). With the saturator (4) in operation, the air delivered to the column had a relative humidity of 100%. The volume of liquid contained within the column was maintained at 2400 ml by adjustment of the height of the

residual overflow.

A 20ℓ volume of stock surfactant solution was prepared in the reservoir (11), an encapsulated magnetic follower added, and mixed on the stirrer (10) for one hour prior to use. The stock phage suspension was prepared by addition of 0.1 ml of crude phage lysate to 1ℓ of distilled water and overnight storage at $4^{o}C$. This suspension was stored on ice (16) and stirred (15) continuously throughout each experimental run.

At time zero minutes, the two feed pumps (12) and (17) were activated. The flow rates were controlled by adjustment of the pumps' speed and by the regulators (13) and (18) which damped the pulsating output of these pumps. The ratio of the flow rates of surfactant solution to phage suspension was maintained at 100 to 1, giving a phage concentration entering the column of 1% of that of the stock phage suspension.

The foam produced by aeration passed up the column into the expansion chamber where entrained liquid drained off and was either recycled or run to waste via the control valve (7). On passing out of the expansion chamber, the foam was collapsed by the foam breaker (8) and the liquid collected at the sampling port. Exhaust air from the column was passed through the ultrafilter (9) to avoid possible aerial contamination of the laboratory with phage-containing aerosols.

To ensure steady-state conditions had been attained, the unit was allowed to operate until 18 litres of surfactant solution had passed through the column before taking any of the samples.

After each experiment, the feed lines and the column were flushed with distilled water and drained.

2.2. Determination of Arquad Concentration

The following methods were based on those used by Guy (67). Both relied on the neutralization of cationic material by anionic surfactant and detection of the excess anionic surfactant as a methylene blue complex, soluble in chloroform.

2.2.1. Titrimetric Method

This was an adaptation of a technique described in 'Recommended Methods for the Analysis of Trade Effluents' (79). Methylene blue indicator (see Appendix IV) was added to the Arquad sample and titrated against anionic surfactant, the end-point being taken as the first appearance of blue colour in the chloroform layer.

Standard solutions of anionic Teepol 610 and cationic Arquad T50 were prepared containing 1 mgl^{-1} of active material.

One ml of the cationic surfactant solution was placed in a 150 mm x 15 mm Pyrex test tube followed by 2 ml of chloroform and 2 ml of methylene blue indicator solution.

The anionic surfactant was added from a burette, with constant, gentle swirling until the first appearance of blue colour in the chloroform layer, and the volume used noted. A correction factor was calculated by dividing the product of the volume (ml) and concentration ($mg.l^{-1}$) of anionic surfactant by the product of the volume (ml) and concentration ($mg.l^{-1}$) of cationic surfactant. Test samples, suitably diluted as necessary to give a concentration of surfactant less than 2 $mg.l^{-1}$, were similarly assayed. The concentration of active cationic material was then calculated using the following formula:

$$\text{Concentration of cationic surfactant } (mgl^{-1}) = \frac{\text{Number of ml anionic surfactant x concentration } (mgl^{-1}) \text{ of anionic surfactant}}{\text{Number of ml of actionic surfactant x correction factor}}$$

2.2.2. Colorimetric Method

This technique was adapted from one described in 'Standard Methods of Chemical Analysis' (127). The cationic surfactant sample was reacted with a known excess amount of anionic surfactant, the methylene blue complex extracted into chloroform and its optical density measured at a wavelength of 652 nm. The surfactant concentration was then compared with a standard graph relating Arquad concentration in mgl^{-1} to optical density. This was prepared by treating standard Arquad solutions containing 0.0, 5.0, 10.0, 15.0 and 20.0 mgl^{-1} active matter as follows: Aliquots of 2 ml were reacted with 2 ml of a 20 mgl^{-1} Teepol 610 solution in a 10 ml volumetric flask and made up to volume with distilled water. The resultant solution was transferred to a 250 ml separating funnel, two drops of 1% $^{w}/_{v}$ phenolphthalein indicator added and made slightly alkaline with 0.1M sodium hydroxide. The solution was then just acidified by dropwise addition of 0.1M sulphuric acid. One ml of chloroform and 2.5 ml of the methylene blue indicator solution were added and the stoppered funnel gently swirled for 30 seconds. After allowing separation, the chloroform layer was drawn off. Two further extractions using one ml aliquots of chloroform were performed, and the methylene blue - surfactant solution discarded. The combined chloroform extracts were replaced in the separating funnel and back extracted with 5 ml of wash solution (Appendix IV). After separation and removal of the chloroform layer, the wash solution was re-extracted with a further 1 ml of chloroform which was again separated and removed. After discarding the wash solution, the separating funnel was rinsed with 1 ml of chloroform. All the chloroform extracts were combined in a 10 ml volumetric flask and made to volume with chloroform.

The optical density of the methylene blue-surfactant complex in the chloroform was determined at a wavelength of 652 nm using an Eel 'Spectra' Spectrophotometer. Each standard solution was similarly treated and the standard graph prepared (see Appendix I). Test solutions were diluted to within the concentration range of this graph and after preparation of the complex and measurement of its optical density, the Arquad concentration was determined from the standard curve.

2.3. Determination of Ionic Concentration

2.3.1. Calcium Ions

Atomic absorption spectrophotometry was employed using an Eel 240 Atomic Absorption Spectrophotometer. Illumination was provided by a Cathodeon Hollow Cathode lamp Type 4 BS/Li, operated at a filament current of 3mA. The support gas (air) and fuel gas (acetylene) were delivered to the burner at flow meter settings of 5 and 1 respectively. At a slit width value of 2, readings were taken in the integrated mode at a wavelength of 423 nm.

To determine calcium concentration in the samples, a standard curve was first prepared relating absorption to concentration of calcium in $mg.l^{-1}$. Standard solutions were prepared by dissolving calcium sulphate in distilled water to give calcium concentrations of 2.5, 5.0, 7.5, 10.0, 15.0 and 20.0 $mg.l^{-1}$, which were measured against a distilled water blank. The standard curve was prepared and is shown in Appendix II.

Prior to their assay, samples were suitably diluted with distilled water to bring their optical density readings within the range of the standard curve.

2.3.2. Sulphate Ions

A gravimetric method, based on that outlined in 'Standard Methods of Chemical Analysis' (127) was adopted. This involved reacting test samples with excess barium chloride and weighing the resulting barium sulphate precipitate.

Specifically, 5.5 ml of a 0.1 M barium chloride solution and 5 ml of 3.0 M hydrochloric acid were placed in a 500 ml conical flask, and heated to 95°C. Two hundred and fifty ml of test solution was slowly added with stirring and the temperature maintained at 95°C for

1 hour. The flask was then allowed to stand at room temperature for 18 hours when the suspension was filtered through a Gooch sintered glass crucible, previously dried to a constant weight. The precipitate was washed with 200 ml of distilled water and dried in an oven at 180°C for 4 hours. After allowing the crucible to cool to room temperature in a desiccator, it was reweighed and the weight of precipitated barium sulphate determined. The weight of sulphate in the precipitate was calculated using the following formula:

$$\text{Concentration of sulphate in sample } (mgl^{-1}) = \frac{\text{Weight of precipitate(mg)} \times 4 \times \text{molecular weight } SO_4^=}{\text{molecular weight } BaSO_4}$$

Where less than 250 ml of collapsed foam sample was available for analysis, the total volume was used and the volume correction factor in the formula [4] was replaced by $^{1000}/_{\text{sample volume used (ml)}}$.

2.3.3. Phosphate Ions

A spectrophotometric method was employed, as described in 'Analysis of Water' (115). This involved reacting the phosphate with ammonium molybdate, reducing the resultant phosphomolybdic complex with ascorbic acid and measuring the blue colouration produced colorimetrically.

Sulphomolybdic reagent was prepared as detailed in appendix IV. A stock solution containing 1 gl^{-1} potassium dihydrogen phosphate was made up and diluted to 10 mgl^{-1} to provide a working solution, used for plotting the calibration curve. Table 5 shows the quantities of reagents added to a numbered series of 150 ml conical flasks.

Table 5 Preparation of Standard Curve

Solution Components	Flask Number				
	B	1	2	3	4
10 mgl^{-1} PO_4^{3-} solution (ml)	0	4	8	12	16
Distilled water (ml)	40	36	32	28	24
Equivalent to x mgl^{-1} PO_4^{3-}	0	1	2	3	4
Sulphomolybdic reagent (ml)	4	4	4	4	4
Ascorbic acid (g)	0.1	0.1	0.1	0.1	0.1

The flasks were heated and maintained at 100°C, with swirling, for exactly 1 minute. After cooling, the contents were transferred to 50 ml volumetric flasks and made to volume with distilled water. Using an Eel spectrophotometer, the optical densities of the solutions were read at 608 nm against the blank solution. A standard curve of phosphate concentration versus optical density was then plotted and will be found in appendix III.

For sample assays, constituents were added as shown in table 5 with the phosphate solution and distilled water replaced by 40 ml of sample, suitably diluted to bring the phosphate concentration within the range of the standard curve. The samples were treated as before and their optical densities measured against the blank. The equivalent phosphate concentration was determined from the calibration curve and the sample concentration calculated.

2.4. Preparation and Maintenance of Cultures of Escherichia coli

The specific host strain used was Escherichia coli K_{12} Hfr H Catalogue number NCIB 10235 obtained from the National Collection of Industrial Bacteria. Stock cultures were maintained on slopes of Oxoid Blood Agar Base, previously prepared in 28 ml Universal bottles. These were subcultured at monthly intervals and stored at 4°C following overnight incubation at 37°C. Prior to use, a heavy loopful of one of these cultures was aseptically transferred into a Universal bottle containing 15 ml of Oxoid Nutrient Broth and incubated without shaking for 4 hours at 37°C. After this time the culture had entered its logarithmic growth phase and attained a concentration of approximately 10^8 cells $ml.^{-1}$.

2.5. Agar Overlay Technique

This was used for the preparation of phage stocks and for the assay of phage samples. The method consisted of overpouring seeded soft agar onto basal agar plates and was first described by Eisenstark (38).

The basal agar plates were prepared by addition of 12 ml aliquots of molten, Oxoid Blood Agar Base to the required number of plastic Petri dishes and allowing these to solidify. Prior to use, they were stored at room temperature in an inverted position for 24 hours to allow excess moisture to evaporate. For each experiment, the required number of sterile, capped tubes (100 mm x 10 mm) were placed in a Grant JB3 water bath, maintained at a temperature of 47°C ± 0.1°C. Into each tube a 2.5 ml aliquot of 0.6 $^{w}/_{v}$ Oxoid Agar No. 3 containing 1% $^{w}/_{v}$ Oxoid Bacteriological Peptone was dispensed. Five drops (approximately 0.12 ml) of the actively-growing host culture were then added by Pasteur pipette, followed by 0.1 ml of phage suspension, or its dilution, dispensed with a Sigma MP-100 micropipette. The contents of each tube were mixed by gentle swirling and rapidly poured and evenly distributed over each of the agar plates. A maximum of six overlay tubes were seeded at any one time before being poured to avoid significant heat inactivation of both host and phage. After allowing the soft agar overlay to solidify, the plates were inverted and incubated for 18 hours at 37°C.

2.6. Preparation of Cultures of MS-2 Bacteriophage

The method used was a modification of the agar overlay technique (section 2.5.) in which the phage inoculum was obtained from a single plaque, isolated from a sample assay plate. A small piece of soft agar containing the discrete MS-2 plaque was removed with a sterile scalpel and transferred to 5 ml of distilled water in a Bijou bottle. The bacteriophage were eluted from the agar by maceration with a glass rod followed by storage for 3 hours at a temperature of 25°C. Aliquots of 0.1 ml of the decanted supernatant were then added to 10 seeded overlay tubes and poured onto basal agar plates.

Following incubation, the soft agar overlay was removed from each plate with a glass spreader and poured into a hand homogenizer. Thirty ml of distilled water and 5 ml of chloroform were then added and the whole thoroughly emulsified. Following centrifugation at 30,000 g for 15 minutes in an MSE 18 centrifuge the phage-containing supernatant was decanted and stored in a 100 ml screw-capped bottle at 4°C until required. The infective titre of these preparations was approximately 10^{11} plaque-forming-units per ml ($pfuml^{-1}$). This generally declined to approximately 10^{9} $pfuml^{-1}$ after 4 months, necessitating the preparation of fresh stocks after this period.

Prior to use, 0.1 ml of this crude 'lysate' was diluted into 9.9 ml of distilled water in a Universal bottle and stored at 4°C overnight. This was found by experiment (section 3.1.) to be a suitable method of ensuring deaggregation and the preparation was referred to in the text as a stock phage dilution.

2.7. Sampling and Enumeration of MS-2 Bacteriophage

The sample to be assayed was diluted as required in logarithmic steps (1 ml into 9 ml or 0.1 ml into 9.9 ml) into pre-cooled ($4^{o}C$) distilled water in Universal bottles. Thorough mixing was achieved by agitation for 20 seconds on a vortex mixer. Aliquots of 0.1 ml of the appropriate dilutions were plated out as described in section 2.5. each dilution being assayed in triplicate. After over-pouring, the plates were set aside with the lids ajar for 20 minutes to allow excess moisture from the overlay to evaporate before incubation. This was found to be necessary to prevent condensation on the agar surface which resulted in large coalescing plaques causing enumeration difficulties. After incubation, the number of plaques per dilution was determined and the infective titre ($pfuml^{-1}$) of the sample calculated.

2.8. Bacteriophage Deaggregation Procedures

These were assessed by determining the infective titre before and after treatments designed to disrupt any bacteriophage aggregates.

2.8.1. Dilution

A variety of diluents were used which are described in detail in section 3.1. Aliquots of 9.9 ml of the appropriate diluent were added to each of 4 Universal bottles and cooled to $4^{o}C$ in a refrigerator. At time zero minutes 0.1 ml aliquots of phage lysate were added to each replicate and after 30 seconds agitation on a vortex mixer, samples were taken for assay of the initial bacteriophage titre. The Universal bottles were returned to the refrigerator and at the specified time intervals, samples were withdrawn for bacteriophage assay.

2.8.2. Sonication

Ten ml of bacteriophage suspension at $4^{o}C$ was added to a Universal bottle and placed in a 400 ml beaker containing crushed ice. A 0.1 ml aliquot was assayed for the initial bacteriophage titre of the suspension. Sonication was carried out in an MSE 100 watt ultrasonic disintegrator with a titanium vibrator probe having a cross-sectional area at the tip of 7.1 mm^2. With the probe submerged in the phage suspension to a depth of 1 mm, a frequency of 20 kcs^{-1} at a peak to peak amplitude of 8 microns was applied for 20 seconds. The Universal bottle was removed from the disintegrator, the contents agitated on a vortex mixer for 30 seconds and the infective titre assayed. This was repeated six times to give a total sonication time of 120 seconds.

2.9. Arquad Toxicity Experiments

2.9.1. Sampling Intervals of 5 Minutes

A stock solution of Arquad was prepared containing 1000 mgl^{-1} active matter and aliquots suitably diluted to make up the test solutions. One hundred ml of each solution was added to as many 100 ml rimless Erlenmeyer flasks as were required. These were then equilibrated to 30°C in a water bath. For each test the relevant flask was removed from the water bath and placed in a 400 ml insulated beaker of water at 30°C on a magnetic stirrer. An encapsulated magnetic follower was added to the flask and the stirrer speed adjusted to give adequate mixing without causing foaming of the contents. At time zero minutes, 0.1 ml of a stock phage dilution was added to the flask and a stopclock started.

At 5 minute intervals for 30 minutes, 0.1 ml aliquots were removed and immediately assayed for remaining bacteriophage.

For experiments involving pH control of the solutions, adjustments were made by dropwise addition of 0.1 M solutions of hydrochloric acid or potassium hydroxide and monitored using an EIL 7030 pH meter and combined electrode 1160-200. For experiments involving assays at longer time intervals, the flasks were returned to the water bath for incubation after an initial mixing period of 5 minutes. In all cases, the initial titre at time zero minutes was determined by assay of the stock phage dilution before addition to the flask.

2.9.2. Sampling Intervals of Less Than 1 Minute

To investigate the initial, rapid rate of inactivation the following method was adopted. One ml of surfactant solution at twice the concentration under investigation was added to a 100 mm x 10 mm

test tube. This, together with a stock phage dilution were equilibrated to 30°C in a water bath. At time zero seconds, 1 ml of the phage dilution was added to the tube which was rapidly agitated on a vortex mixer. At 30 second intervals thereafter 0.1 ml aliquots were removed and added to 9.9 ml of sterile, 1% peptone at 4°C. These samples were collected and stored on crushed ice in a Thermos flask for bacteriophage assay at the end of the experiment.

2.10. Isoelectric Focussing of MS-2

2.10.1. By Electrophoresis on Cellulose Acetate Strips

A technique was devised to establish a stable pH gradient with carrier ampholytes in a supporting medium of Shandon Celagram cellulose acetate. Strips of this material 100 mm x 25 mm, were immersed for 5 minutes in 10 ml of a stock phage dilution containing 0.2 ml of 40% $^{w}/_{v}$ Ampholine (LKB) with a pH range of 3.5 - 10.0. Six such strips were set up in a Shandon Model U77 Electrophoresis Apparatus with 0.2% $^{v}/v$ sulphuric acid and 0.4% $^{v}/v$ ethanolamine in the anodic and cathodic compartments respectively. The apparatus was connected to the Shandon d.c. power supply unit which was operated in the constant current mode at values of between 1mA and 3mA per strip for period of 1 to 5 hours duration. At the end of each run, the power supply was switched off and the strips removed and cut into 10 equal sections. The pH of each section was determined by placing a small piece of Universal pH indicator paper on the section and reading the pH by colour matching with the test chart. Each section was then placed in a Bijou bottle containing 1 ml of a 1% $^{w}/v$ peptone solution at pH 7.0. These were then stored overnight at $4^{o}C$ to allow elution of the bacteriophage and assayed following vigorous mixing on a vortex mixer.

2.10.2. By Disc Gel Electrophoresis

This technique necessitated the prior removal of interfering soluble proteins and salts present in the crude bacteriophage lysate. This was achieved by centrifugation in an MSE Super Speed 50 Centrifuge. Twenty-five ml of lysate was added to each of two, 25 ml polypropylene tubes which, together with their cap assemblies, were balanced to within 10 mg of each other. They were then placed in the previously-

cooled, 8 x 25 ml aluminium angle rotor and centrifuged for 150 minutes at 40,000 rpm, corresponding to 135, 000 g. The supernatant from each tube was discarded and the pellets resuspended in 25 ml of distilled water. After a repeat centrifugation, the supernatant was again discarded and each phage pellet resuspended in 1 ml of a 20% $^{w}/_{v}$ sucrose solution. The suspensions were pooled in a Bijou bottle and sonicated for a total of 60 seconds (as described in section 2.8.2.), to disrupt any virus aggregates formed by sedimenting the phage. This treatment yielded MS-2 suspensions with infective titres of approximately 10^{12} pfuml.$^{-1}$.

The isoelectric focussing of MS-2 was performed in a Shandon Analytical Gel Electrophoresis Apparatus, by an adaptation of the method of Rice and Horst (112). Two hundred ml of a stock solution containing 2.8% $^{w}/_{v}$ acrylamide and 0.12% $^{w}/_{v}$ N,N' - methylenebis-acrylamide was prepared and stored at 4^{o}C until required. At the start of an experiment, 10 ml of this solution was transferred to a 50 ml Quickfit flask to which was added 0.5 ml of 40% $^{w}/_{v}$ ampholine, pH range 3.5 - 10.0 and 10 µl of the purified phage suspension. Three mg of ammonium persulphate was then added and the flask connected to a vacuum pump via a Quickfit stopcock and gently shaken for 30 seconds. This degassing was essential as oxygen prevented complete polymerization of the gel. The mixture was poured into 60 mm x 6 mm glass or plastic tubes which had one end sealed with parafilm; 10 mls being sufficient to form 3 gels each approximately 55 mm long. These gels were incubated at 30^{o}C for 30 minutes to complete polymerization and the parafilm sealing the bottom of the tubes replaced by muslin to retain the gels. The tubes were fixed into the electrophoresis apparatus and the top (anodic) buffer

compartment filled with 5% phosphoric acid and the bottom (cathodic) compartment with 5% ethylene diamine. The apparatus was connected to the Shandon d.c. power supply unit and placed in a refrigerator to maintain a low temperature. Initially, the current was maintained at a constant 2mA per tube until the voltage had risen to the required level whereupon the supply was switched to the constant voltage mode and this value maintained for the required time interval. At the end of the run, the power supply was switched off and the tubes removed from the buffer reservoirs. The gels were removed from the glass tubes by 'rimming' which involved interposing a syringe needle between the walls of the tube and the gel and injecting distilled water. This was not necessary when plastic tubes were used as the polyacrylamide gel did not adhere to the plastic.

Because of the semiliquid nature of the gels, it was found necessary to maintain their shape for sectioning by laying them in plasticine moulds lined with Parafilm. Using a razor blade, each gel was cut into a series of slices, each 3 mm thick, which were placed in numbered, plastic Universal bottles each containing 2 ml of distilled water. The slices were macerated by rapidly pipetting up and down within a pasteur pipette and stored overnight at $4^{o}C$ to allow the ampholine and bacteriophage to elute from the gel. The contents of each bottle were then vigorously agitated for 30 seconds on a vortex mixer and the pH measured using an EIL 7030 pH meter with a combined electrode. Cross-contamination of the gel slices with ampholine or bacteriophage was avoided by washing the electrode assembly with methanol and distilled water between samples. The bacteriophage titre of the slice eluants was then assayed.

2.11. Mechanical Agitation of Bacteriophage Suspensions

The method used was based on one described by Campbell-Renton (25). A 350 ml aliquot of 0.2 M phosphate/citric acid buffer pH 6.0 (prepared as in Table 7) was added to a 500 ml conical flask containing an 8 mm by 60 mm encapsulated magnetic follower, and equilibrated to 30°C in a water bath. A 0.1 ml aliquot of a stock phage dilution was added to the flask and the contents magnetically stirred for 5 minutes. A sample was then assayed for initial bacteriophage concentration and the suspension rapidly dispensed into numbered Universal bottles as indicated in Table 6.

Table 6 Conditions Applied to Buffered Phage Suspensions

TEST CONDITIONS	UNIVERSAL NUMBER				
	1-4	5-8	9-12	13-16	17-20
Completely filled - no air space	✓	✓			
10 ml added			✓	✓	✓
8 x 4 mm glass beads added	✓	-	✓	-	✓
Agitation	✓	✓	✓	✓	-

These were then fixed to the table of a Gallenkamp Orbital Incubator, such that they were horizontal to the plane of movement. The temperature control was set at 30°C and the shaker operated at 300 rpm for 1 hour. The Universals were then removed and the treatments assayed for remaining bacteriophage.

2.12. pH Inactivation Experiments

2.12.1. Unaerated Suspensions

A 0.1 ml aliquot of a stock phage dilution was added to 9.9 ml of test solution in a Universal bottle, previously equilibrated to 4°C in a refrigerator. The contents were thoroughly mixed by agitation on a vortex mixer, assayed for bacteriophage and a stopclock started. The Universal was returned to the refrigerator for 5 hours and then resampled for bacteriophage. Tests were performed in duplicate for each pH value examined.

This procedure was used to examine pH inactivation in distilled water and a buffer system. The pH of the distilled water was adjusted by addition of 0.1 M hydrochloric acid or 0.1 M potassium hydroxide and determined with an EIL 7030 pH meter with a combined electrode.

The buffer system used was di-Sodium hydrogen phosphate/citric acid, as described in 'CRC Handbook of Chemistry and Physics' (24). Stock solutions were prepared containing 35.61 $g l^{-1}$ $Na_2HPO_4.2H_2O$ (0.2 M) and 21.01 $g l^{-1}$ $H_3C_6H_5O_7.H_2O$ (0.1 M) and mixed in the volumes shown in table 7 to give the required pH.

Table 7 Values of pH corresponding to Mixed Volumes of Disodium Hydrogen Phosphate and Citric Acid as Shown

pH	Na_2HPO_4 (0.2 M)	citric acid (0.1 M)
	(ml)	(ml)
2.2	0.40	19.60
3.0	4.11	15.89
4.0	7.71	12.29
5.0	10.30	9.70
6.0	12.63	7.37
7.0	16.47	3.53
8.0	19.45	0.55

These values were verified with a pH meter and adjusted as necessary with the appropriate stock solution.

2.12.2. Aerated Suspensions

For these experiments, four batch flotation columns were constructed from glass tubing 300 mm in length and with an internal diameter of 24 mm. Sintered glass discs as described in section 2.1.1. were affixed to one end of each column with Araldite. 35 mm lengths of similar glass tube had one end drawn out to 5 mm outside diameter and were attached to the sparger end of the columns with Araldite. These formed connections for attachment of the air lines. The columns were installed in the water bath described in section 2.1.1. and connected to its metered air supply. In all experiments the air flow rate to each column was maintained at 1 ℓmin^{-1} and the water bath temperature adjusted to $30^{o}C \pm 0.1^{o}C$. At the start of an experiment, 50 ml of each test solution was added to 100 ml conical flasks containing 6 mm by 50 mm encapsulated magnetic followers and equilibrated to $30^{o}C$ in a water bath. To each flask was added 0.1 ml of a stock phage dilution and the contents magnetically stirred for 5 minutes to ensure mixing. At time zero minutes samples were taken for bacteriophage assay and the contents of each flask rapidly added to the columns. After aeration for 60 minutes the solutions were again assayed for remaining bacteriophage.

In addition to distilled water and Na_2HPO_4/citric acid buffer, a Na_2HPO_4/NaH_2PO_4 buffer system was investigated. This was also prepared as described in the 'CRC Handbook of Chemistry and Physics' (24). Stock solutions containing 35.61 gl^{-1} $Na_2HPO_4.2H_2O$ (0.2 M) and 31.21 gl^{-1} $NaH_2PO_4.2H_2O$ (0.2 M) were prepared and appropriate volumes mixed to give the required pH as shown in Table 8.

Table 8 Values of pH Corresponding to Mixed Volumes of Disodium Hydrogen Phosphate and Sodium Dihydrogen Phosphate as Shown

pH	Na_2HPO_4 (0.2 M) (ml)	NaH_2PO_4 (0.2 M) (ml)	Dilute to 200 ml with distilled water
6.0	12.3	87.7	"
7.0	61.0	39.0	"
8.0	94.7	5.3	"

2.13. Estimation of Bubble Diameter by Photography

A Praktica LLC camera, mounted on a tripod, was fitted with a 52 mm auto-extension tube and 1:1.8/50 mm Pancolar Lens and focussed on the bubbles rising within the column. The metric scale of a plastic ruler, held in a clamp stand, was set up alongside the column. A piece of black card was placed behind the column and a white card perpendicular to this on the opposite side of the column to the scale. Illumination was provided by a Philips Number 2 Photoflood bulb in a Paterson General Purpose Reflector, directed onto the column from a position behind the clamp stand and scale. The film used was Ilford HP_4 rated at 400 ASA which at a selected speed of $^1/_{1000}$ second required an aperture of f5.6 to f8 for correct exposure.

The negatives were slide-mounted and displayed on a screen 3 metres from the projector lens, allowing accurate comparison of the measured bubble diameters with the calibration scale.

2.14. Surface Tension Measurements

These were undertaken using a Du Nüöy Tensiometer. The solutions under examination were contained in glass dishes of 80 mm diameter previously cleaned by soaking in chromic acid and thoroughly rinsed in distilled water. These were filled to a depth of 15 mm and the solutions allowed to equilibrate for 1 hour. A 10 mm platinum wire loop was cleaned by immersion in chromic acid, rinsing in distilled water and finally flaming to red heat in a bunsen flame for 30 seconds. This was attached to the torsion bar of the tensiometer and the instrument calibrated against distilled water. One of the dishes containing test solution was placed on the instrument table and the wire loop placed on the liquid surface with the minimum of disturbance. The force required to just remove the loop from the surface was measured and expressed as the surface tension. The mean of six such readings was recorded as the surface tension of that particular solution.

3. RESULTS AND DISCUSSION

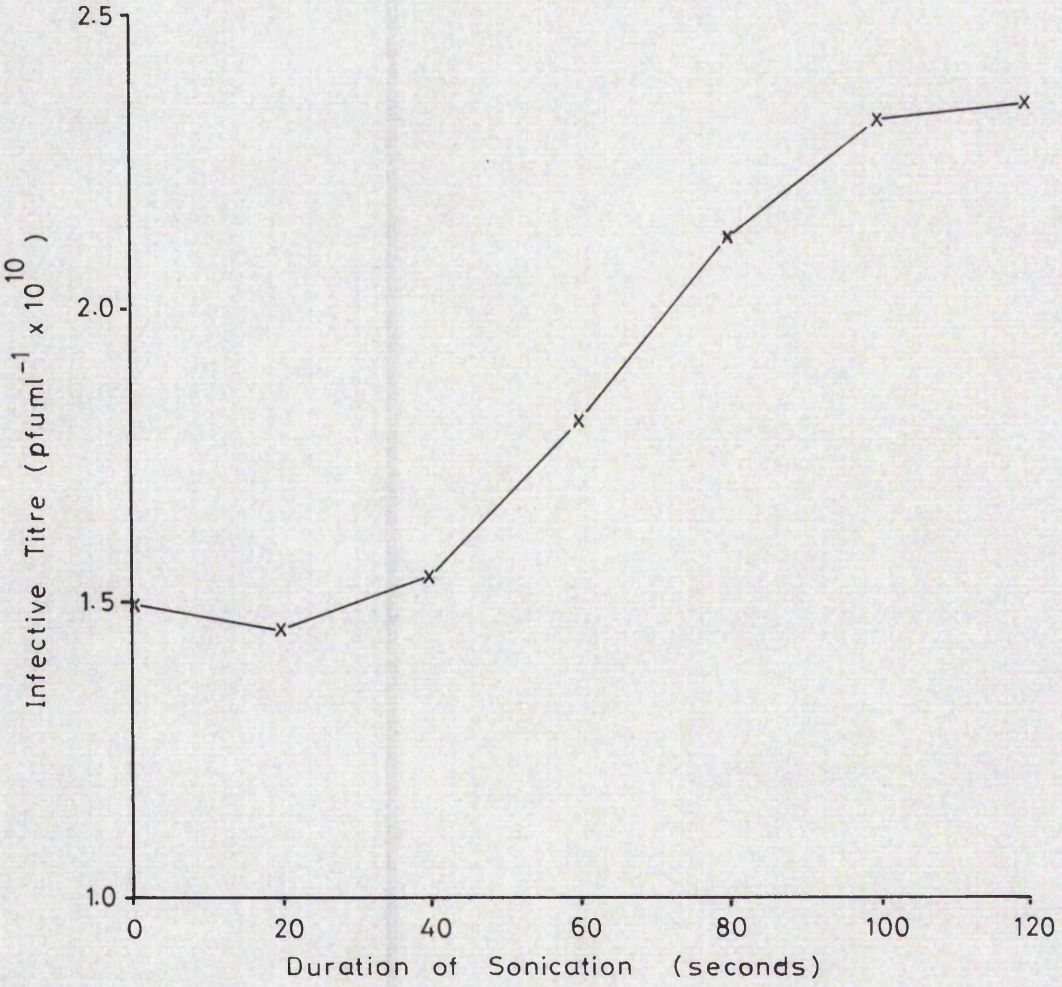
3.1. Bacteriophage Deaggregation Experiments

The replication of bacteriophage within the host bacterium and their subsequent release by cellular lysis inevitably results in a proportion of the phage particles being aggregated by attachment to host cell debris or to each other. Since aggregates are indistinguishable from single particles by viable cell counting techniques, it is possible for significant inactivation of particles within aggregates to occur with no detectable loss of infective titre from the phage suspension. Similarly, dispersion of such aggregates can cause significant errors in the assessment of the infective titre of viral preparations, as has been demonstrated with certain plant viruses (111, 122). Before undertaking inactivation studies it was therefore essential that all viable phage particles were singly dispersed throughout the preparation.

Since virus aggregates were comparable with protein complexes, it was reasoned that procedures commonly used for cellular disruption would prove effective in causing their deaggregation. Thus ultrasonic vibrations, widely used for the disruption of cellular membranes and the lysis of cells, was recently employed by Sime and Bedson for the dispersion of vaccinia virus (124). Accordingly a method based on their techniques and described in section 2.8.2. was used to sonicate an undiluted, crude phage lysate for a total of 120 seconds. The ultrasonics were applied in periods of 20 seconds duration to avoid excessive heating of the suspension and the results of initial, intermediate and final assays are shown in figure 7.

Analysis showed a significant increase in titre only occurred after a cumulative sonication period of approximately 60 seconds.

Figure 7. Effect of Sonication on the Mean Titre of Undiluted MS-2 Phage Lysate.

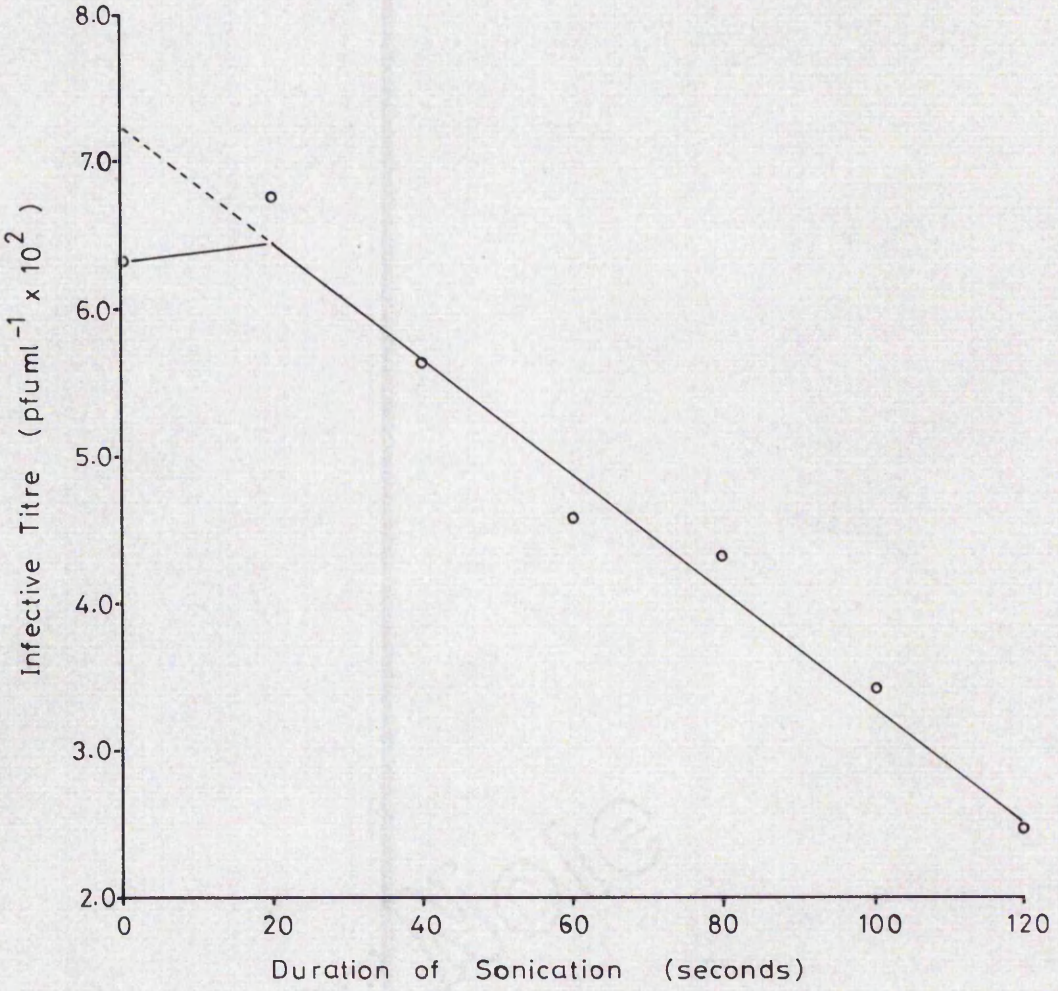


A further increase was noted after each of the three successive sonication periods, the total increase in titre amounting to 56.7%.

The crude phage lysate used in this experiment contained a relatively high concentration of extraneous proteins and salts which could possibly have affected the degree of deaggregation achieved by either enhancing or suppressing the disruptive effect of the ultrasonics. This hypothesis was tested by repeating the experiment with a phage suspension which had been diluted 10^{-8} in distilled water prior to sonication (see figure 8). In this case there was a reduction in titre for each successive treatment after the first sonication period, thus demonstrating phage inactivation resulting from sonication. The increase in titre after the initial sonication period suggested that aggregate disruption, as well as inactivation, had occurred during this period. Regression analysis of the data omitting the initial titre, gave a straight line relationship having a correlation co-efficient of -0.98. Extrapolation of this line to the y-axis (dotted line on the graph) yielded an intercept value for the initial titre of 7.22×10^{2} pfu ml^{-1}, which was 14% higher than the measured initial titre.

From the results of these two experiments it was proposed that the extraneous protein in the undiluted lysate had protected the phage from inactivation but had not prevented disruption of the aggregates, as shown by the increased titre in figure 7. Dilution of the lysate in distilled water had reduced the concentration of protein allowing phage inactivation and at least some deaggregation, as shown in figure 8. If this was correct, it followed that sonication of bacteriophage in a protein solution after they had been previously sonicated as a suspension in distilled water, would have no effect

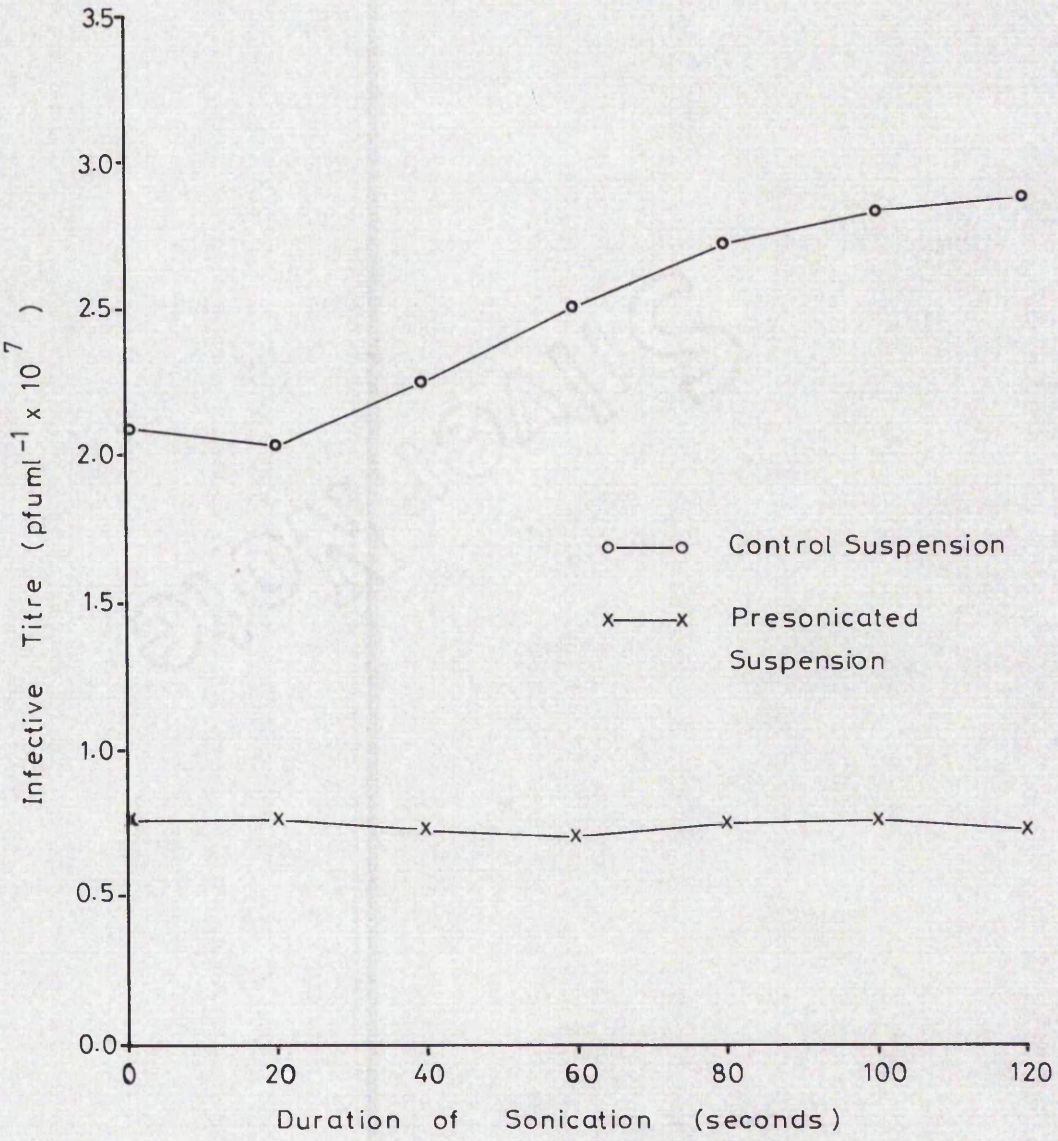
Figure 8. Effect of Sonication on the Mean Titre of Diluted MS-2 Phage Lysate.



upon the titre. Accordingly, two aliquots of a phage lysate were diluted 10^{-2} in distilled water one of which was sonicated for a total of 120 seconds as before. Both were then diluted 10^{-2} in 1% w/v peptone and sonicated for six, 20 second periods. The results are shown in figure 9. Once again the untreated suspension showed a significant increase in titre due to deaggregation. As predicted, however, the pre-sonicated sample showed no significant variation in titre during sonication in peptone and thus deaggregation must have occurred during the sonication in distilled water. Furthermore, the lack of inactivation during the second sonication demonstrated that peptone did protect deaggregated particles from the lethal effects of sonication. The mechanism by which such protection occurred may have been similar to that by which peptides prevented inactivation of T4 during freezing and thawing (128), although the precise mode of action in both cases is unknown.

Sonication of the control sample in the above experiment produced an increase in titre of 40% compared with 56.7% obtained earlier by sonication of a crude lysate (figure 7) and 14% obtained by extrapolation of the data from sonication in distilled water (figure 8). This variation was unexpected since the method of sonication and of phage lysate preparation were the same in each case. In fact, the greatest increase in titre was found on sonication of the undiluted phage lysate and the lowest increase from the sample pre-diluted 10^{-8} in distilled water. This suggested that dilution in distilled water may itself have caused some deaggregation. This was investigated and the results together with a possible mechanism will be discussed later.

Figure 9. Effect of Sonicating a Pretreated Suspension of MS-2 in 1% Peptone.

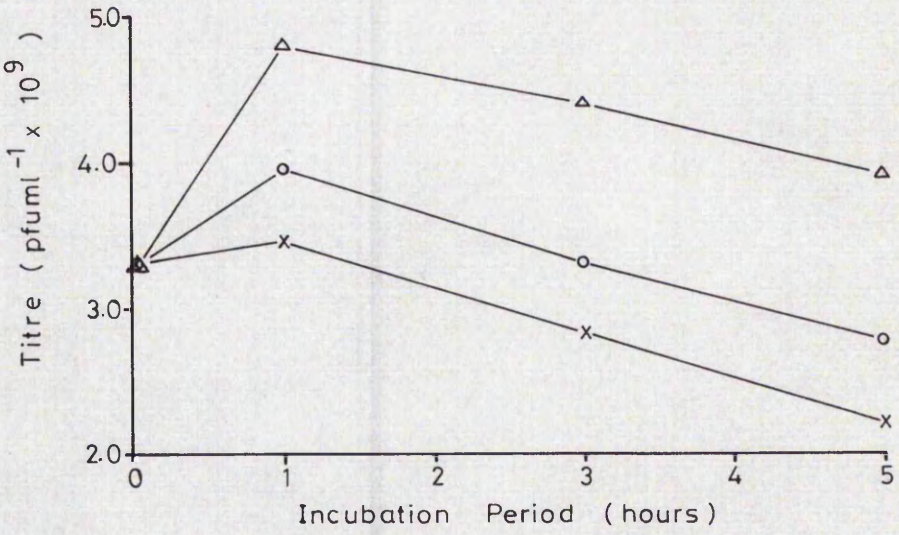


As an alternative to sonication, treatment with detergents has been used for the disruption of cellular components. Furthermore, Brakke (18) has shown that aggregates of Barley Stripe Mosaic virus could be dispersed by various detergents at concentrations of between 0.01 and 0.5% w/v. At the higher concentration of 1% w/v, however, sodium dodecyl sulphate has also been used in conjunction with 1% β mercaptoethanol to disintegrate various bacteriophages in order to characterise their constituent coat proteins (130). Clearly, therefore, the type of detergent used and its concentration were critical in order to achieve deaggregation without inactivation or disruption of the phage particles. Figure 10 shows the results of experiments in which MS-2 was suspended in various concentrations of non-ionic (Nonidet P40) and anionic (Teepol 610) surfactants and sampled during incubation at $4^{o}C$. The data show that all concentrations of both detergents produced significant phage inactivation when incubated beyond one hour. Whilst Teepol 610 had little effect on the titre during the first hour of incubation, Nonidet P40 produced an increase in titre over this period. The magnitude of this increase was proportional to the detergent concentration and reached 45.5% in the 0.50% detergent solution.

It is interesting to note that the rate of inactivation during one to five hours incubation was very similar for each concentration of the particular detergent. The mean inactivation rates for Nonidet P40 and for Teepol 610 were 2.8×10^{8} pfu ml^{-1} hr^{-1} and 1.9×10^{8} pfu ml^{-1} hr^{-1} respectively. The lack of dependence of inactivation rate on concentration suggested that a 'saturation level' had been exceeded even at the lowest concentration. This saturation level may have been related to the maximum amount of detergent which the phage

Figure 10. Deaggregation of MS-2 in Solutions of Detergent at 4°C.

1. Nonidet P 40

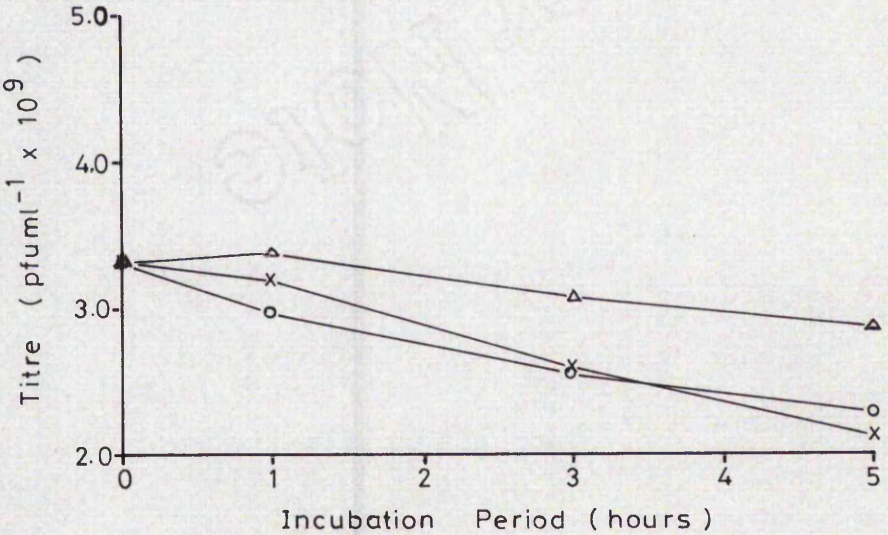


△——△ 0.5% w/v active matter

o——o 0.1% w/v active matter

x——x 0.05% w/v active matter

2. Teepol 610



capsids were capable of adsorbing; the difference in inactivation rates between the two detergents being due to their differing toxicities.

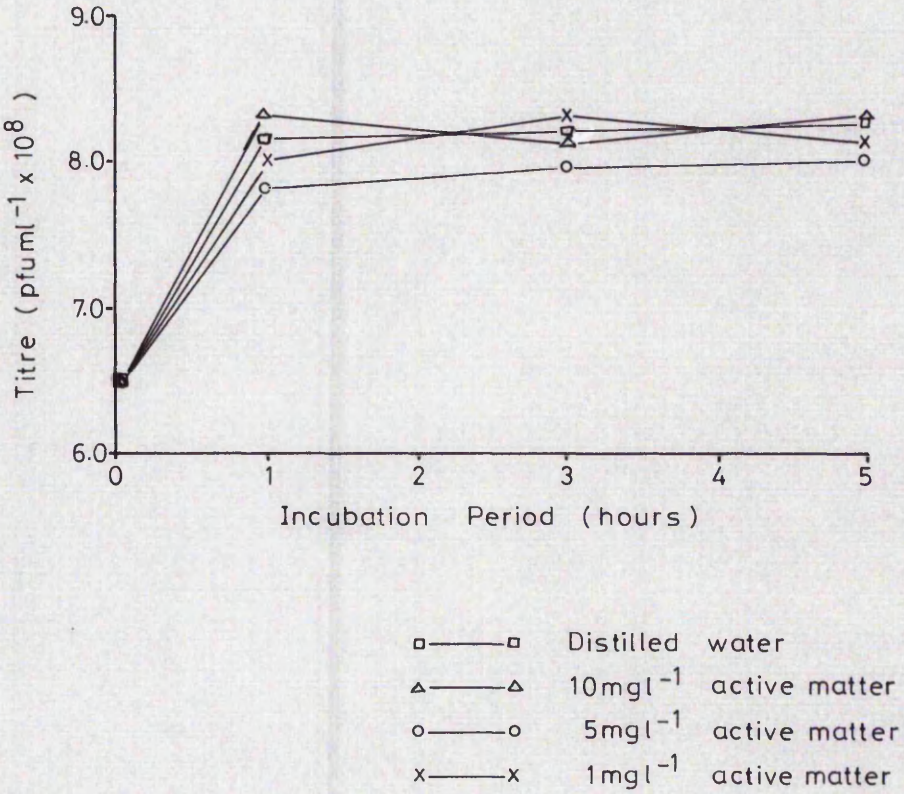
During further experiments using considerably reduced detergent concentrations, the maximum attainable increase in titre was found to be of a similar magnitude to that produced by dilution in distilled water (see figure 11). At the concentrations used ($\leqslant 10\ mgl^{-1}$) the increase in titre was independent of concentration, and after 5 hours incubation reached 26.7% in Nonidet P40, and 32.8% in Teepol 610. These compared with a mean increase in titre of 31.6% in the distilled water controls.

It was suggested earlier that the greater increase in titre during sonication of a crude lysate compared with that of a distilled water suspension may have resulted from 'dilution deaggregation'. Sonication of 10^{-8} and 10^{-2} dilutions of lysate caused 14% and 40% titre increases respectively whereas sonication of undiluted lysate caused a 56.7% increase. Thus 10^{-8} and 10^{-2} dilutions apparently caused 42.7% and 16.7% titre increases respectively. These compare favourably with the 31.6% increase following 10^{-2} dilution for 5 hours obtained in the above experiment. Clearly the most complete deaggregation was obtained with the highest dilution factor. However an extended incubation period at the lower dilutions may have increased the degree of deaggregation achieved as will be shown later.

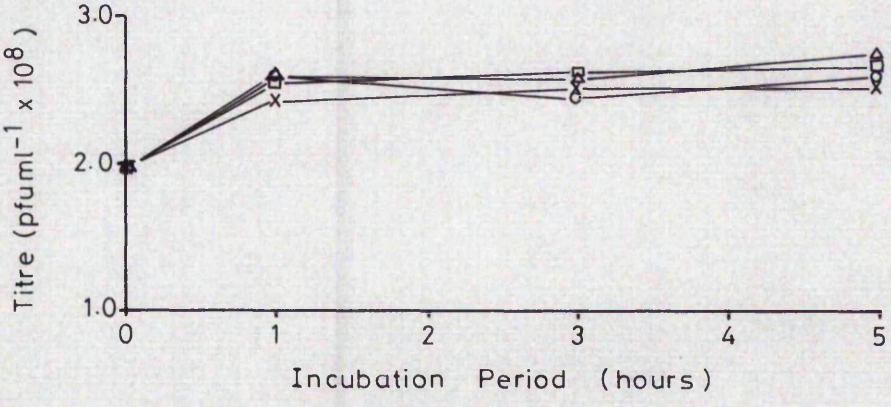
The stability of virus aggregates in low ionic strength media has been investigated for potato X virus (111) and tomato spotted-wilt virus (16). Reichmann found that the irreversible end-to-end aggregation of potato X virus could be minimized by suspension in 0.05 M sodium citrate (111) whilst Black *et al* found that reversible

Figure 11. Deaggregation of MS-2 in Detergent Solutions of Low Concentration.

1. Nonidet P40



2. Teepol 610



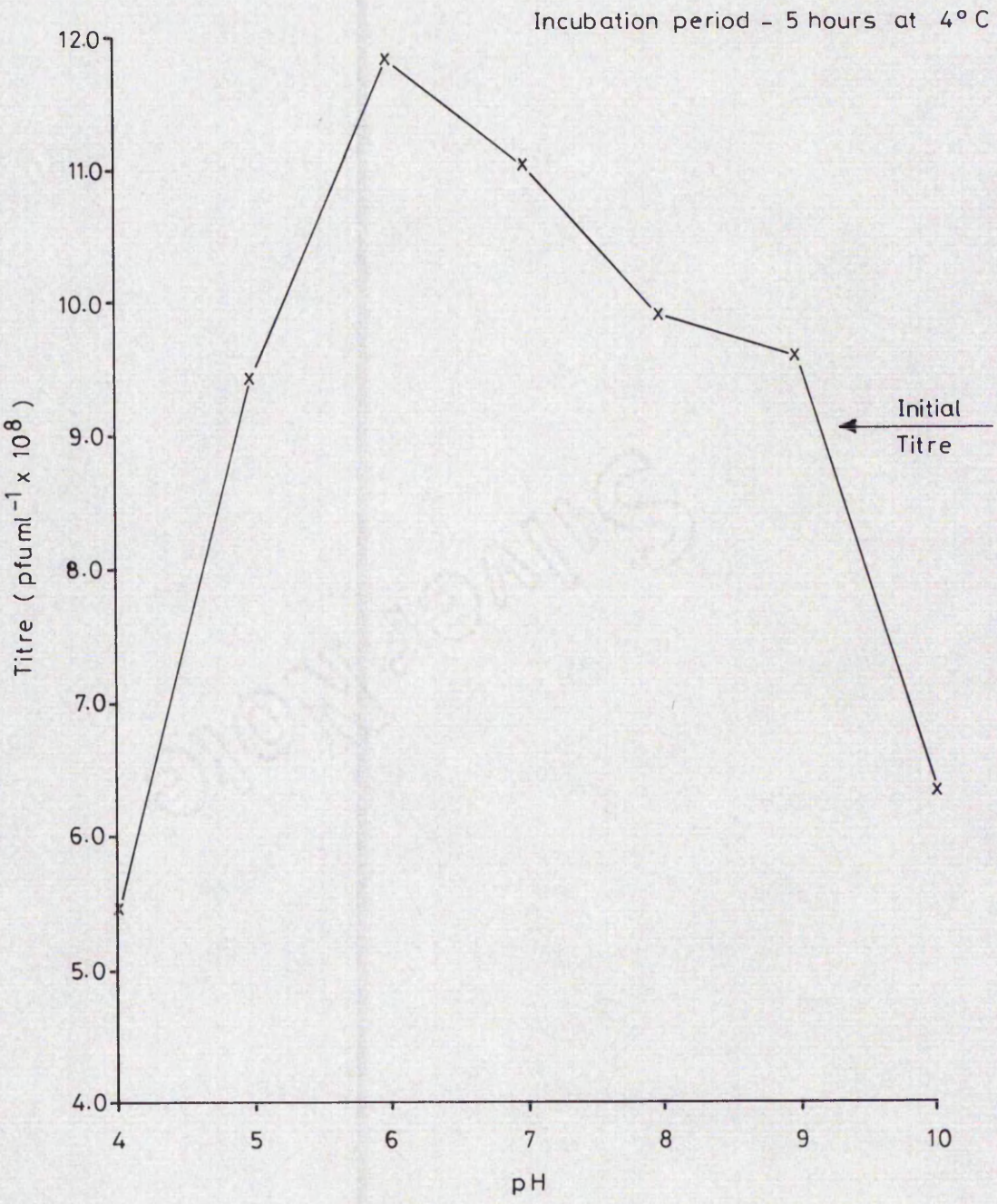
aggregation of tomato spotted-wilt virus was reduced in water containing an ionic concentration of less than 0.01 M (16). Reichmann, however, found that suspension of viruses in distilled water resulted in almost complete, reversible aggregation. This apparently conflicting evidence was obtained with large, relatively complex plant viruses and none of the authors attempted to explain their results in terms of reaction mechanisms.

Brakke (20) and Loring and his co-workers (95) have shown the importance of calcium and magnesium ions in the stabilization of brome mosaic virus and tobacco mosaic virus respectively. Such divalent cations may have been responsible for aggregate stability by forming 'bridges' between adjacent, negatively-charged particles. Suspension of such aggregates in diluents of low ionic strength may have caused these ions to diffuse from the aggregates resulting in their dispersion by mutual repulsion of the particles.

Since the degree of ionization of protein molecules is dependent upon the pH of the solution, it was considered feasible that deaggregation of virus particles would be influenced by pH. Accordingly the effect of 10^{-2} dilution of a crude phage lysate in pH-adjusted distilled water was examined using the method described in section 2.12.1. Following incubation for 5 hours at $4^{o}C$, the results shown in figure 12 were obtained. Analysis of the data revealed that a significant titre increase only occurred in the samples incubated at pH values of 6.0 - 8.0. No significant change in titre was recorded at pH values of 5.0 and 9.0, whilst a significant reduction was noted at pH values of 4.0 and 10.0.

These results suggested that deaggregation only occurred at pH values of between 6.0 and 8.0. However, it was possible that

Figure 12. Deaggregation of MS-2 in Distilled Water at Various pH Values.



deaggregation occurred throughout the range examined but simultaneous pH inactivation progressively obscured this effect at the pH extremes. To verify this alternative, stock phage was diluted 10^{-2} in distilled water at pH values of 4.0 and 10.0 and incubated for 1 hour at 25°C. After assay, each suspension was adjusted to a pH of 6.0, incubated for 5 hours at 4°C, and re-assayed. If deaggregation had occurred at the pH extremes, no further increase in titre would be found during the second incubation period under conditions known to cause deaggregation.

Table 9 Estimation of Aggregation in MS-2 Suspensions Incubated for 1 Hour at pH Values of 4.0 and 10.0

Sample	Mean Titre (pfu ml^{-1})	Standard Error
Initial (0 Hours)	3.47×10^7	0.37×10^7
pH 10 (1 Hour)	5.25×10^6	0.65×10^6
After incubation @ pH 6	5.10×10^6	0.59×10^6
pH 4 (1 Hour)	1.90×10^7	0.13×10^7
After incubation @ pH 6	1.89×10^7	0.21×10^7

The data (see Table 9) revealed no significant increase in titre after further incubation and it was therefore concluded that deaggregation occurred at all pH values in distilled water and was not a pH-dependent function as had earlier been suggested. Thus whilst varying the pH may have governed the dissociation of ionizable groups on the virus capsid, the forces involved in aggregation were apparently unaffected by the concentration of hydrogen ions present in the surrounding medium.

Inactivation in distilled water, however was pH-dependent and

plotting survival ratios (i.e. Residual titre (R)/Initial titre(I)) against pH for aggregated and deaggregated suspensions was therefore expected to yield curves of similar shape. Accordingly, an aliquot of crude phage lysate was deaggregated by sonication prior to incubation in pH-adjusted distilled water and the results compared with those previously obtained for an untreated lysate.

As figure 13 shows, the pH sensitivity profiles of the treated and untreated lysates were basically identical, the vertical displacement of the untreated lysate confirming that dilution deaggregation ocurred to the same extent at all the pH values tested. It is probable that deaggregation in distilled water resulted from diffusion of essential, binding ions from the aggregate and consequent expansion of the hydrated layers surrounding the particles, thus causing their mutual repulsion. Such phenomena have been demonstrated for mineral particles, as discussed in the Introduction.

In the sonication experiments, the maximum increase in titre obtained was 56.7% whereas in the dilution experiments this was 35.4% at pH 6.0 (see figure 12). This discrepancy was due either to variations in the extent of aggregation in the stock phage lysates used, or to the relative efficiencies of the two processes in causing deaggregation. If the latter was correct, then sonication of a 'dilution deaggregated' phage suspension would be expected to cause further deaggregation. Thus an aliquot of crude phage lysate was diluted 10^{-2} in distilled water at $4^{o}C$ and stored for 5 hours. The results of sonicating this suspension are shown in figure 14. As can be seen, the extrapolated regression line bisected the y-axis within the range of the experimentally determined initial titre. Thus no further deaggregation was achieved by sonication, showing that dilution

Figure 13. Comparison Between the Survival of Aggregated and Deaggregated MS-2 at Various pH Values.

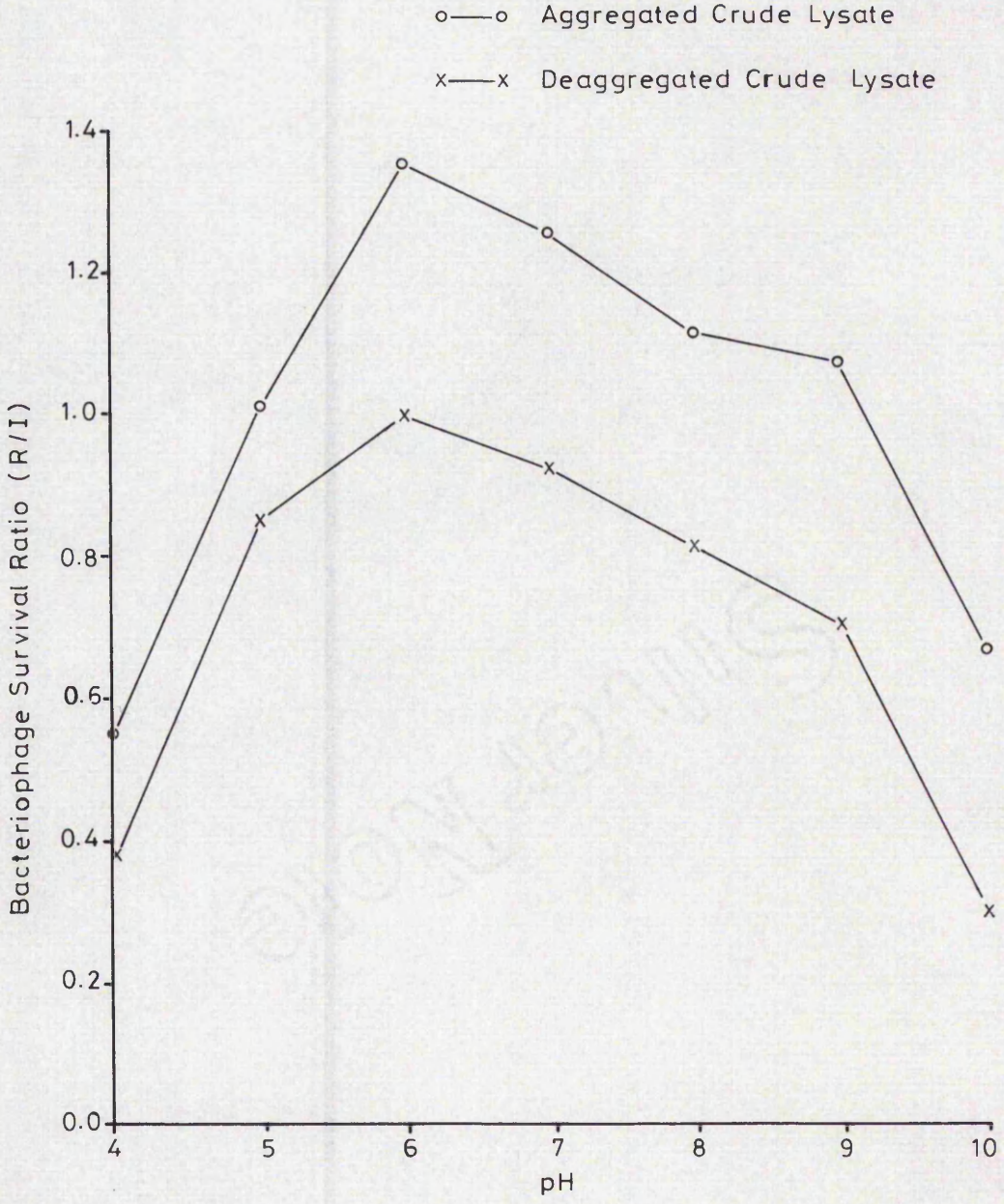
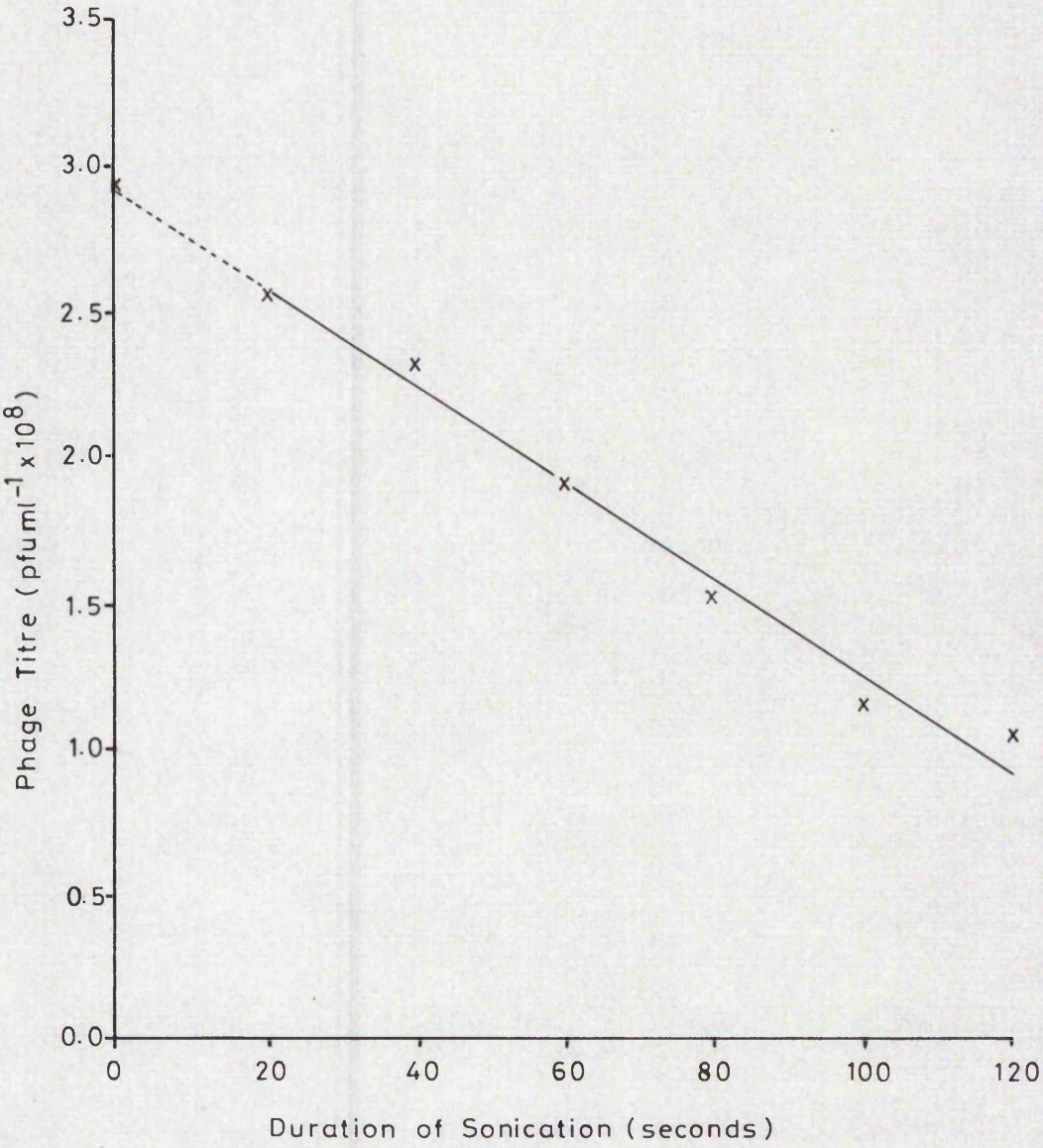


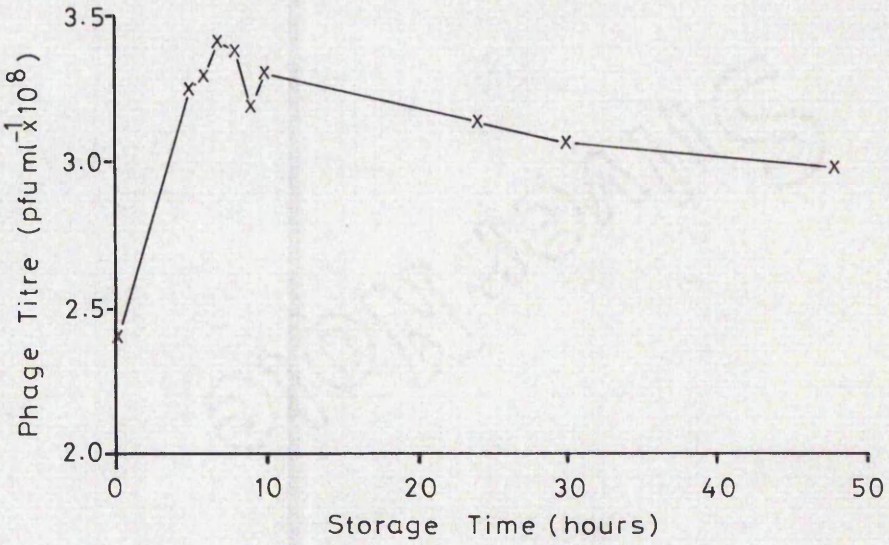
Figure 14. Effect of Sonication on a Phage Suspension Stored in Distilled Water.



and storage of phage in distilled water was sufficient to cause complete deaggregation. This result was supported by D.B. Jones (80) who found that ultra violet irradiation of crude phage lysates yielded inactivation curves with a characteristic initial lag phase as shown in figure 8. This was thought to result from undetected inactivation of particles in aggregates and since this lag phase was not found when using suspensions diluted in distilled water, complete aggregate dispersion had evidently taken place.

Since deaggregation by dilution in distilled water required no elaborate procedures, nor any added detergents, it was adopted as a routine pre-treatment of the phage prior to use. Since experimentation had shown that dilution to 10^{-2} would cause complete deaggregation after approximately 8-10 hours and that no significant loss of titre occurred after prolonged storage at $4^{o}C$ (see figure 15), overnight storage of a 10^{-2} dilution in a refrigerator was routinely adopted for phage deaggregation.

Figure 15. Effect of Prolonged Storage on a Phage Suspension in Distilled Water at 4°C.

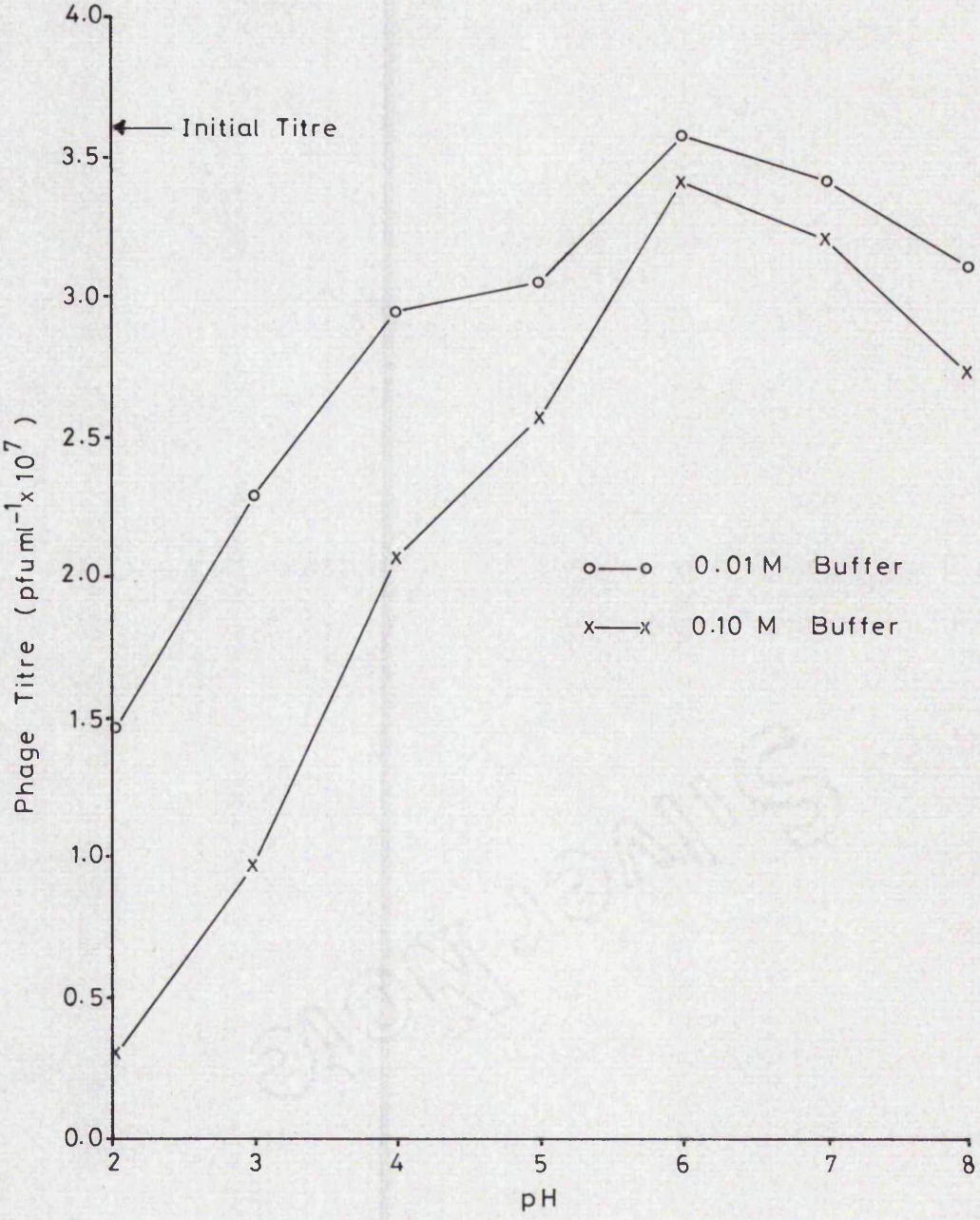


3.2. Inactivation of MS-2 in Buffer Solutions

Whilst MS-2 was relatively resistant to pH inactivation within the range pH 4.0 - 10.0 in distilled water, it was recognized that the presence of ions could affect bacteriophage sensitivity by adsorption to the capsid. The stability of several plant viruses during extraction and purification in buffers of varying concentration and pH has been investigated by a number of researchers (16, 19, 20, 95, 122). In general, the results showed maximum virus stability at pH 6.0 - 7.0 and least aggregation at buffer concentrations of 0.005 M or less. However pH and ionic strength were interrelated thus Brome Mosaic Virus was more stable at pH 7.0 in a 0.01 M phosphate buffer than in 0.1 M, whilst at pH 6.0 the virus was more stable at the higher concentration (20). Ionic interactions with plant viruses were clearly complex phenomena and none of the authors cited attempted to explain their findings.

To investigate the effects of pH and molarity on the structurally simpler and more resistant MS-2, 0.1 M and 0.01 M phosphate-citrate buffers of various pH values were prepared (see section 2.12.1) and aliquots of deaggregated phage added. Following storage for 5 hours at $4^{o}C$, the results (see figure 16) showed that over the pH range 5.0 to 7.0 inclusive there was no significant difference between the phage titres at the two buffer concentrations. Furthermore as with storage in distilled water, least phage inactivation occurred at pH 6.0 in both concentrations. At the lower pHs of 2.0 and 4.0, however, significantly greater inactivation occurred in the 0.1 M buffer. Evidently the susceptibility of MS-2 to high concentrations of H^{+} ions increased with the molarity of the buffer.

Figure 16. Inactivation of MS-2 in Phosphate-Citrate Buffer at Various pH Values.



Mineral flotation theory predicts that increasing the ionic strength of the medium results in a condensation and stabilization of the hydrated layers surrounding a mineral surface. Assuming such a phenomenon occurred around the phage capsid, it would be reasonable to expect a change in the sensitivity of the virus towards pH inactivation, whilst not explaining the mechanism by which such inactivation proceeds.

An alteration in the stability and thickness of surface hydrated layers also affects the rate and tenacity of attachment of mineral particles to air bubbles (84), as discussed earlier. Thus it was postulated that the rate of virus inactivation in aerated suspensions would be dependent upon ionic concentration and pH. Studies by others, (137), had shown that MS-2 was rapidly inactivated in aerated suspensions of 1.0 M NaCl, although no loss of phage was detected in 0.1 M NaCl. Three alternative inactivation mechanisms were proposed which depended upon enhanced adsorption to the interfaces and increased forces on the adsorbed capsids as the ionic strength was increased.

Throughout these studies, however, no consideration was given to the possible effect of pH. The stability of the hydrated layers surrounding bubbles and mineral particles is influenced by H^+ and OH^- ions. In unaerated suspensions, relatively high concentrations of these ions were required before phage inactivation became significant, i.e. below pH 5.0 (see figure 16). During aeration, however, it was considered that the effect of H^+ ions on particle-bubble attachment might render surface inactivation of MS-2 more sensitive to pH. To verify this proposal, various experiments were carried out using the batch flotation columns described in section 2.12.2.

Preliminary tests with pH-adjusted distilled water were inconclusive due to the difficulties encountered in maintaining a constant pH. However experimentation with phosphate-citrate buffer had shown that at a concentration of 1.0 m M, the pH inactivation of phage did not differ significantly from that found in distilled water. At this concentration, the buffer was also capable of maintaining a stable pH during aeration, thus enabling inactivation in aerated and unaerated suspensions to be compared at various pH values. The results (see figure 17) showed no significant difference between the pH inactivation of MS-2 in aerated and unaerated suspensions. Apparently therefore the influence of hydrogen ion concentration on particle-bubble attachment was insufficient to enhance surface inactivation. However, under the same experimental conditions, increasing the buffer concentration at a constant pH of 6.0, markedly increased the degree of inactivation caused by aeration (see figure 18). This was in agreement with the findings of Trouwborst et al (137) described earlier, for the inactivation of MS-2 in aerated sodium chloride solutions.

These experiments however did not preclude the possibility of inactivation due to the violent agitation caused by an ascending bubble stream. Such a mechanically-induced inactivation mechanism, if correct, would have to be strongly dependent upon the ionic strength of the suspending medium since no such inactivation was demonstrated in a 1 m M buffer (see figure 17). Nevertheless, mechanical agitation has been implicated in virus inactivation by a number of researchers (1, 25, 95, 100) although only Adams (1) recognized the possibility of inactivation by adsorption to the interfaces generated during agitation. Using similar methods to those described in

Figure 17. Comparison Between Inactivation of MS-2 in Aerated and Unaerated Suspensions.

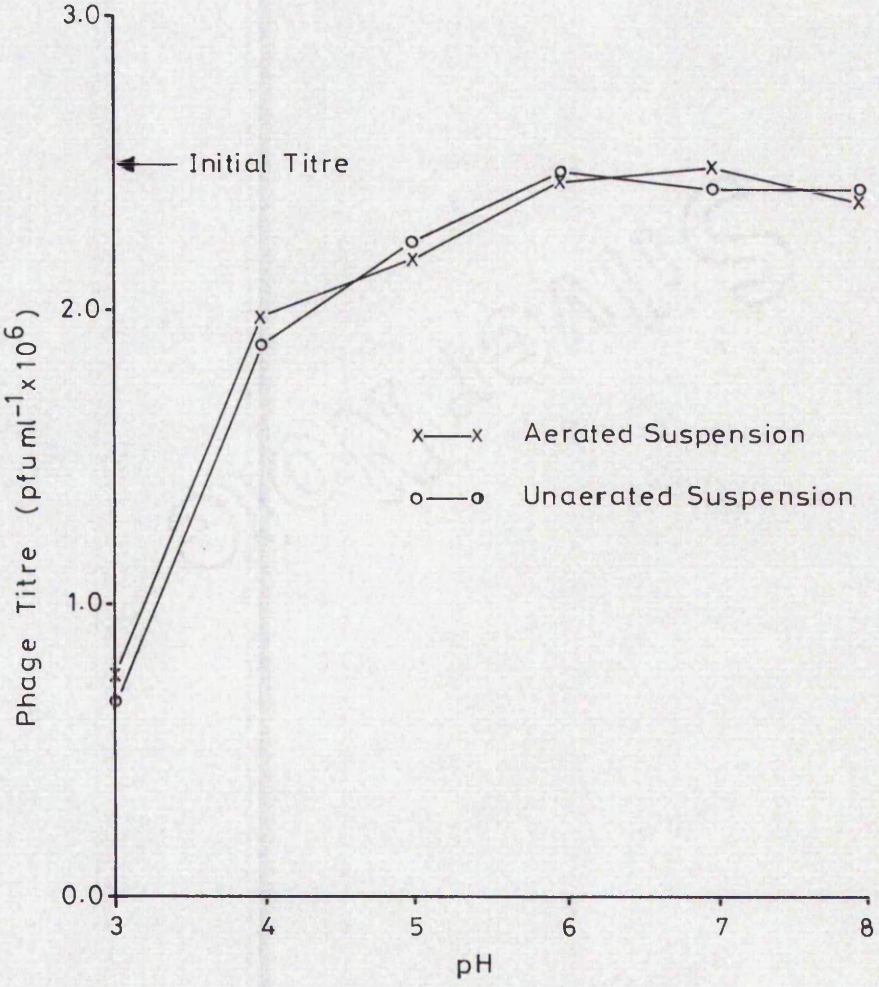
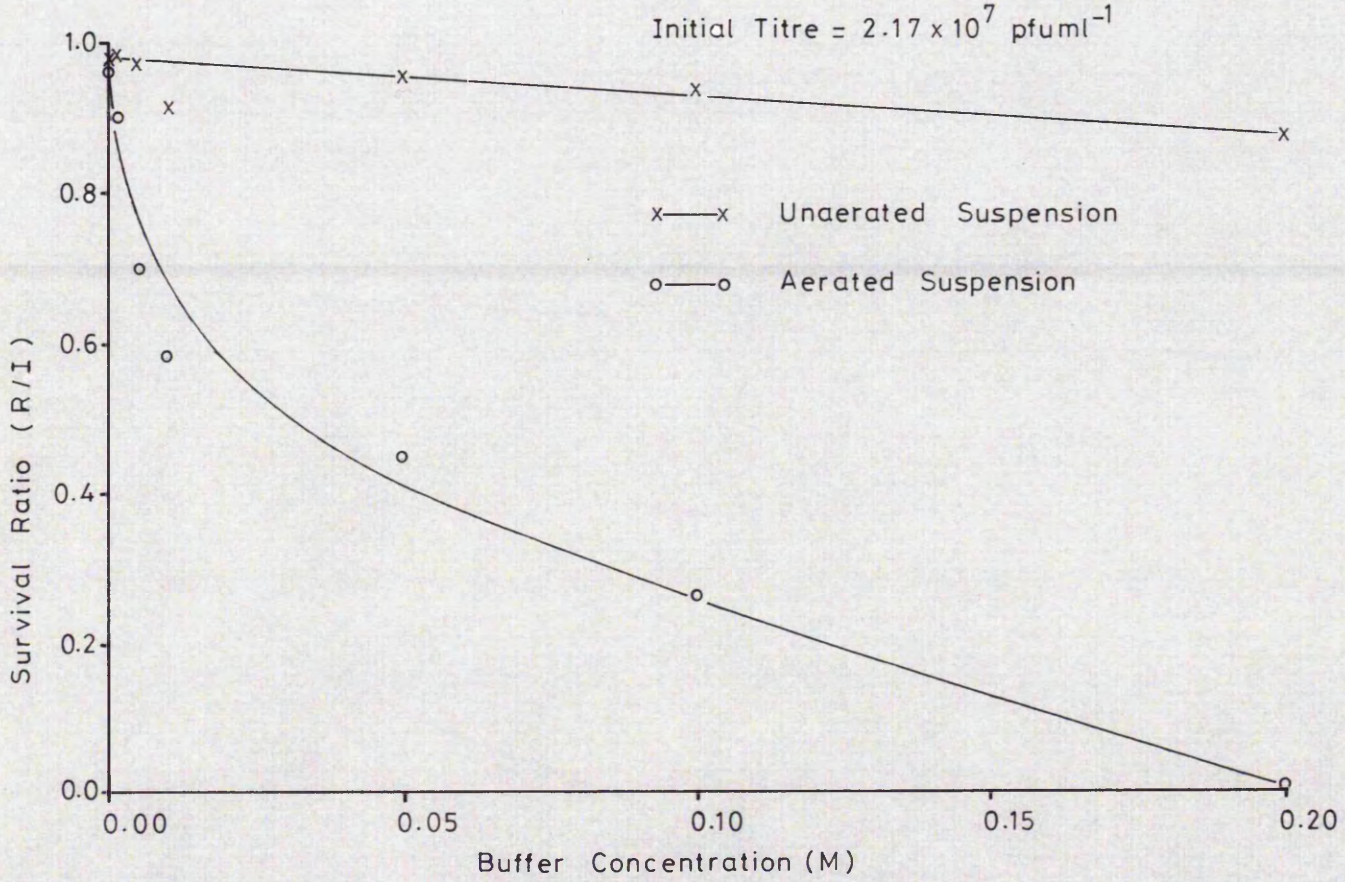


Figure 18. Variation in Inactivation with Buffer Concentration.



section 2.11., he was able to show that mechanical agitation alone did not cause inactivation of a number of T-bacteriophages of Escherichia coli. In order to verify his findings for the more stable picornaviruses, aliquots of an MS-2 suspension in various volumes of 0.2 M phosphate-citrate buffer (pH 6.0) in Universal bottles were agitated as described in section 2.11. The results (table 10) showed that agitation of completely filled Universal bottles did not significantly increase the degree of inactivation compared with the unagitated control. However, agitation of bottles containing only 10 ml of suspension, i.e. approximately half-filled, significantly reduced the infective titre, suggesting that the large surface area in conjunction with the high ionic concentration, was responsible for the inactivation. However, it was noticed that greater agitation of the suspension occurred in the half-filled bottles, and thus mechanical agitation could still have been responsible for the apparent surface inactivation. An alternative method of agitation was therefore devised in which the bottles were fixed radially to a disc 250 mm in diameter, rotating at approximately 10 r.p.m. and tilted 10 degrees to the vertical. 'Full' bottles had no airspaces but contained glass beads whilst the bottles containing 20 ml had an airspace but no glass beads. The passage of the beads and the air bubbles up and down the bottles during rotation was considered to cause approximately the same degree of agitation. The incubation time was increased to 2 hours, however, to compensate for the less vigorous agitation.

Once again, agitation of the bottles containing an air space significantly increased inactivation compared with the unagitated control, whilst agitation of the full bottles did not affect the phage titre (Table 10).

Table 10 The Effect of Agitation on the Infective Titre of Suspensions of MS-2 in 0.2 M Phosphate-Citrate Buffer pH 6.0

Sample	Mean Titre pfu ml^{-1}	Standard Error
Agitation by Orbital Shaker		
Initial	1.61×10^5	0.07×10^5
Unagitated Control	1.49×10^5	0.04×10^5
Agitated "Full" Sample	1.51×10^5	0.08×10^5
Agitated "10 ml" Sample	9.72×10^4	0.38×10^4
Agitation by Rotating Disc		
Initial	3.48×10^5	0.09×10^5
Unagitated Control	3.10×10^5	0.17×10^5
Agitated "Full" Sample	2.95×10^5	0.19×10^5
Agitated "20 ml" Sample	2.54×10^5	0.10×10^5

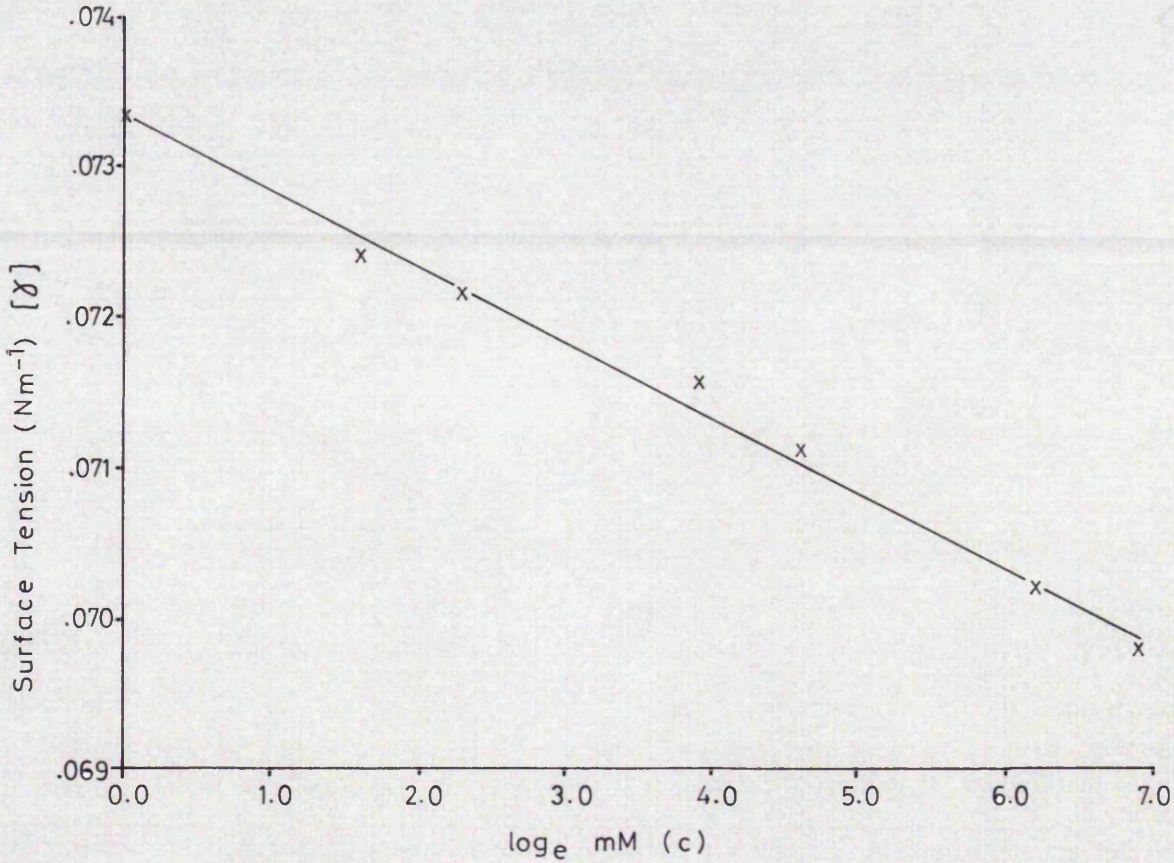
These 2 experiments therefore confirmed the findings of Adams that mechanical agitation did not cause inactivation unless associated with a gas-liquid interface. Thus the loss of infective titre during aeration shown in figure 18 was caused by a surface inactivation mechanism, related to the buffer concentration.

Conceivably the buffer may have affected the properties of the gas-liquid interface and accordingly the surface tension of buffer solutions at various concentrations was measured using a Du Nüoy Tensiometer as described in section 2.14.

The results (figure 19) showed a linear relationship between the logarithm of the buffer concentration (c) and surface tension (γ) of the form:

$$\gamma = 0.0733 - 0.000494 \log_e c$$

Figure 19. Relationship Between Concentration and Surface Tension for a Phosphate - Citrate Buffer of pH 6·0.



Addition of inorganic salts to a solution usually results in an increase in the surface tension as found with the sodium chloride solutions used by Trouwborst et al (137). Thus in this case it was concluded that the observed reduction was due to the citric acid which possessed surface active properties due to its hydrocarbon chain structure.

A reduction in surface tension under constant aeration conditions was expected to cause a reduction in the size of bubbles generated since a forming bubble detaches from the sparger pore when its buoyancy just overcomes its adhesion to the sparger. These two forces are related to bubble radius and surface tension respectively, and the following relationship has been shown to apply for a single capillary opening (84):

$$r = k^4 \, r_c^2 \gamma \qquad (1)$$

where r = bubble radius

r_c = internal radius of capillary

γ = surface tension

k = constant related to bubble shape (normally ≈6).

During the aeration experiments described earlier (figure 18), it was noticed that as predicted, the bubbles were smaller and more uniform in shape at the higher buffer concentrations. Furthermore they appeared to be more closely packed which suggested that the total surface area of the bubbles per unit volume of solution had increased. To confirm this, the mean diameters of bubbles generated in buffers of various concentration were measured using the photographic technique described in section 2.13. Examples of the bubbles obtained in distilled water and 0.2 M buffer are shown in plates 1 and 2

respectively. By measuring the difference in height (h) between the aerated and unaerated liquid levels in the columns, the volume of air (V) contained within the liquid at any instant could be determined from the equation:

$$V = \pi R^2 h$$

where R = internal radius of the columns.

The average volume of each bubble (ν) was determined from:

$$\nu = \frac{4}{3}\pi r^3$$

where r = mean radius of the bubbles.

Dividing the total volume of air (V) by the average bubble volume (ν) produced the number of bubbles contained in the bulk solution at any instant. The product of the number of bubbles by the surface area of each bubble (i.e. $4\pi r^2$) divided by the volume of bulk solution in the column, yielded the total surface area generated per ml of solution. Plotting this against the molarity of the phosphate-citrate buffer yielded the relationship shown in figure 20.

Using equation (1) the expected values for the bubble radii were calculated by substituting a value of 5.0μ for sparger porosity (r_c), as supplied by the manufacturers, and surface tension values derived from figure 19, for γ. These calculated values for bubble radius differed significantly from the measured values as shown in Table 11.

Plate 1.

Photograph Showing the Bubble Pattern Produced During the Aeration of Distilled Water.

0
7 0
8 0
9 0
10 0
11 0
12 0
13 0

Plate 2.

Photograph Showing the Bubble Pattern Produced During the Aeration of 0·2M Phosphate - Citrate Buffer.

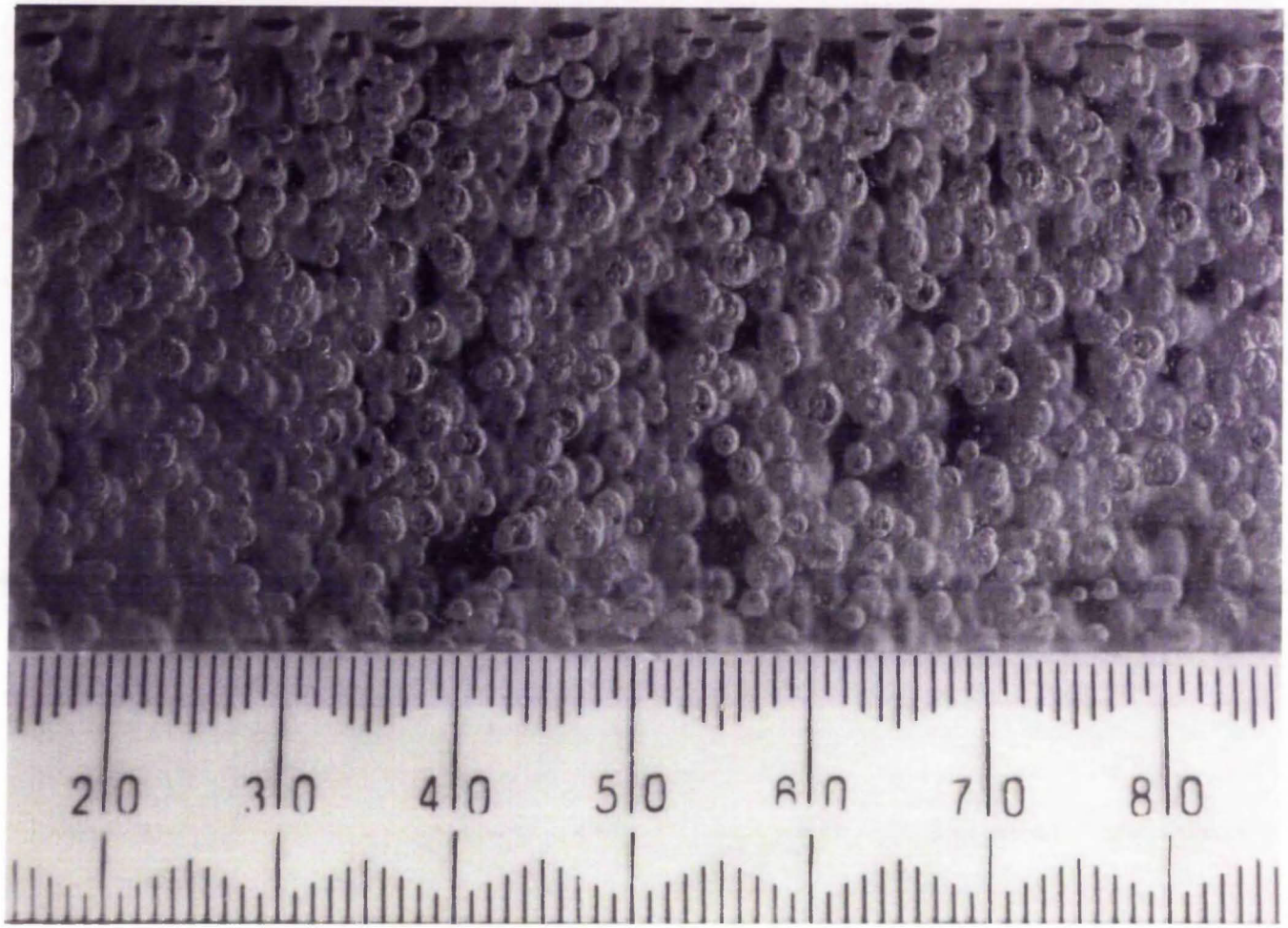

20
30
40
50
60
70
80

Figure 20. Relationship Between Buffer Concentration and Generated Surface Area.

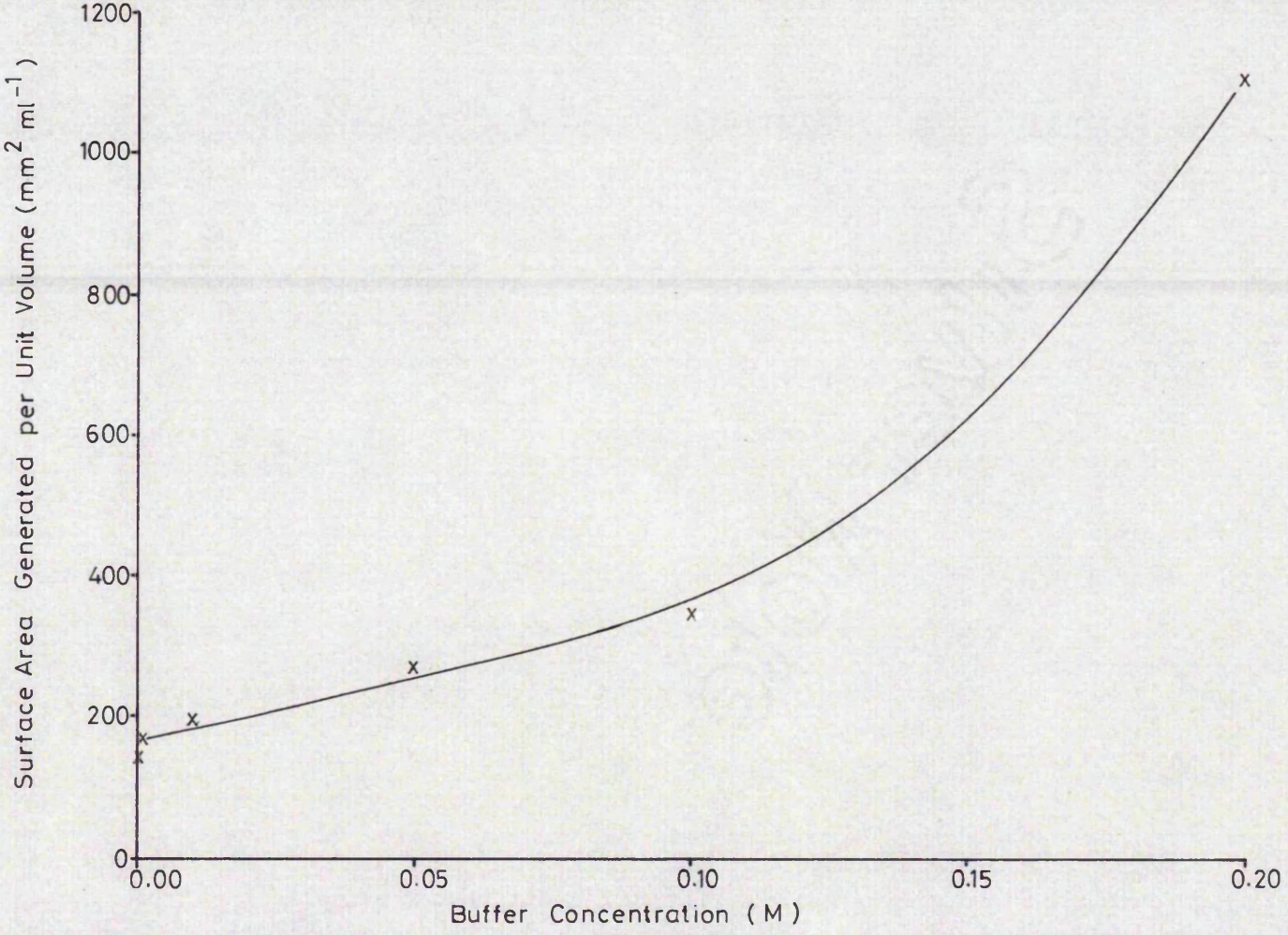


Table 11 Comparison Between Measured and Calculated Bubble Radii at Various Buffer Molarities

Buffer Molarity (mM)	Measured Bubble Radius (mm)	Calculated Bubble Radius (mm)
Distilled Water	3.37	1.75
1	3.19	1.75
10	3.00	1.74
50	1.97	1.73
100	1.21	1.73
200	0.75	1.72

Clearly bubble size was influenced to a greater extent by ionic concentration than by surface tension in aerated salt solutions. This phenomenon, referred to in the Introduction, was due to the stabilizing effect of the ions on the hydrated layers surrounding the bubbles. This prevented bubble coalescence; a factor which significantly increased bubble size at salt concentrations below 50 mM.

At higher molarities the measured radii were unexpectedly lower than the calculated values. This was thought to be due to the increased turbulence above the sparger, resulting from the greater bubble density, which caused premature shearing of forming bubbles. Equation (1) was derived for bubbles issuing singly from a capillary opening which therefore avoided interference from factors such as turbulence and coalescence.

Plotting phage inactivation during aeration (obtained from figure 18) against surface area generated per unit volume (obtained

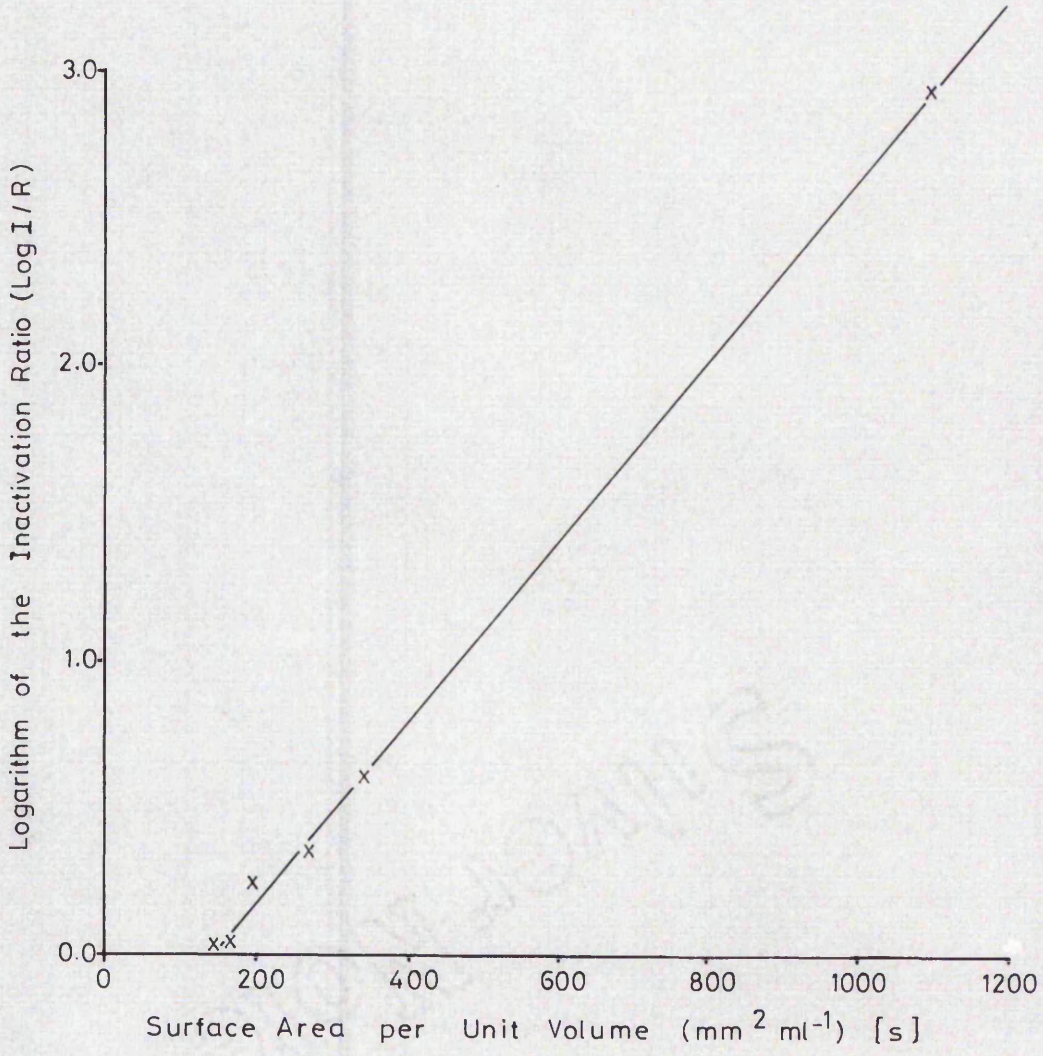
from figure 20) at various buffer concentrations, the relationship shown in figure 21 was derived. This was of the form:

$$\log_{10} {}^{I}/_{R} = -0.446 + 0.0035$$

Thus phage inactivation in aerated buffers was directly proportional to the interfacial surface area generated per unit volume of suspension. However no significant inactivation occurred at zero buffer concentration (i.e. distilled water) despite a generated surface area/unit volume of 140 mm^2ml^{-1}. This suggested that ions were actively involved in surface inactivation and that their role was not confined to increasing interfacial density. This hypothesis could be tested by either increasing bubble density in distilled water or by increasing buffer concentration whilst maintaining a constant surface area per unit volume. Unfortunately the former method was impractical owing to the inability of the pump to supply sufficient air and the construction of the flotation columns and spargers which were unable to withstand the higher air pressures required.

The alternative experiment was therefore devised which allowed the phage particles to be exposed to various concentrations of buffer at a constant surface area: volume ratio. This technique consisted of incubating phage dilutions in thin films supported by wire loops. Ten such loops were prepared using 60 mm lengths of 25 swg Nichrome wire each with a 3.5 mm diameter ring formed at one end. When dipped into a phage suspension, these loops supported a film of liquid which was assumed to possess two planar surfaces having a total surface area of 19.25 mm^2. To ascertain the volume of liquid held within each film, the dry weight of each loop was determined and subtracted from the mean of ten measurements of the weight of the loop containing films

Figure 21. Relationship Between MS-2 Inactivation and Generated Surface Area.



of distilled water. Assuming a specific gravity of 1.00 for distilled water, the mean volume of liquid contained within each film was found to be 0.0039 ml, and no significant difference (at the $\alpha = 0.005$ level) was found between the mean volumes for each of the 10 loops. The surface area per unit volume of the film of liquid held within a loop was therefore 4961 $mm^2 ml^{-1}$.

By calculation, it was determined that phage suspensions containing approximately 3×10^4 pfu ml^{-1} would yield about 100 plaques per plate if "loopfuls" of suspension were added directly to soft agar assay tubes. Accordingly, two such suspensions were prepared and each assayed by the routine dilution technique and also by immersing each of the 10 loops, mounted in bacteriological loop holders, into the suspension and distributing each 'loopful' into one of 10 seeded assay tubes. The results (Table 12) showed no significant difference between the mean titres determined by each method and the 'loop assay' was therefore considered suitable for assaying these surface films of phage suspensions.

Table 12 Comparison Between the 'Dilution Method' and the 'Loop Method' for Assaying Phage Suspensions

Assay Method	Mean Titre pfu ml^{-1}	Standard Deviation
Suspension 1		
Dilution	3.10×10^4	0.24×10^4
Loop	3.08×10^4	0.43×10^4
Suspension 2		
Dilution	2.59×10^4	0.17×10^4
Loop	2.61×10^4	0.22×10^4

By experiment, it was found that the films would remain intact for up to 5 minutes when the loops were placed inside a 37°C incubator containing a large dish of water to raise the relative humidity to approximately 100%. It was reasoned that increasing the incubation temperature would accelerate the inactivation rate, in common with many other chemical and physical processes, thus accentuating possible differences between the treatments. Such "incubated" films showed no loss in weight due to evaporation over the 5 minute period and using this technique, the inactivation of phage in films of phosphate/citrate buffer at various concentrations was examined. Typical results (Table 13) showed no inactivation in the control samples incubated in 10 ml aliquots of the buffer solutions. However, approximately 85% of the phage was inactivated after 5 minutes incubation in the thin films and this titre reduction was independent of the buffer concentration.

Table 13 Inactivation of MS-2 in Surface Films of Phosphate/Citrate Buffer at pH 6.0

Buffer Concentration	Initial Titre pfu ml^{-1}	S.D. $\times 10^5$	Control* pfu ml^{-1}	S.D. $\times 10^5$	5 Minute Incubation pfu ml^{-1}	S.D. $\times 10^4$
Distilled Water	1.93×10^5	0.29	1.82×10^5	0.24	2.69×10^4	0.41
0.001 M	1.93×10^5	0.29	1.99×10^5	0.31	3.09×10^4	0.36
0.010 M	1.93×10^5	0.29	1.88×10^5	0.19	2.93×10^4	0.39
0.200 M	1.93×10^5	0.29	1.92×10^5	0.22	2.82×10^4	0.42

* 1 ml of phage suspension added to 9 ml of buffer, incubated at 37°C for 5 minutes and assayed by the dilution method.

Thus phage inactivation during aeration apparently did not require the presence of ions in solution. However static films differed from aerated suspensions in the mechanism of phage-surface attachment. In the films, the energy for attachment was presumably derived from the Brownian motion of the phage particle whereas in an aerated system, attachment was promoted by the turbulence and bubble-particle collision. The latter presumably resulted in higher impact velocities and therefore required more rapid thinning of the hydrated layers surrounding the bubble and particle. Ions have been shown to promote such thinning and to greatly reduce the time required for bubble attachment to a mineral surface (84). Thus during aeration increasing the buffer concentration may have promoted phage inactivation by facilitating attachment as well as by increasing the bubble density. During further studies to be reported later it was found that the type of ion affected the rate of surface inactivation and this phenomenon will be discussed in greater detail at that stage.

For phage particles to attach to an interface they must have possessed a degree of surface activity. This was considered feasible in view of the similarity between the spherical protein subunits of the phage capsid and certain globular proteins, which have been shown to fractionate during aeration of dilute solutions (121).

As discussed in the Introduction, one of the surface inactivation mechanisms proposed by Trouwborst et al was the lethal deformation of adsorbed virus particles by the surface energy of the interface (137). They suggested that the increase in inactivation with sodium chloride concentration resulted from the increased surface energy, caused by

the sodium and chloride ions in solution, acting on the adsorbed viruses. This proposal apparently contradicted the results presented here which showed that using a phosphate-citrate buffer, inactivation increased with a decrease in surface tension. However inorganic salts also increase the degree of frothing as has been demonstrated, and probably enhance particle-bubble attachment as discussed. These factors undoubtedly contributed to the increased inactivation observed by Trouwborst and his co-workers and the inactivation mechanism was not therefore solely one of physical disruption by interfacial forces as proposed.

Trouwborst and others have also studied the inactivation of several viruses in aerosols (35, 134, 135, 136, 139). They found that for bacteriophages MS-2 and T1, greatest inactivation occurred in aerosols formed from various salt solutions when these were maintained at a relative humidity corresponding to that of a saturated solution of the salt. This was also the value at which the surface: volume ratio of the aerosol droplets was maximal since at lower relative humidities evaporation proceeded beyond saturation, the salts crystallized and the droplets solidified. This therefore supported the hypothesis that inactivation was dependent upon the surface area: volume ratio. However, Akers and Hatch (3) cited several examples of aerosol inactivation which were independent of relative humidity. Such conflicting evidence probably resulted from experimental inaccuracies caused by inactivation during aerosol formation, rehydration during collection and/or shearing during penetration of the impinger collecting fluid, all of which were recognized as potential sources of error by Barlow (12) and by De Jong (35).

The nature of the changes induced by surface adsorption were also investigated by Trouwborst and his co-workers. They found that during surface inactivation bacteriophage T1 released its nucleic acid although no morphological differences between viable and inactivated phage particles could be detected by electron microscopy (138). In order to verify these findings for the smaller, simpler, RNA-containing MS-2, phage suspensions were examined by electron microscopy before and after surface inactivation. A suspension containing 3.2×10^{10} pfu ml^{-1} in 0.2 M phosphate-citrate buffer was divided into two aliquots. One was aerated for 5 hours as described earlier, after which time the titre was reduced to 5.1×10^{2} pfu ml^{-1}. Samples of this and the unaerated control were prepared for electron microscopy under the guidance and with the assistance of Dr. D.B. Jones in this laboratory. Unfortunately the suspensions were not sufficiently concentrated, yielding less than 1 particle per field of view and it was not possible, with the facilities available, to significantly increase this concentration. Thus although quantitative analysis of the results was not possible, the particles did not appear to have been altered in size or shape by aeration of the sample. However it was noticed that a higher proportion of phage particles from the control sample had electron-opaque centres which has been suggested as representing intact, viable viruses containing RNA (personal communication, D.B. Jones). If this was correct, the higher proportion of 'empty' capsids seen in the aerated sample demonstrated the loss of RNA during surface inactivation, in agreement with the findings of Trouwborst.

The lack of visible morphological changes to the phage capsid during surface inactivation controverts the suggested mechanisms

involving physical disruption by interfacial forces. However, RNA release resulting from surface inactivation may be similar to the mechanism of RNA injection following phage adsorption to the sex pilus of the host bacterium. As noted in the Introduction the A-protein of the capsid has been suggested as the site of this adsorption and this then presumably undergoes a conformational change which initiates RNA injection.

Such alterations in protein structure can be induced by a number of physical and chemical factors such as heat, extremes of pH or high ionic concentrations. Evidence for such changes induced by surface adsorption has also been obtained for enzymes by measuring the decrease in their activity (77). It is therefore feasible that, whilst not causing gross physical disruption of the capsid, surface adsorption may so alter the A-protein structure as to initiate RNA release. Unfortunately further investigation of this mechanism was considered beyond the scope of this work.

3.3. Isoelectric Focussing of MS-2

The results reported earlier demonstrated that MS-2 was relatively resistant to pH inactivation. However in common with other proteins, the solution pH determined the electrical charge on the phage coat proteins by regulating the dissociation of free ionizable groups. This would affect the adsorption of charged molecules such as surfactants and thus it was considered necessary to determine the isoelectric point of the phage.

Using cellulose acetate strips as described in section 2.10.1, a number of difficulties were encountered. At current values above 1 mA, the strips tended to heat up causing them to dry out and the combination of these effects resulted in no viable phage being eluted from the strips. The current was therefore restricted to 1 mA and the whole apparatus placed in a refrigerator at $4^{o}C$ to minimize overheating. Under these conditions it was found that visible protein bands appeared on the strips after 3 hours, showing that isoelectric focussing had occurred. Prolonging the incubation period beyond 3 hours caused a reduction in the voltage generated across the strips from a maximum attained value of 180 V. This resulted from the diffusion of ampholine from the strips and the inflow of ethanolamine from the cathodic buffer compartment, as evidenced by the high pH recorded throughout each strip.

Further difficulties were encountered when attempting to measure the pH gradient formed. Elution of segments of sectioned strip with distilled water was impractical due to the extremely small volume of ampholine contained within each segment and hence its very poor buffering capacity. However this was resolved by pressing universal indicator paper against the strip and reading the pH by colour matching to a

test chart. Whilst lacking accuracy, the results showed an irregular, non-linear gradient with a pH rise from 3.0 to 8.0 within 25 mm of the cathode end, while the remaining 60 mm of the strips showed a constant pH of approximately 8.5. However, sectioning of the strips into 10 equal segments and eluting the phage in 1% w/v peptone resulted in approximately 25% of the phage titre occurring in the second segment of each strip. As these segments had pH values between 3.5 and 4.5 as determined by pH indicator papers, this suggested that the isoelectric point of MS-2 was within this range.

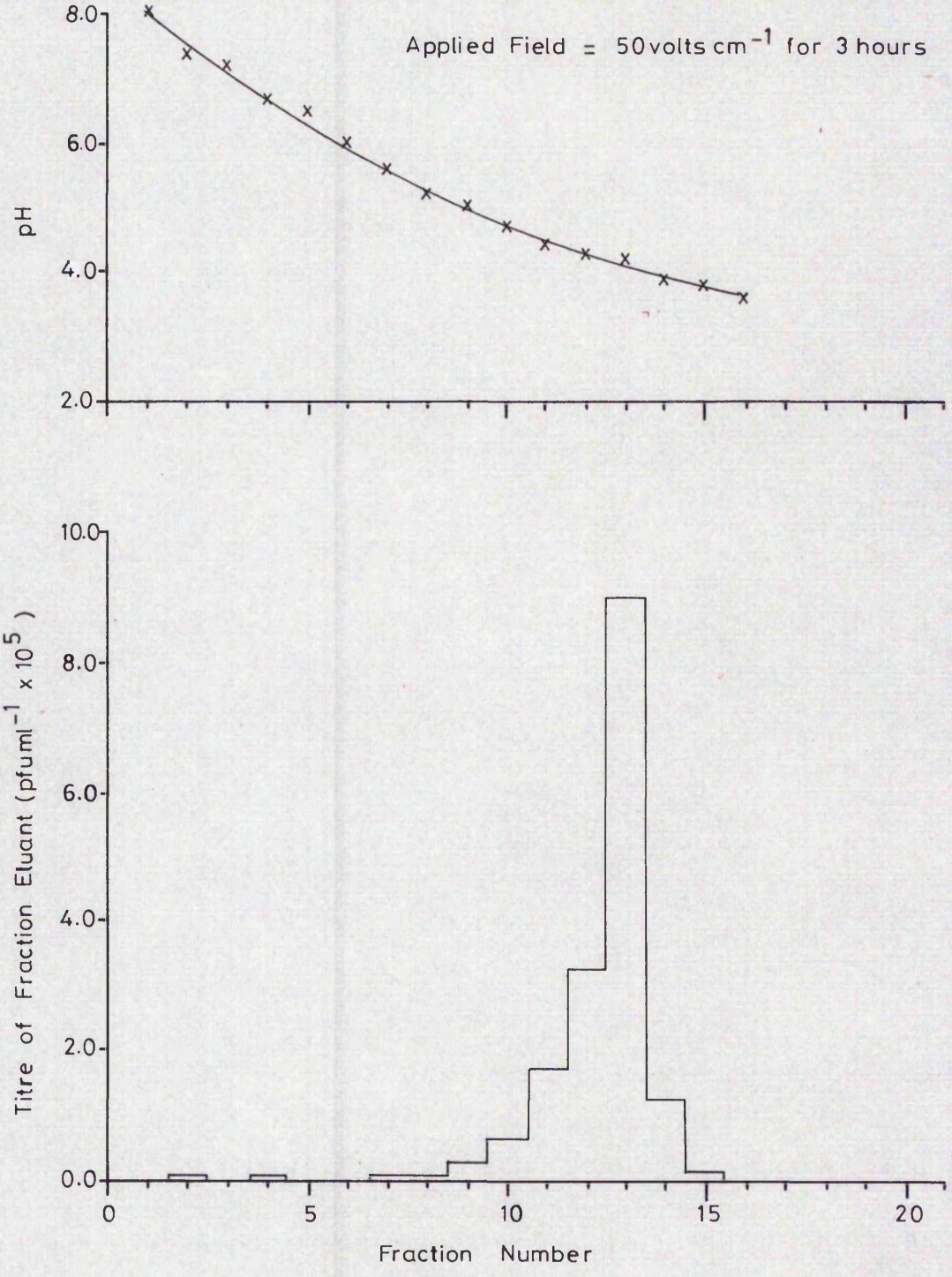
Subsequent experiments were conducted using the Shandon Analytical Gel Electrophoresis Apparatus as described in section 2.10.2. Initially 2% w/v polyacrylamide gels containing 0.5% w/v agarose were used, as described by Peacock and Dingman (109). The required amount of agarose was dissolved in water with heating, cooled to 40^{o}C, and mixed with a second solution containing the acrylamide, bisacrylamide, ampholine and phage sample, also at 40^{o}C. The ammonium persulphate was then added, the solution degassed and the gels cast at room temperature (20^{o}C). In practice as the pH gradient became established and the voltage increased, the gels shrunk considerably. This allowed the sulphuric acid in the cathode compartment to permeate between the gel and the tube, diffusing into the gel and consequently destroying the pH gradient. Attempts to overcome this problem by increasing the amount of agarose up to 1.2% w/v proved unsuccessful as did altering the acrylamide: bisacrylamide ratio and varying the amount of ammonium persulphate added. However, limited success was obtained by casting a "plug" of 10% w/v polyacrylamide, 5 mm in length, at each end of the gels. These adhered strongly to the sides

of the glass tubes, and although some gel shrinkage was noted, a uniform pH gradient was maintained. A typical set of results obtained from these runs is shown in figure 22. As can be seen, the majority of the phage recovered (54%) was found in fraction 13 which had a pH of 4.1, suggesting that this represented the isoelectric point of MS-2. However, approximately 3×10^7 phage particles were added to the gel which, assuming 100% recovery, should have yielded a titre of approximately 9.0×10^5 pfu ml^{-1} for each fraction eluant, assuming no phage migration had occurred. This corresponded to the titre of fraction 13 and thus it could be argued that pH 4.1 represented the optimum pH for survival of the bacteriophage. This argument was rejected for two reasons; firstly the pH optimum for survival of MS-2 in earlier experiments had been found to be 6.0 and not 4.1. Secondly, 100% phage recovery was considered unlikely in view of the probable toxicity of the gel components and the unlikelihood of complete elution of phage from the gel fractions.

In a series of experiments to assess the toxicity of the gel components, it was found that acrylamide, N, N′-methylenebisacrylamide and ammonium persulphate were all toxic to MS-2 at the concentrations used during casting of the gels. However addition of slices of polyacrylamide to aliquots of a phage suspension in distilled water did not reduce their titre and it was therefore concluded that once polymerized, the acrylamide and bisacrylamide were non-toxic.

The extent of phage inactivation occurring during electrophoresis was extremely difficult to estimate. During gel formation, the concentration of toxic acrylamide and bisacrylamide monomers decreased with time to reach zero concentration after 30 minutes when the gels had fully polymerized. In addition, the excess ammonium persulphate

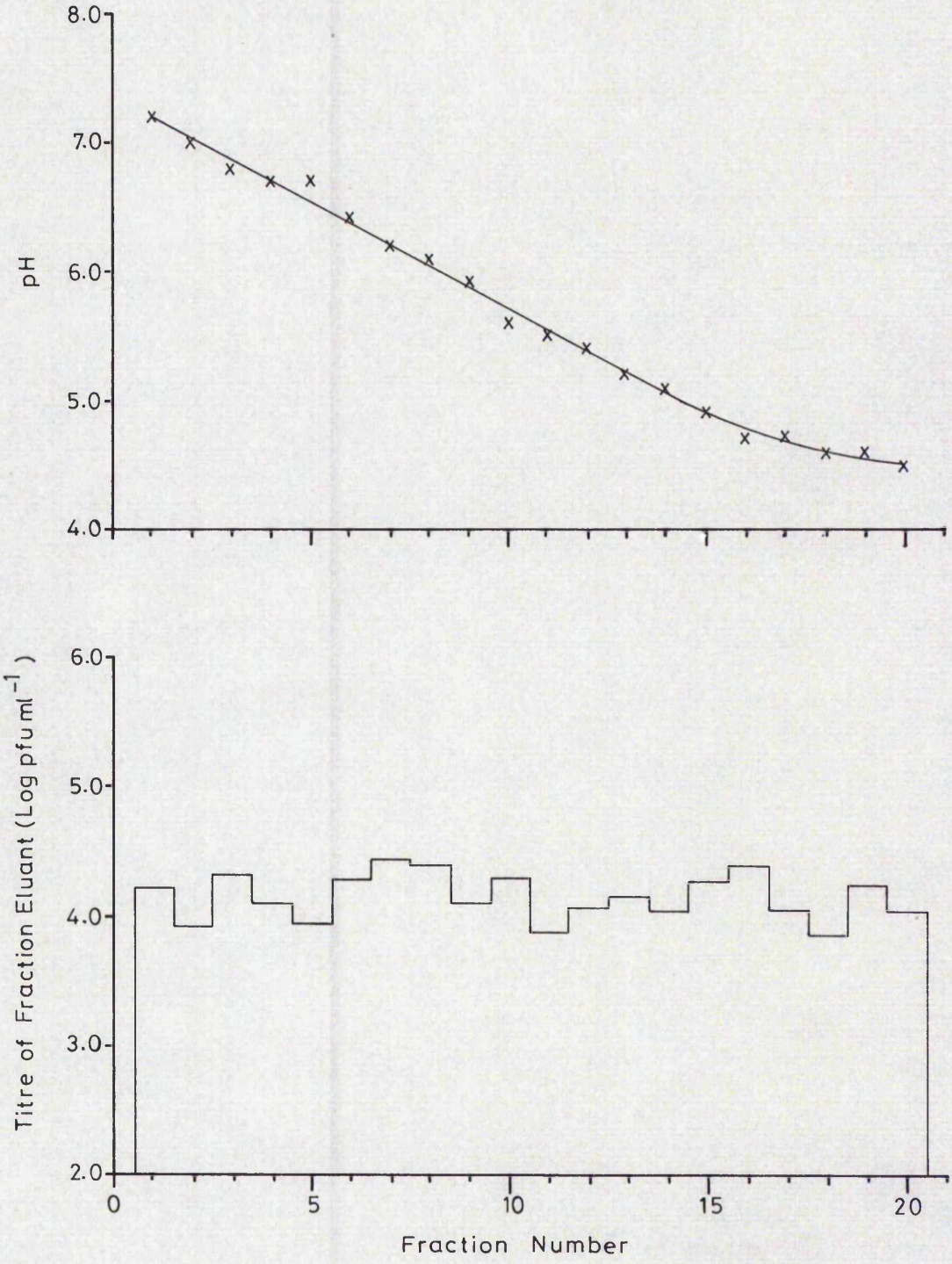
Figure 22. Isoelectric Focussing of MS-2 in 'Plugged' Gels of 2% Polyacrylamide - 0·5% Agarose.



was run off from the gel during the first 30 minutes of electrophoresis as the pH gradient was being established. In order to determine the loss of titre during polymerization of the gel and establishment of the pH gradient, an aliquot of a phage suspension of known titre was dispersed in an acrylamide-agarose mixture which was distributed into gel tubes and allowed to polymerize as before. These were positioned in the electrophoresis apparatus and subjected to a constant current of 2 mA per tube for 30 minutes, by which time the voltage had risen to 150 V and the pH gradient was considered to be established. The gels were then fractionated and eluted as before, and typical pH and phage assay data is shown in figure 23. As can be seen, a pH gradient was established although not extending through the full range of approximately 3.5 to 9.0. Assay of the gel slices showed that no isoelectric focussing had occurred after 30 minutes since no significant difference was found between the phage titres of the gel slice eluants. By converting the titres for each fraction into pfu per gel slice (i.e. the product of the titre and the eluant volume), a cumulative total of 6.20×10^5 pfu were recovered from the gel. Initially, 50 µl of a 1.1×10^9 pfu ml^{-1} phage suspension was added to 10 ml of acrylamide-agarose mixture. Each gel was cast from 3 ml of this mixture and therefore the amount of viable phage added per gel was 1.1×10^9 $(0.05 \times 3/10)$ = 1.65×10^7 pfu. Thus approximately 96% of the infective titre was lost during the process of casting the gel, establishing the pH gradient, and eluting from the crushed gel slices.

At this stage the use of composite polyacrylamide-agarose gels was discontinued due to the problems associated with gel shrinkage. Since this does not occur during electrophoresis in a single buffer

Figure 23. Loss of Infective Titre from Gel Fractions During Polymerization and Establishment of the pH Gradient.



system, for which the mixed gel was developed, it was assumed that the phenomenon was related to the pH gradient formed by the ampholytes. The remaining studies were carried out using polyacrylamide gels following the method devised by Rice and Horst (112) and described in section 2.10.2. Using glass tubes it was found that the gels were extremely difficult to remove intact. The usual methods of 'rimming' with a hypodermic needle and applying water pressure were unsuccessful due to the plasticity and adhesion of the low concentration polyacrylamide gels. After experimenting with various coatings on the glass tubes, these were discarded in favour of tubes made from sections of disposable, 10 ml plastic pipettes (obtainable from Sterilin). The gels did not adhere to these and were therefore readily removable.

After preliminary tests, it was decided to standardize the operating conditions such that the electrophoresis equipment was initially run at a constant current of 2 mA per tube until the voltage had risen to 150 V. This usually required between 30 and 50 minutes, after which time the supply was switched to the constant voltage mode and maintained at 150 V for the required time. These represented optimum current and voltage levels for rapid focussing without excessive heating of the gels. Initially 3% w/v polyacrylamide gels were prepared and electrofocussed for periods of between 2 and 5 hours. Figures 24 and 25 show typical results for a 2 hour and a 5 hours run respectively. As figure 24 shows, a linear gradient between pH 2.7 and 9.3 was formed after 2 hours. However there was little evidence of any bacteriophage migration, although the highest titre was recorded in fraction 6 which had a pH of 4.0, corresponding to the value found earlier (see figure 22). It was also noted that the first

Figure 24. Isoelectric Focussing of MS-2 in a 3% Polyacrylamide Gel After 2 Hours.

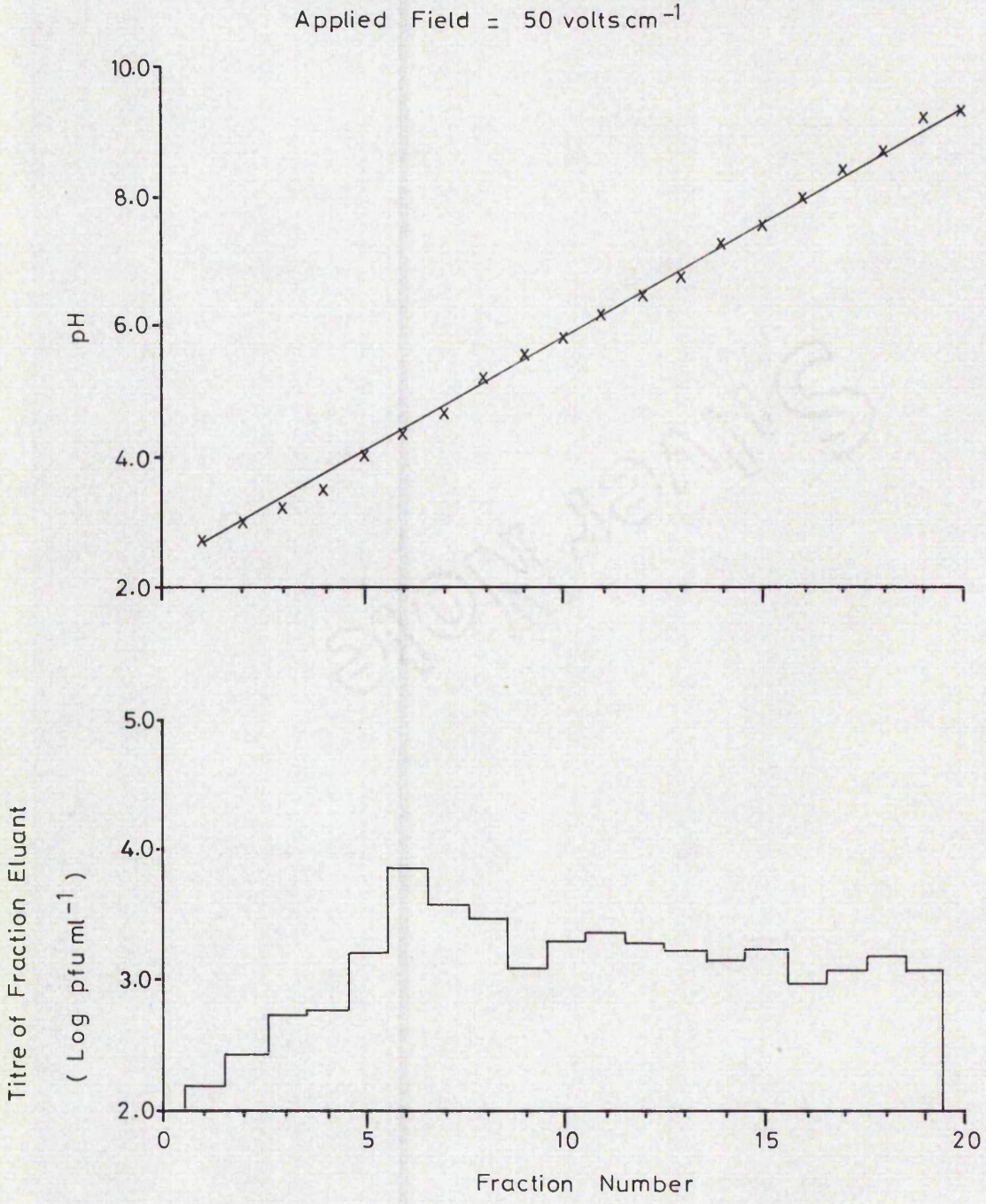
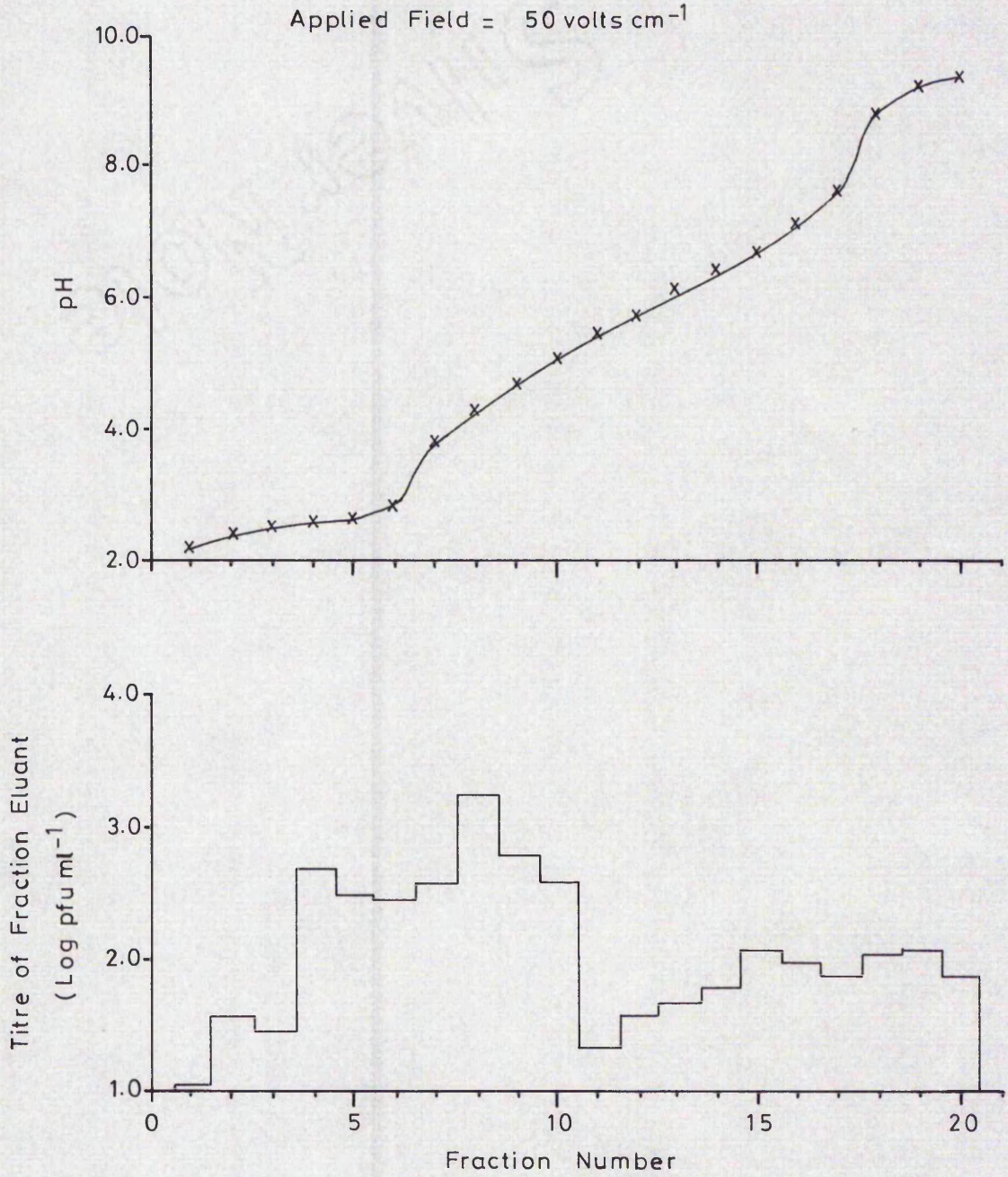


Figure 25. Isoelectric Focussing of MS-2 in a 3% Polyacrylamide Gel After 5 Hours.



4 fractions had significantly lower titres than the rest which was possibly due to inactivation resulting from their low pH. Summation of the total viable phage count for all the fractions and comparison of this value with the phage input, yielded a total phage recovery of 2.1%.

After 5 hours electrophoresis, the pH gradient was no longer linear. This was probably due to the ampholine buffer diffusing from the extremities of the gel and the ingress of alkali and acid from the buffer compartments which respectively increased and decreased the pH values at either end of the gel. Furthermore the total phage recovery from the gel was reduced to 0.16% of the input, probably as a result of the longer regions of extreme pH at either end of the gel. However there was a significant difference between the titres of the fractions, with 83% of the phage recovered in fractions 4 to 10 inclusive. The proportion of phage particles concentrated in these fractions was probably much greater but pH inactivation, particularly within fractions 4, 5 and 6 may subsequently have reduced the infective titre. The highest titre was found in fraction 8 which had a pH of 4.3, although this result should be treated with caution for the reasons outlined above.

From these experiments it was concluded that the maximum duration for a run consistent with a linear pH gradient was 4 hours. However, the mobility of phage particles within a 3% w/v gel was insufficient to allow complete electrofocussing in this time period. To overcome this problem attempts were made to prepare gels of a lower polyacrylamide concentration and it was found that by increasing the amount of bisacrylamide from 0.1% to 0.12% w/v, a satisfactory gel containing 2.8% w/v polyacrylamide could be prepared. To evaluate

these modifications 2.8% w/v gels were cast from a solution to which had been added an aliquot of purified phage suspension as described in section 2.10.2. These were electrofocussed for 4 hours at 150 volts and the results (figure 26) showed a linear pH gradient with a significant proportion of the phage in fractions 4 and 5. From a total of 4.15×10^9 pfu added per gel 0.62% was recovered with 78% in fractions 4 and 5, which had pH values of 3.9 and 4.0 respectively.

The low phage recovery was again thought to be at least partly due to the overnight elution of the gel slices with distilled water. The majority of the slices had pH values outside the optimum for survival reported earlier and thus pH inactivation would be expected to lower the phage titre. To overcome this problem, duplicate gels were run simultaneously and sectioned as before. One was eluted with distilled water for pH measurement and the other eluted with 1% w/v peptone adjusted to pH 7.0 with sodium hydroxide. After storage of the latter at $4^{\circ}C$ for 18 hours, the eluants were assayed for phage. This was performed in quadruplicate (8 gels in all) and the mean of the results showed a phage recovery of 19% with over 92% in fraction 6 at a pH of 4.0 (figure 27). Assuming 100% recovery and no phage migration within the gel, each slice would have contained 3.6×10^8 pfu. Fraction 6 contained a total of 9.8×10^8 pfu showing that an increase in concentration had occurred in that fraction. It was shown earlier (figure 23) that 96% of the phage added to acrylamide-agarose mixtures was inactivated during polymerization and elution of the slices. Thus although the acrylamide concentration was higher and the duration of the run was longer in the above experiment, the degree of inactivation was less (81%). Therefore

Figure 26. Isoelectric Focussing of MS-2 in a 2·8% Polyacrylamide Gel After 4 Hours.

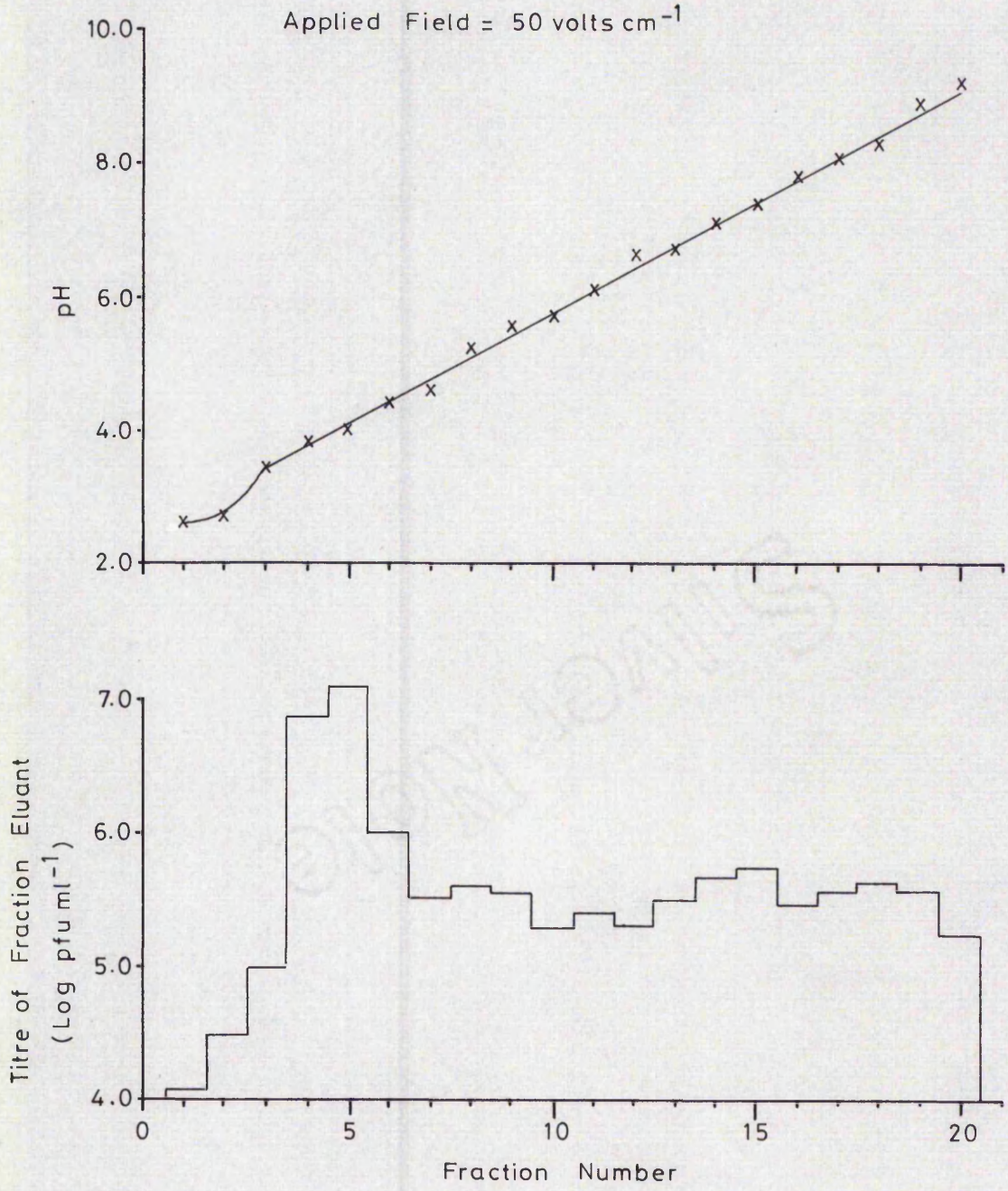
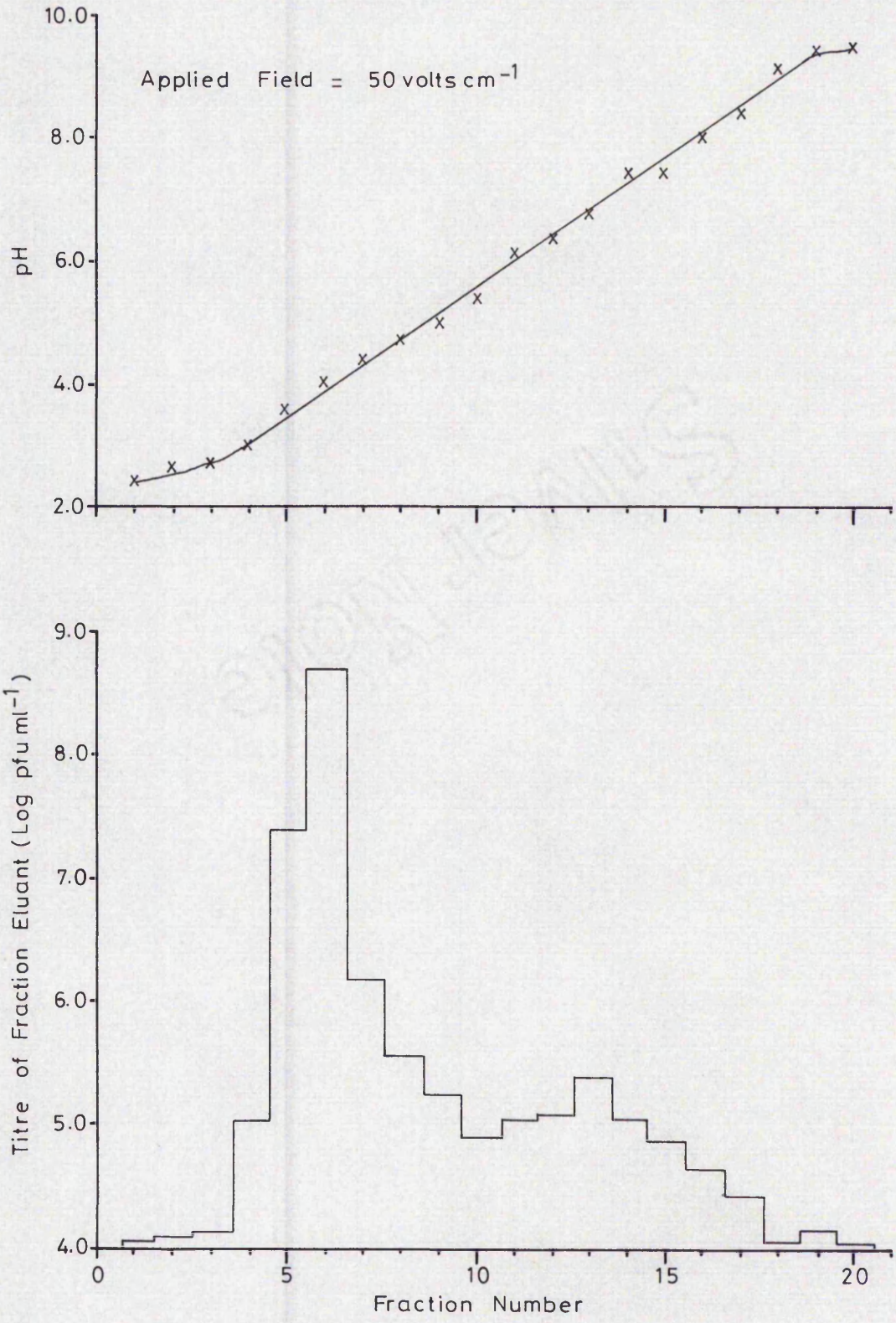


Figure 27. Isoelectric Focussing of MS-2 Followed by Elution of the Gel Slices with 1% Peptone.



eluting the slices with peptone reduced the inactivation by at least 15% although the greatest loss in titre occurred during gel polymerization.

Assuming the same recovery rate per slice (i.e. 19% of initial titre) and no electrofocussing of the phage, each fraction would have contained 6.84×10^7 pfu. Thus fraction 6 showed an increase in phage titre of 1433% and despite being unable to recover all the added, viable phage, the results have clearly demonstrated that considerable isoelectric focussing of MS-2 occurred. The isoelectric point of MS-2 obtained by these methods was therefore between pH 4.0 and 4.1. This was in good agreement with the values reported for other viruses, in particular the value of pH 4.1 for the closely related bacteriophage Qβ (112). However it was noticed in most experiments that a second, smaller peak occurred between pH 7.0 and 8.0, as for example fraction 13 in figure 27.

As reported earlier, Mandel had shown by electrofocussing that Poliovirus Type I existed in two conformationally distinct states which had isoelectric points at pH values of 7.0 and 4.5 (99). Using purified, radio-isotope labelled virus Mandel was also able to show that the population was homogenous and that each particle could exist in either state according to pH. However when the viruses were exposed to conditions such as heat, U.V.-irradiation or antibody neutralization, the inactivated particles were irreversibly stabilized in the configuration with the isoelectric point of 4.5.

Unfortunately, radio-isotope labelling of MS-2 was considered beyond the scope of this work and assessment of the isoelectric point of inactivated particles was therefore impossible. However

experiments with the surviving viable fraction suggested strong similarities with Mandel's results. Accumulation of eluants from gel fractions of the high pH peak for a number of experimental runs enabled 10 ml of phage suspension to be collected which, after ultracentrifugation, yielded 50 µl of suspension with a sufficiently high titre for further isoelectric focussing. The results showed that this fraction had an identical electrophoretic pattern to that of the parent populations, i.e. with phage recovery peaks at pH values of 4.1 and 7.6. This confirmed that the states with different isoelectric points were interconvertible since no enhanced recovery at pH 7.6 was found.

3.4. Surfactant Toxicity Studies

The results in the previous section demonstrated that MS-2 could exist in either of two interconvertible states characterized by differing isoelectric points. Clearly the configuration as well as the surface charge of the phage capsid varied with pH and it was therefore considered very likely that the adsorption of ionic detergents would also be pH dependent. This has been overlooked in the majority of studies on surfactant toxicity although Stols and Veldstra (129) reported that quaternary ammonium salts showed a greater toxicity towards Turnip Yellow Mosaic Virus at a high pH.

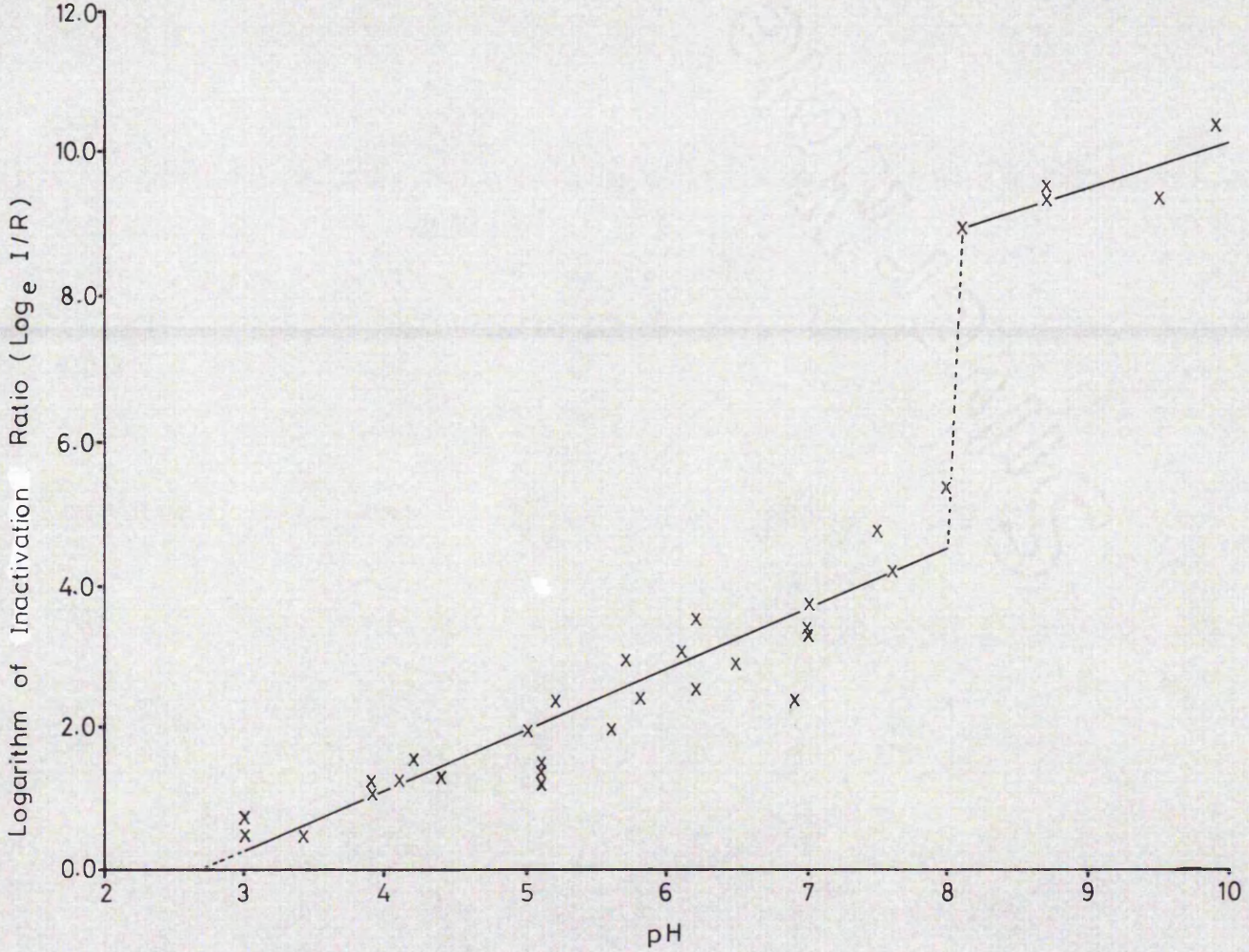
To assess the effect of pH on Arquad toxicity, the method described in section 2.9.1. was devised. It was found that 5 minutes contact time was the minimum required for thorough mixing of the flask contents at a stirrer speed which did not cause foaming. It was also sufficiently short for any loss of titre due solely to pH inactivation to be negligible. The surfactant concentration of 30 mgl^{-1} was found to be sufficient to yield significant inactivation within 5 minutes at all pH values tested without causing foaming problems. The results (figure 28) showed a linear relationship between the logarithm of the ratio of bacteriophage inactivated ($^{I}/_{R}$) and pH within the range 3.0 to 8.0, as described by the following relationship:

$$\text{Log}_e\ {}^{I}/_{R} = 0.834(\text{pH}) - 2.21$$

Between pH values of 8.0 and 8.1, the rate of inactivation rose sharply but above pH 8.1, the rate increased at approximately the same value as before.

Extrapolating the regression line for the lower pH values to its intercept with the x-axis yielded a value of pH 2.65 when $\log_e\ {}^{I}/_{R} = 0$.

Figure 28. Inactivation of MS-2 in Solutions of Arquad at Various pH Values.



Thus at this pH, the Arquad would be expected to show no toxicity towards MS-2. To verify this, the phage was incubated in a distilled water control and in a 30 mgl^{-1} solution of Arquad, both adjusted to pH 2.65 with 1.0M hydrochloric acid. Analysis of the results (Table 14) showed a significant difference between the mean residual titres with the sample incubated in Arquad showing approximately 13% greater inactivation than the distilled water control. This suggested a toxicity mechanism independent of pH which will be discussed later.

Table 14 Inactivation of MS-2 in 30 mgl^{-1} Arquad at pH 2.65

Sample	Initial Titre pfu ml^{-1}	Standard Deviation	Residual Titre pfu ml^{-1}	Standard Deviation
Distilled water	2.85×10^5	0.11×10^5	2.43×10^5	0.11×10^5
30 mgl^{-1} Arquad	2.85×10^5	0.11×10^5	2.12×10^5	0.12×10^5

These results clearly demonstrated an ionic interaction between the Arquad and the phage which was pH dependent. It was not considered likely that pH would affect the Arquad since, as pointed out by Grieves and Bhattacharyya (56), quaternary ammonium salts are almost completely dissociated in dilute solution with insignificant hydrolysis to form quaternary ammonium bases. Therefore the effect of pH was confined to the phage capsid and to the dissociation of the free amino and carboxyl groups residing therein. The ionization of increasing numbers of carboxyl goups as the pH was increased allowed greater adsorption of surfactant to the capsid and therefore increased inactivation. This adsorption occurred over the entire capsid surface and the proportional increase in toxicity with pH up

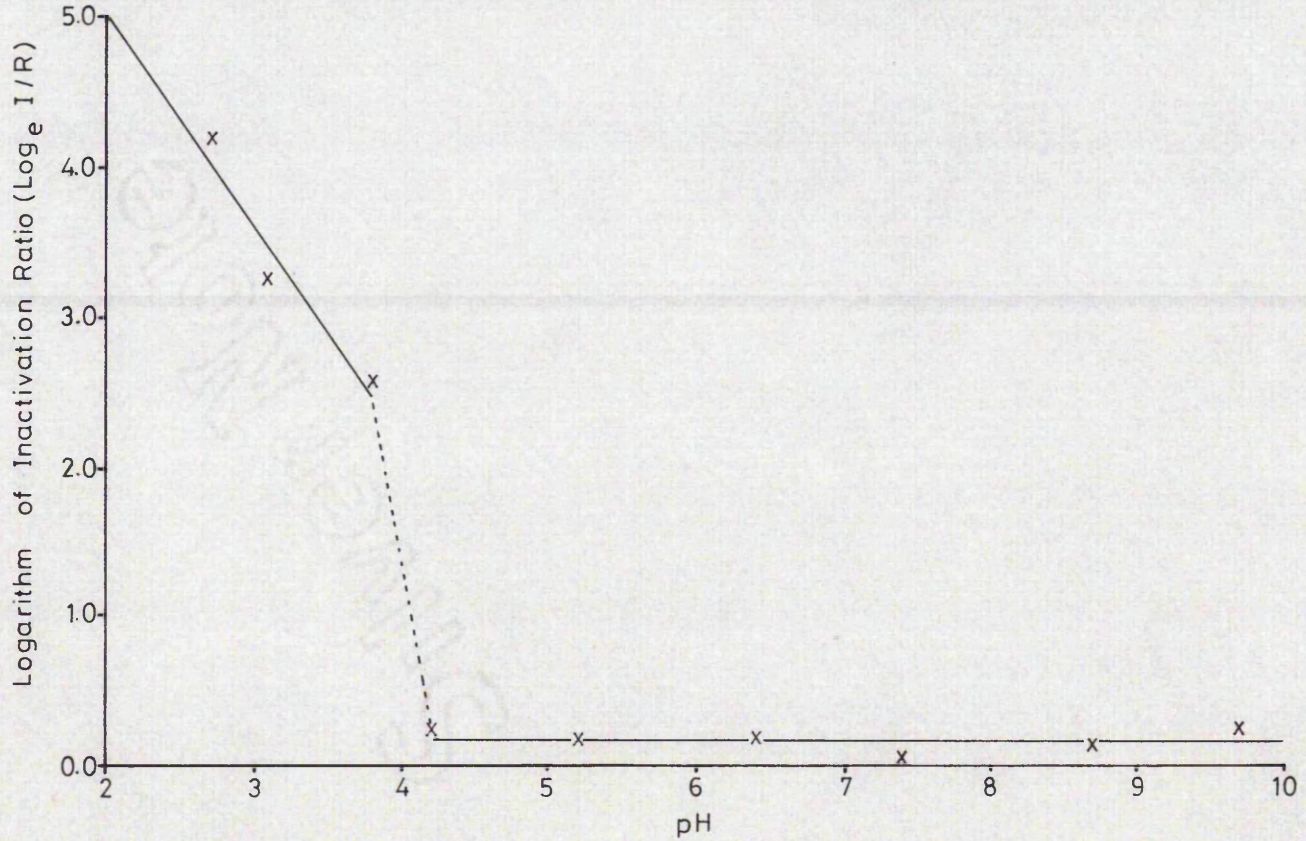
to a value of 8.0, suggested an inactivation mechanism related to the total amount of adsorbed surfactant. This will be discussed in greater detail in a later section.

The rapid increase in inactivation at pH 8.0 suggested a fundamental change in the state of the capsid. However the electrophoretic studies reported earlier demonstrated that a proportion of the phage population existed in a configuration having an isoelectric pH of 7.6. It was postulated that this state was characterized by having exposed ionizable region(s) which at a pH of 8.0, became negatively charged and therefore adsorbed the cationic surfactant. Assuming this region corresponded to the A-protein molecule, it was further postulated that the lethal event resulting from Arquad adsorption was the release of RNA. This corresponded with the proposed mechanism for surface inactivation discussed earlier.

The configuration characterized by an isoelectric pH of 4.1 was more resistant to Arquad inactivation but nevertheless the same mechanism could have been involved in the loss of infectivity. If correct, the similarity between the increase in inactivation up to pH 8.0 and above pH 8.1 could have resulted from a proportion of the population becoming 'fixed' in the configuration having the lower isoelectric pH.

Since adsorption of a cationic surfactant to a negatively-charged capsid caused inactivation, it was postulated that anionic surfactants might cause inactivation if adsorbed to a positively-charged capsid. To verify this hypothesis, the experiment was repeated using the anionic surfactant Teepol 610. As expected, the results (figure 29) showed a high degree of inactivation only at low pH values. Above a pH of 4.2 the degree of inactivation varied randomly about a mean

Figure 29. Inactivation of MS-2 in Solutions of Teepol at Various pH Values.



low level of 15%. Between pH 4.2 and 3.8 the degree of inactivation increased rapidly to 94% and thereafter the relationship between inactivation and pH could be expressed by the following equation:

$$\log_e {}^{I}/_{R} = 7.841 - 1.41(pH)$$

Clearly Teepol was more toxic to the proportion of phage particles having an isoelectric pH of 4.1 (c.f. Arquad). Presumably in this configuration more amino groups were exposed which below pH 4.1 became ionized and thus adsorbed the sulphate groups of Teepol molecules. These amino groups may have been located on or adjacent to the A-protein and the lethal event again may have been RNA release.

It was also apparent in both experiments that a degree of inactivation occurred at pH values at which the net charge on the phage capsid would be the same as that on the surfactant molecules. By extrapolation in figure 28, it was predicted that Arquad would show no toxicity towards MS-2 at a pH of 2.65. In fact the infective titre was reduced by 13% which corresponded closely with the 15% reduction in titre caused by Teepol at pH values above 4.2. This similarity in the degree of inactivation between a cationic and an anionic detergent suggested that the polar groups were not involved. However since the hydrocarbon chains were of approximately the same length in both detergents, it was thought that these would adsorb to hydrophobic regions of the phage capsid to the same extent. It was considered feasible that this could result in inactivation since hydrophobic adsorption of collectors to mineral surfaces has been shown to cause profound changes in their electrokinetic potential (84). If phage particles were susceptible to such changes this would account for inactivation independent of pH since hydrophobic adsorption

is relatively insensitive to pH.

Earlier in this text the results of experiments on phage deaggregation using detergents at concentrations ranging from 5 gl^{-1} to 1 mgl^{-1} were reported (see figures 10 and 11). These experiments showed that, even at a concentration of 5 gl^{-1}, approximately 4 hours were required for Teepol to reduce the phage titre by 15%. At the lower concentration range (figure 11.2) no significant inactivation occurred during 5 hours incubation. The disparity between these sets of results was probably due to the difference between the incubation temperatures used. The deaggregation experiments were conducted at $4^{o}C$ whilst the surfactant toxicity studies were performed at $30^{o}C$. The effects of temperature and concentration on surfactant toxicity will be reported and discussed later.

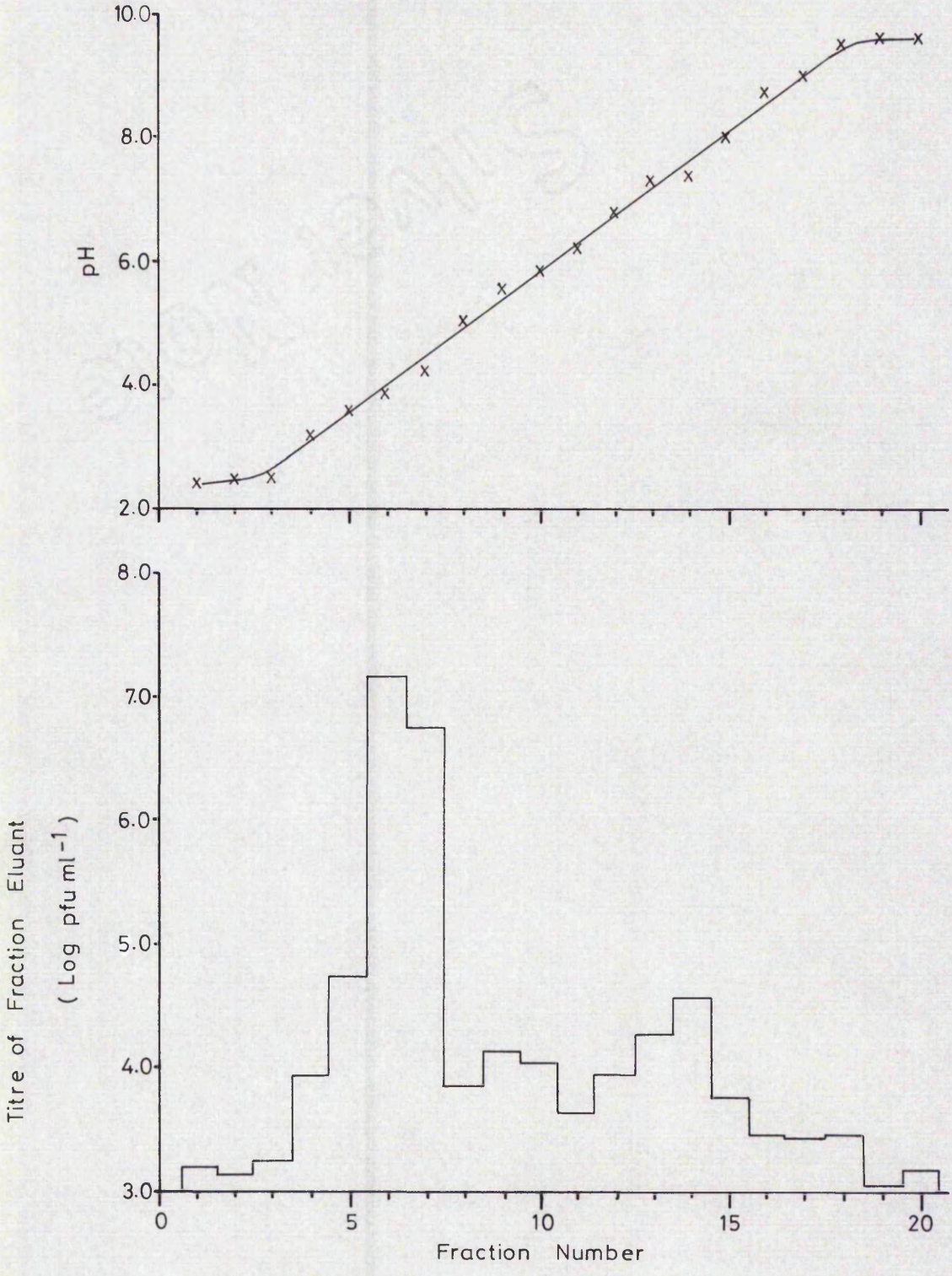
These results confirmed the earlier proposal that pH would affect surfactant toxicity. As expected Arquad toxicity increased at high pH due to the increased numbers of ionized carboxyl groups on the phage surface available for adsorption. At low pH more amino groups became ionized and thus capable of adsorbing the sulphate groups of Teepol molecules. Furthermore the increased toxicity of these surfactants was closely related to the different conformational states of the phage particles.

As reported earlier, Mandel found that inactivation by heat, U.V. and antibody neutralization stabilized poliovirus particles in one configuration (99). Such stabilization in the low isoelectric pH configuration was suggested to account for the proportion of an MS-2 population surviving Arquad inactivation above pH 8.1. If this were correct then electrofocussing of the survivors of Arquad

inactivation at high pH would not be expected to show a second peak of viable phage with an isoelectric pH of 7.6. To verify this hypothesis 250 ml of a phage suspension containing 3.1×10^{10} pfu ml^{-1} was incubated for 5 minutes with Arquad at a concentration of 30 mgl^{-1} adjusted to a pH of 8.5. In order to stop the reaction, 25 ml of an acidified 10% $^{w}/v$ peptone solution was added to yield a final solution with a pH of 6.0. The phage particles were then separated by ultracentrifugation, the supernatant solution discarded and the phage pellets resuspended in 500 µl of distilled water. This yielded a phage suspension with an infective titre of 7.8×10^{8} pfu ml^{-1}. Electrophoresis of this suspension showed somewhat variable results although the majority of the analyses showed concentration of the phage particles at two isoelectric pH values as before (see figure 30).

This indicated that the survivors of Arquad inactivation at high pH were not irreversibly stabilized in one electrophoretically distinct state. Thus either MS-2 differed from Poliovirus Type I in being resistant to irreversible stabilization or, more likely, Arquad treatment differed from heat, U.V. irradiation and antibody neutralization in being incapable of causing such stabilization. Although not electrophoretically distinguishable from a normal population, the survivors of high pH Arquad inactivation could nevertheless have possessed a stabilized, localized region of the capsid such as the A-protein. Electrofocussing of viruses is influenced solely by the overall ionic charge on the capsid and minor, localized variations are not detectable unless they affect that overall charge. Thus the surviving phage fraction might have been capable of undergoing transition to the high isoelectric pH state

Figure 30. Isoelectric Focussing of the Survivors of Arquad Inactivation at pH 8.5.



although the A-protein configuration remained the same as that in the low isoelectric pH state. These particles would therefore show the same Arquad sensitivity as 'normal' particles in the low isoelectric pH configuration.

These pH studies have indicated that inactivation by surfactants can procede via mechanisms involving ionic or non-ionic adsorption to the phage capsid. Further information on these mechanisms can be obtained from the rate of inactivation although a search of the literature revealed few studies on the nature of viral-detergent interactions. Stols and Veldstra found that the rate of inactivation of Turnip Yellow Mosaic Virus in quaternary ammonium salts was proportional to the detergent concentration and to a power function of the virus concentration between 0 and 1 (129). This implied a complicated reaction(s) which they found to involve the RNA inside the virus. This was not considered likely to occur with MS-2 since its nucleic acid was completely encapsulated in a protein shell.

During his toxicity studies with MS-2, Guy (67) found that inactivation in Arquad followed first order reaction kinetics. However the straight line plots of the logarithm of the virus titre versus time did not extrapolate to the initial titre at time zero. It was postulated that this resulted from a time lag between the addition of peptone to the sample aliquots and the inhibition of surfactant toxicity. Presumably the peptone stopped the reaction by combining with free Arquad in solution and by eluting pre-adsorbed Arquad from the phage capsid. Assuming Arquad toxicity and its inhibition to be temperature dependent (in common with other chemical reactions) then inactivation could have been stopped virtually instantaneously by the addition of ice-cold peptone to the reacting

mixture. To verify this hypothesis, the method described in section 2.9.2. was adopted to measure the rate of inactivation of MS-2 during 5 minutes contact with various concentrations of Arquad at a constant pH of 7.0. The results (figure 31) confirmed the logarithmic inactivation rates found by Guy but extrapolated to the initial titre at the origin. Thus as predicted, the addition of ice-cold peptone caused virtually immediate and complete cessation of inactivation.

The rates of inactivation were calculated using regression analysis and plotted against the corresponding Arquad concentration, with the values calculated by Guy (67) shown for comparison (figure 32). Ignoring the inactivation rate of the zero concentration (distilled water) control, both sets of data yielded straight line plots of the form: $y = a + bx$. The data obtained from these studies showed an increase in the inactivation rate of -0.005 log pfu $ml^{-1}min^{-1}$ per unit concentration of Arquad (in mgl^{-1}), which compared very favourably with the value of -0.004 calculated from Guy's data. However the rates of inactivation obtained at all Arquad concentrations were significantly higher than those found by Guy. This was thought to reflect the difference between the use of distilled water (as here) and tap water as used by Guy, as a diluent. As will be shown later in this study, the various ions present in tap water would have reduced the toxicity of the Arquad. Furthermore, Guy's experiments were conducted at room temperature (approximately $20^{o}C$) whereas in these studies an incubation temperature of $30^{o}C$ was maintained and as already indicated, the rate of inactivation was expected to increase with temperature.

The relationship between the inactivation rate and surfactant

Figure 31. Inactivation of MS-2 During 5 Minutes Contact with Arquad.

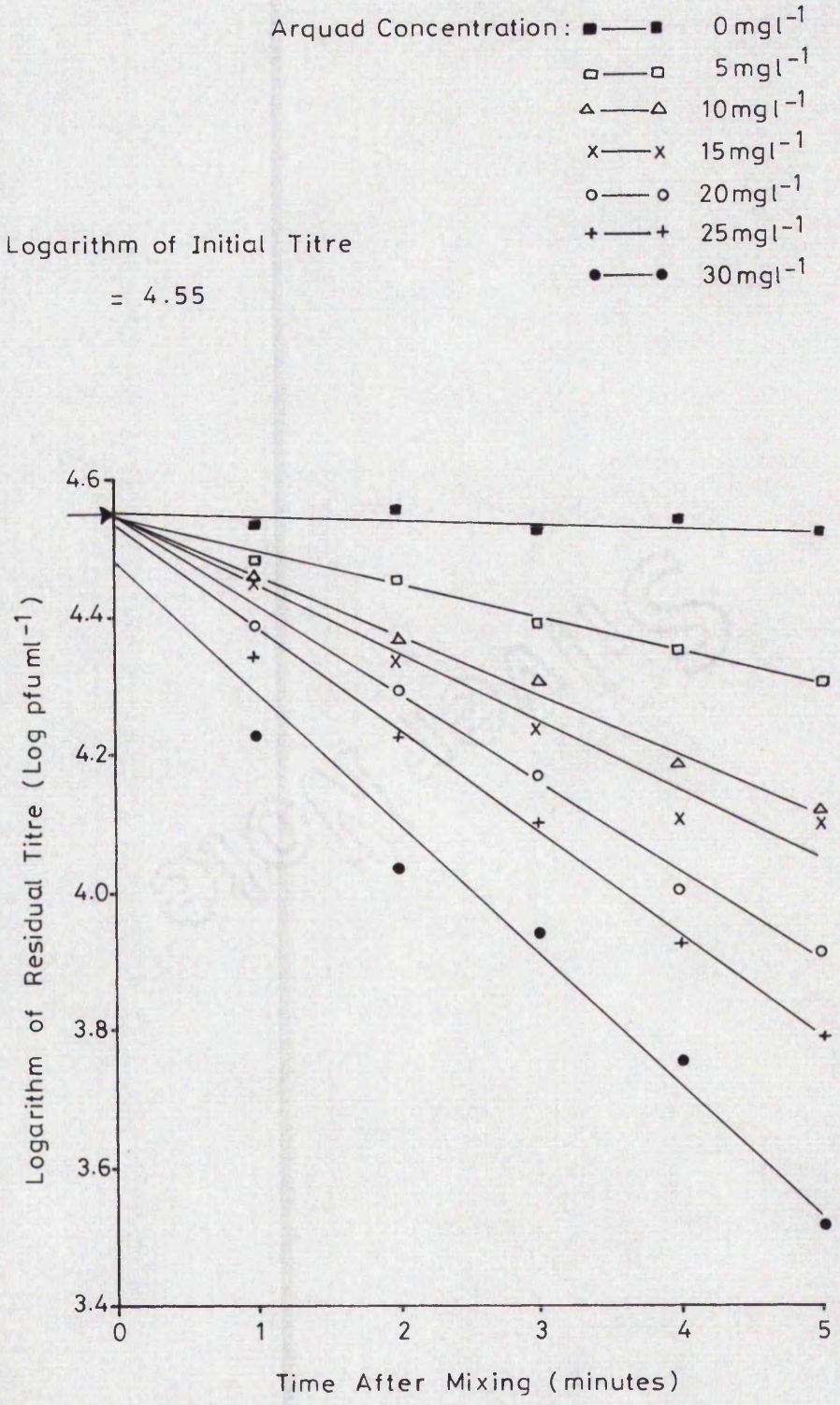
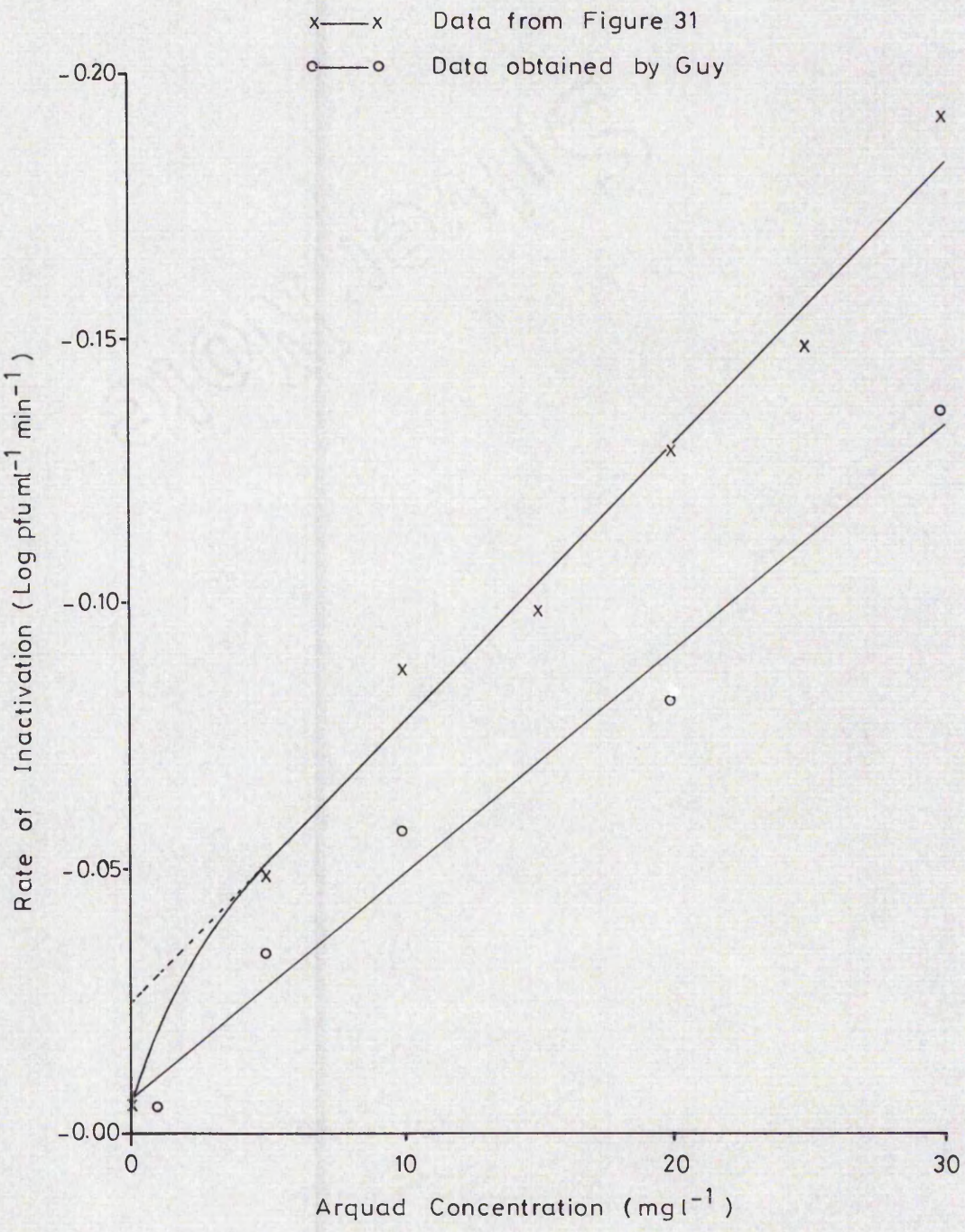


Figure 32. Comparison of Inactivation Rates Calculated From Figure 31 with Those Obtained by Guy (67).



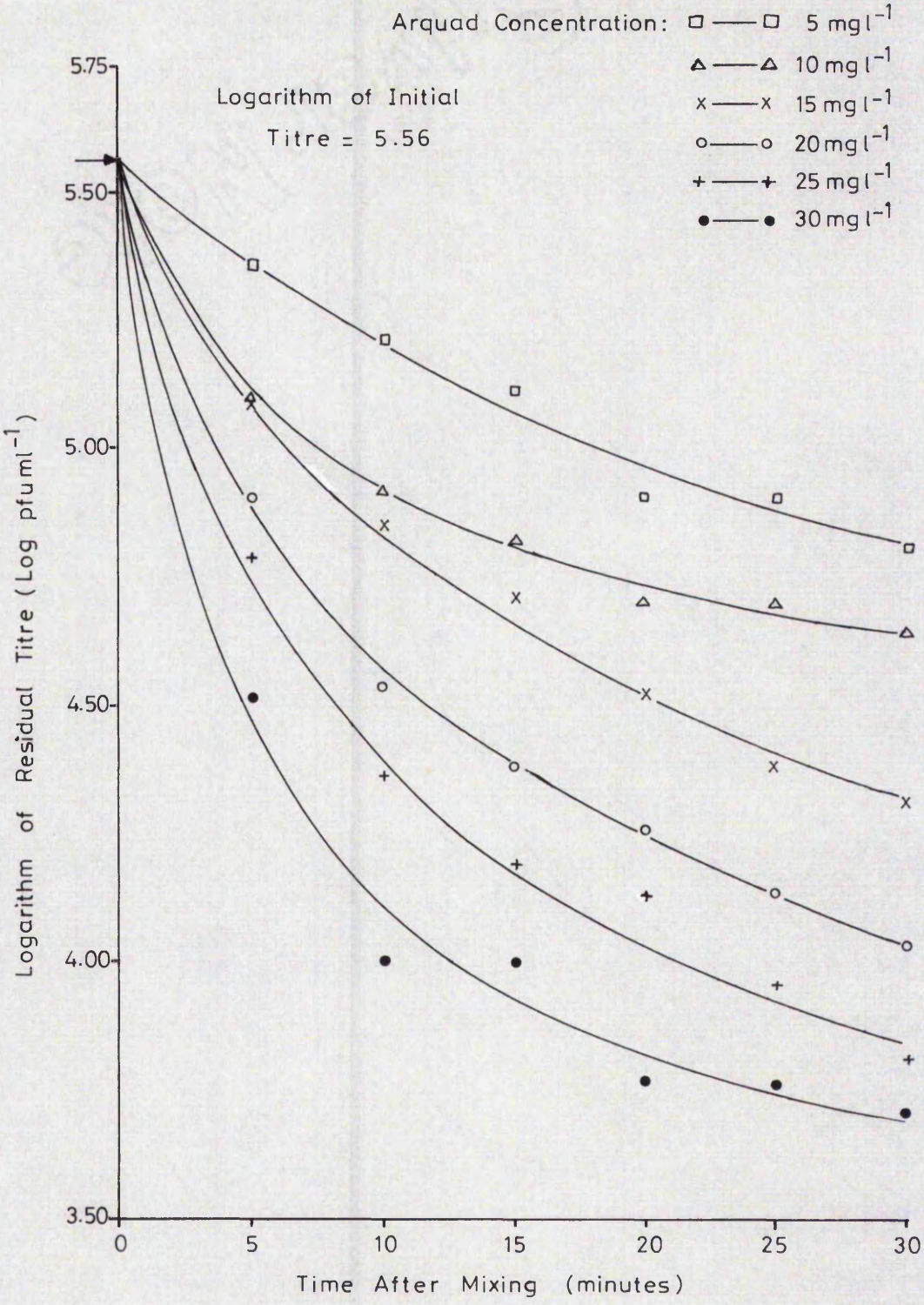
concentration in distilled water was not maintained at concentrations below 5 mgl^{-1}. Thus at zero mgl^{-1} Arquad the rate of inactivation was found to be -0.004 log pfu $ml^{-1}min^{-1}$ compared with the calculated value of -0.024 log pfu $ml^{-1}min^{-1}$ obtained from figure 32.

Although the reaction kinetics were apparently first order during the initial stages of contact, this would not necessarily be maintained during longer incubation periods. Timm and his co-workers reported a reduction in the rate of inactivation of Poliovirus during prolonged incubation (>50 hours) in formalin (132). Later, more exhaustive studies by Gard showed that the rate of formaldehyde inactivation of Poliovirus proceeded in a regularly decreasing manner over a 10 day period (45). He suggested that this was not peculiar to formaldehyde but that most chemical agents acted upon viruses in a similar manner. Despite detailed analysis of his and other researchers data, Gard was unable to suggest a likely inactivation mechanism.

In order to determine whether the rate of Arquad inactivation decreased similarly, the method described in section 2.9.1. was devised to study inactivation during prolonged incubation periods. As figure 33 shows, the apparent first order reaction rate was not maintained over a 30 minute contact period and the curves showed a regularly decreasing reaction rate as found by Gard for prolonged formaldehyde inactivation.

A possible explanation of this phenomenon was heterogeneity with respect to Arquad sensitivity within the phage population. Thus as the more susceptible fraction or fractions were destroyed the resistance of the remaining population effectively increased and the rate of inactivation was reduced. This hypothesis was proposed by

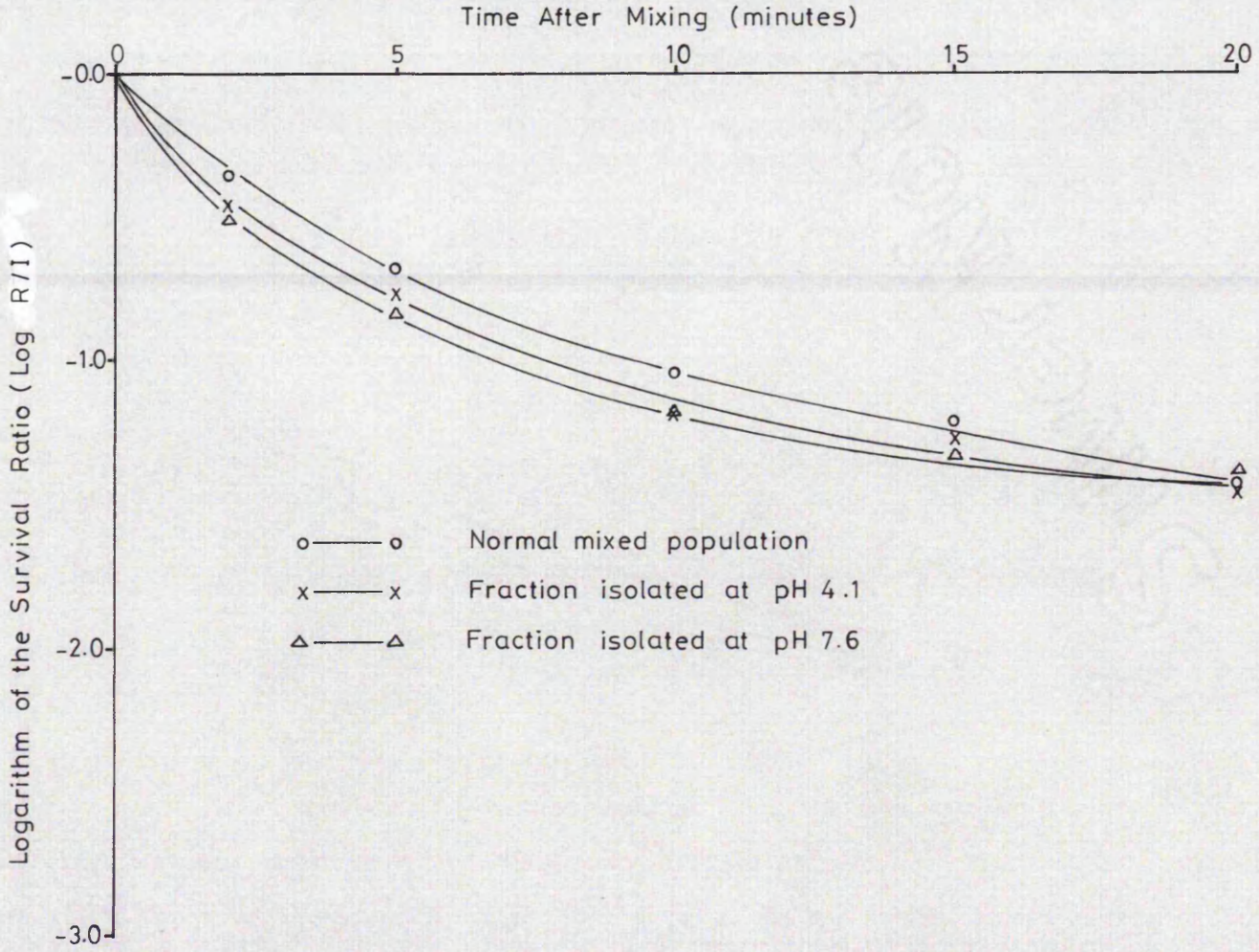
Figure 33. Inactivation of MS-2 During 30 Minutes Contact with Arquad.



Kaplan to explain the two-component curves he obtained for the heat inactivation of Vaccinia virus (81). Data obtained from the electrophoretic studies presented earlier demonstrated the existence of two interconvertible states within a phage population. However electrophoresis of a population treated with Arquad at high pH revealed that the survivors had the same electrophoretic pattern as the parent population and were not stabilized in a resistant state (see figure 30). This implied that fractions of differing sensitivity towards Arquad were not separable by electrophoresis. To verify this fractions isolated from the high and low isoelectric pHs were treated with Arquad as described in section 2.9.1. and the results compared with a 'normal' phage population, as shown in figure 34. As expected the results demonstrated that the rates of inactivation of the two fractions did not differ significantly from that of the normal, 'mixed' population.

Although fractions with differing isoelectric points were interconvertible and therefore showed the same sensitivity towards Arquad, stable resistant fractions could exist which were electrophoretically indistinguishable, as has already been discussed. If these differed in their Arquad sensitivity it was argued that they may also differ in other characteristics. A number of experiments were therefore devised to attempt to demonstrate any such heterogeneity. Assuming different fractions were genetically determined, then plaque cloning would enable them to be isolated. Accordingly a population of MS-2 was grown from a single survivor of 30 minutes contact with 30 mgl^{-1} Arquad using the technique described in section 2.6. As figure 35 shows, no significant difference was found between the original and cloned populations, demonstrating that Arquad

Figure 34. Inactivation of Electrophoretically Separated Fractions of an MS-2 Population in 30mgl^{-1} Arquad.



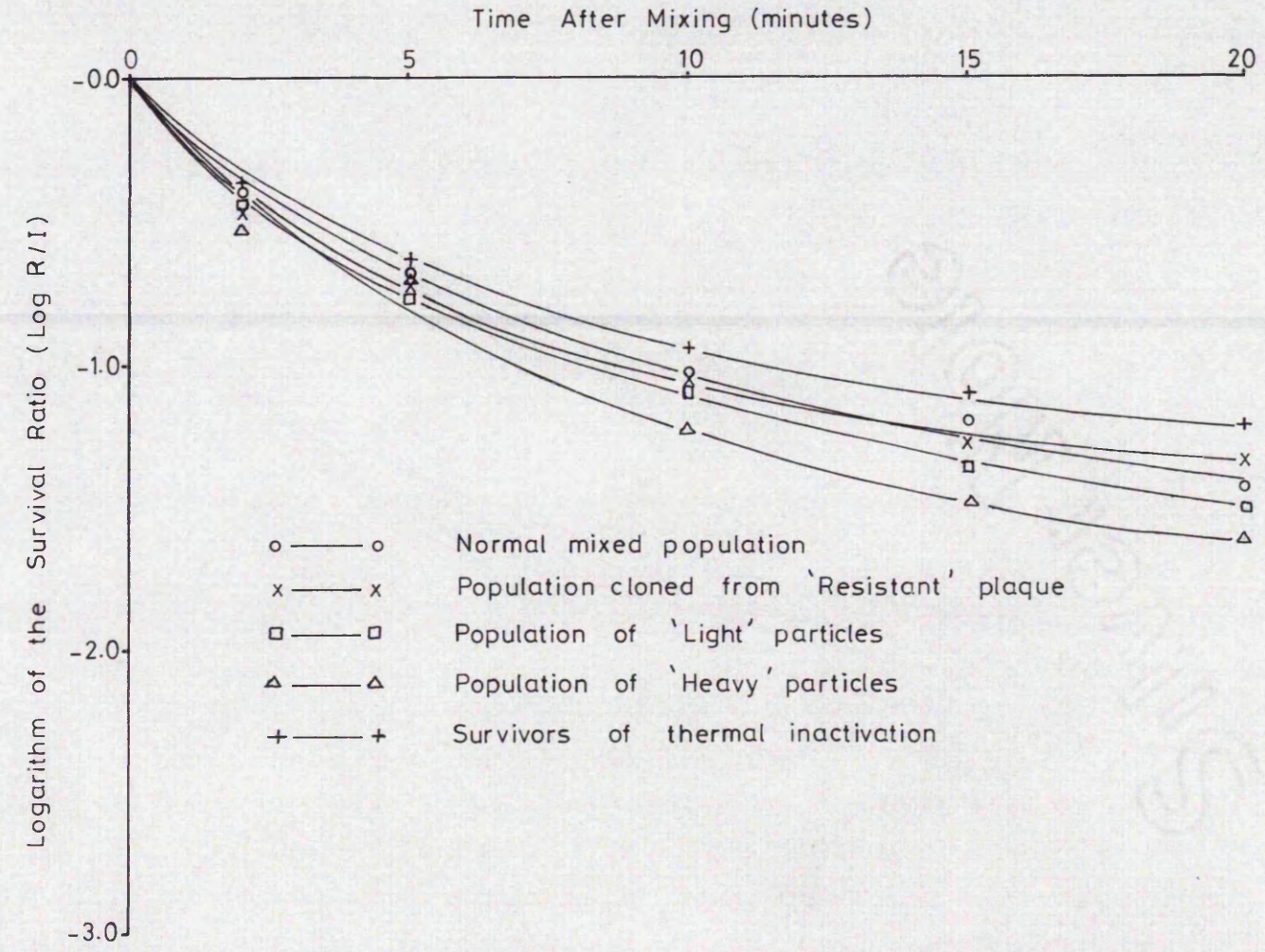
resistance was not a heritable factor.

Although not genetically determined, Arquad resistance could have been a phenotypic factor resulting from variations occurring during assembly within the host bacterium. Such structural variants have been separated in this laboratory by D.B. Jones (80) using density gradient centrifugation. 'Heavy' and 'light' particles, differing in the packing densities of their RNA cores, were separated and found to vary in their resistance to U.V. irradiation. Purified suspensions of these two fractions were kindly provided by D.B. Jones and reacted with Arquad. The results showed both fractions to have identical survival curves to those of a mixed population (figure 35). Assuming both types of particle contained the same amount of RNA, their volumes would have differed. This would have necessitated either a variation in the number of capsomeres per capsid or an alteration in the binding between the sub-units. Whichever alternative was correct however, the structural alterations did not affect the sensitivity towards Arquad.

Alternatively assuming the particles contained different quantities of RNA within capsids of the same structure, the results showed that Arquad toxicity was not due to interaction with the nucleic acid core. If correct this confirmed the earlier assumption that the inactivation mechanism differed from that proposed by Stols and Veldstra to explain the toxicity of quaternary ammonium salts to Turnip Yellow Mosaic Virus (129).

During their research into the heat inactivation of Lambda and ϕ80 phages, Yamagishi and his co-workers (148, 149) produced two component inactivation curves similar to those of Kaplan for Vaccinia virus (81) and Patch (108) for T5 bacteriophage. However, they were

Figure 35. Inactivation of Various Populations of MS-2 in 30mgl^{-1} Arquad.



also able to demonstrate that the lethal event in thermal inactivation was the release of DNA from the phage capsid. Heat resistance was therefore attributed to a greater resistance to head rupture. If resistance to heat and Arquad were determined by the same physiological or morphological properties, it was postulated that the survivors of thermal inactivation might show greater resistance towards Arquad. To test this hypothesis, a phage suspension was heated to 65°C for 75 minutes by which time the titre had been reduced from approximately 10^8 to 10^4 pfu ml^{-1}. Reacting the surviving fraction with 30 mgl^{-1} Arquad as before yielded the inactivation curve shown in figure 35. Although these heated-treated survivors appeared to be slightly more resistant to Arquad, nevertheless they showed a decreasing rate of inactivation with time as did the untreated population.

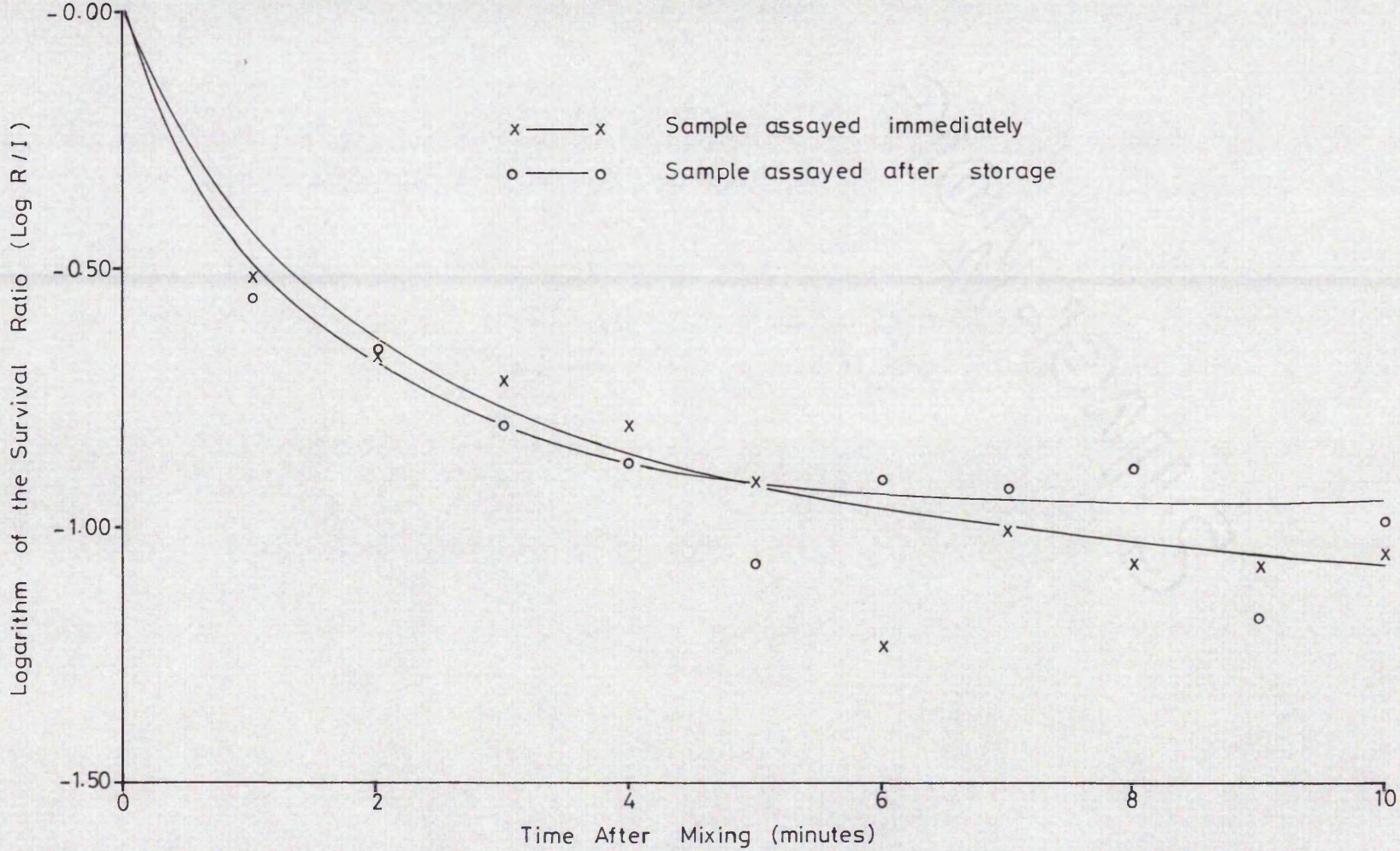
These three, unrelated techniques for isolating detergent-resistant MS-2 (i.e. cloning, centrifugation and thermal inactivation) had thus proved unsuccessful. It was therefore considered probable that no stable, resistant fractions existed and that the population was homogenous with respect to Arquad sensitivity. Thus the decreasing rate of inactivation was not due to phage heterogeneity but probably reflected a complex, time-dependent interaction between the phage capsid and the detergent.

In his review of the literature, Gard cited several researchers who found a decreasing reaction rate with chemical agents such as formaldehyde, phenol and anibine (45). However several of them reported that, following formaldehyde inactivation considerable reactivation of both bacteriophages and viruses could be achieved by incubation with formaldehyde acceptors such as bisulphite, serum, amino acids, etc. A possible explanation for these two observations

was that rapid, initial adsorption of formaldehyde to the capsid caused a reversible loss of infectivity. This was followed by a slower, irreversible inactivation phase. Addition of formaldehyde acceptors caused desorption of formaldehyde from the capsid surface and consequent reactivation. If correct, it was postulated that the initial rapid loss of titre in Arquad might also have been due to a reversible loss of infectivity caused by adsorption of surfactant to the capsid. Removal of adsorbed Arquad might therefore reactivate the phage. As already shown (figure 31) 1% w/v peptone rapidly stopped inactivation due to Arquad by complexing with the surfactant. This was therefore used as a desorbing agent for attempted reactivation of the phage which was reacted with 30 mgl^{-1} Arquad and assayed at various times. Duplicate samples were placed in 1% w/v peptone at $4^{o}C$ and stored for 24 hours before re-assaying. As figure 36 shows, no significant reactivation occurred after incubation in peptone. It was therefore unlikely that the initial rapid loss of titre was due to prevention of host adsorption by the Arquad but resulted from irreversible inactivation.

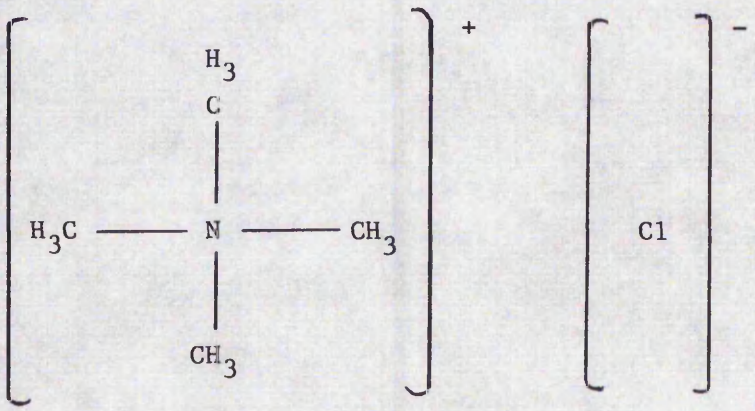
The toxicity of quaternary ammonium compounds towards bacteria (9, 106, 119), their spores (114), yeast cells (5, 44, 113) and viruses (4, 26, 67, 85, 129, 133) is well recorded and has generally been attributed to the cationic quaternary ammonium group. However if this were solely responsible it was considered probable that inactivation would show greater similarities with that produced by other ionic compounds such as halogens, where the mechanism has been shown to follow first order reaction kinetics (120). Studies reported earlier demonstrated that at pH levels at which ionic adsorption of surfactant to the capsid was unlikely to occur, some

Figure 36. Effect of Storage in 1% Peptone on the Infective Titre of Phage Samples Taken During Arquad Inactivation.



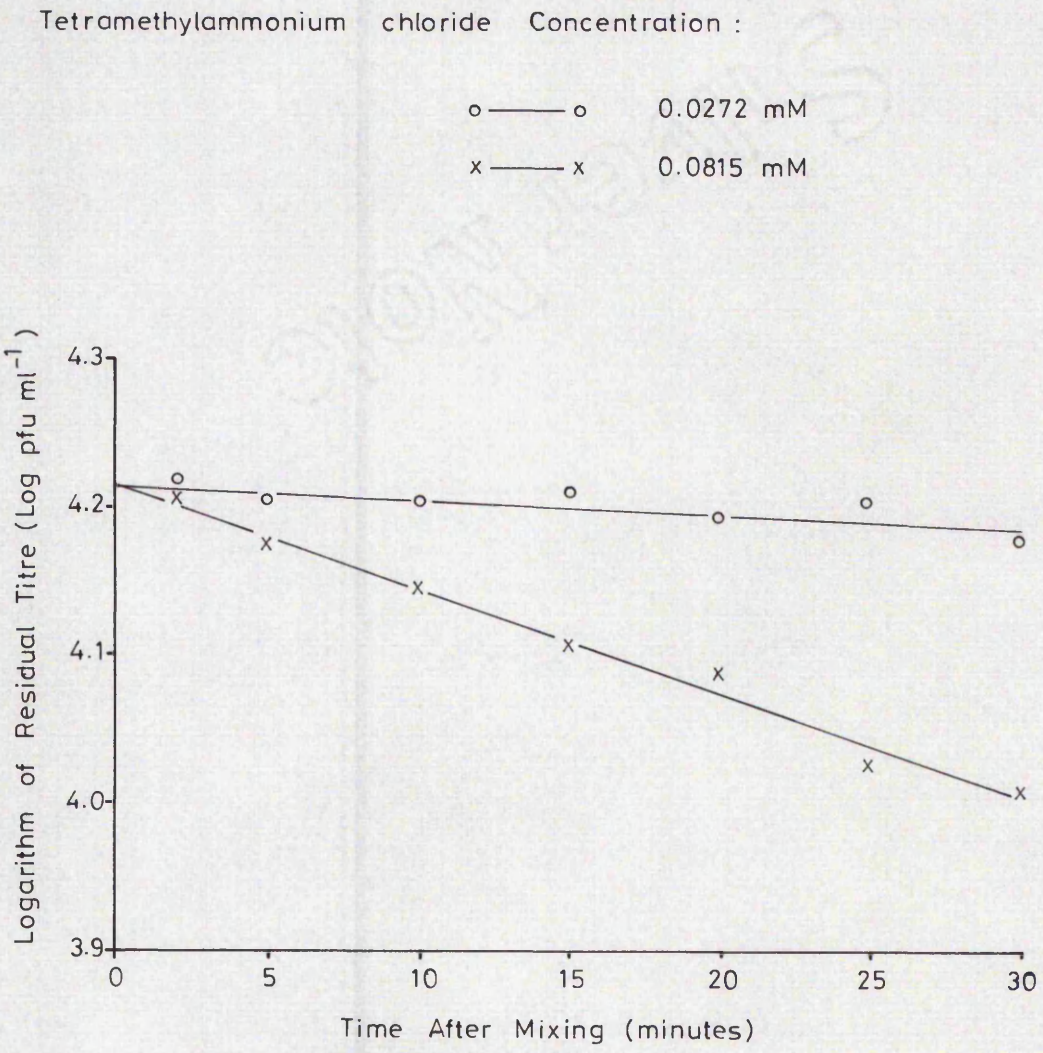
toxicity was evident and this suggested the involvement of the hydrocarbon portion of the detergent molecule. This supported the findings of Stols and Veldstra who demonstrated that varying the length of the hydrocarbon chain attached to a quaternary ammonium salt affected its toxicity to Turnip Yellow Mosaic Virus (129).

The suggested involvement of the hydrocarbon chain in the toxicity of Arquad was confirmed by the use of tetramethylammonium chloride. This compound has the long chain 'R' group of Arquad replaced by a single methyl group thus:



This proved to be considerably less toxic than the equivalent concentration of Arquad. Figure 37, for example, shows typical inactivation curves obtained for concentrations of tetramethylammonium chloride equivalent to 10 and 30 mgl^{-1} Arquad, i.e. 0.0272 mM and 0.0815 mM respectively. Comparing these with the earlier Arquad inactivation curves (figure 33), it was found that 10 mgl^{-1} Arquad caused 87% inactivation after 30 minutes compared with 6.7% for the equivalent concentration of tetramethylammonium chloride and the respective values obtained at 30 mgl^{-1} concentration were 98.6% and 38.3%. Evidently addition of the hydrocarbon chain to the quaternary ammonium ion considerably enhanced the overall toxicity of the compound.

Figure 37. Inactivation of MS-2 in Tetramethylammonium Chloride.



The percentage inactivation occurring after 5 minutes in 0.0815 mM (≡ 30 mgl^{-1} Arquad) tetramethylammonium chloride was 24.8% compared with 90.6% occurring in 30 mgl^{-1} Arquad (obtained from figure 31). Under the same conditions of temperature, Arquad concentration and incubation time, 13% inactivation was attributed solely to the hydrocarbon chains. The greater toxicity of the Arquad molecule compared with that of its individual component groups clearly indicated a synergistic relationship in the overall inactivation mechanism. This probably resulted from the surface activity of the Arquad molecule which increased the concentration of the cationic group at the capsid surface. This would therefore be expected to increase the rate of reaction without affecting the mechanism. However figure 37 shows that tetramethylammonium chloride toxicity followed first order reaction kinetics during 30 minutes contact. Thus the enhanced inactivation resulting from the addition of the hydrocarbon chain, as in Arquad, was not solely due to the increased surface concentration of the polar group resulting from the confered surface activity.

Before postulating an inactivation mechanism, an attempt was made to visualize the lethal event resulting from exposure to surfactants.

Accordingly a suspension of phage particles at a titre of 5.3×10^{10} pfu ml^{-1} was exposed to Arquad at a concentration of 100 mgl^{-1} for one hour. This resulted in a reduction in the infectivity to 4.9×10^{2} pfu ml^{-1} and the suspension was then examined by electron microscopy. Once again the field density of particles was too low for a quantitative assessment but detergent-treated particles appeared identical to those in the untreated control. This suggested

that the lethal event was release of RNA from the phage capsid by a similar mechanism to that proposed earlier for surface inactivation.

At this stage a summary of the results and implications of the experiments on Arquad toxicity so far reported may prove useful:

1. Survival curves appeared linear for the first 5 minutes when plotted on a semilogarithmic scale (figure 31), indicating a first order reaction. However, over 30 minutes these proved to be continuous, smooth curves showing a decreasing rate of inactivation and the proportion of the population inactivated after a given time was directly proportional to the concentration of Arquad.

2. Resistance to Arquad toxicity was not a genetically determined factor (figure 35).

3. Phage particles separated on the basis of their density and heat resistance (figure 35) and isoelectric point (figure 34), did not differ from a normal population in their reaction to Arquad. Thus no permanent heterogeneity in Arquad resistance was demonstrated in the population, although a reversible, pH-dependent interconversion between two forms differing in sensitivity was indicated.

4. Attempts to reactivate the phage following inactivation were unsuccessful, showing that loss of infectivity was irreversible. This was contrary to the effect of agents such as formaldehyde on Poliovirus (45).

5. The initial rate of inactivation was inversely proportional to the hydrogen ion concentration and theoretically reduced to zero at pH 2.65 (figure 28). This showed that interaction between the quaternary ammonium ion and the phage capsid was involved in the inactivation mechanism. Experiments using tetramethlyammonium chloride confirmed this and showed that the interaction followed

first order reaction kinetics (figure 37).

6. The similarity between the residual toxicities of Teepol and Arquad at high and low pH values respectively demonstrated the involvement of the hydrophobic group in the inactivation mechanism. This was not solely the result of increasing the concentration of the polar group on the capsid surface.

As a consequence of these findings it is proposed that the shape of the inactivation curves was dictated by the complex, surface interactions between the phage particles and surfactant molecules and not by an inherent heterogeneity in detergent resistance within the population. Assuming RNA release to be the lethal event, it was considered probable that this was triggered by reaction of the surfactant polar group with the site of adsorption to the host bacterium, i.e. the A-protein. The toxicity of the hydrocarbon chain and its influence on polar group interactions was a more complex phenomenon interpretation of which required an understanding of surface chemistry.

In the field of mineral ore flotation, considerable research has been undertaken into the nature of the adsorption of various substances to mineral surfaces (e.g. 84). In general, such adsorption will affect, or be affected by, the electrical composition of the surface, which is determined by the electrochemical and electrokinetic (zeta) potentials. Figure 38 shows a schematic diagram of the composition of the electrical double layer associated with the surface of lead sulphide in deionized water.

Line AB represents the boundary of the undisturbed crystal lattice. The row of ions, 3, represents the layer in the lattice from which the Pb^{++} cations have been removed and line CD represents the surface

Figure 38. Structure of the Electrical Double Layer on the Surface of Galena in Water.

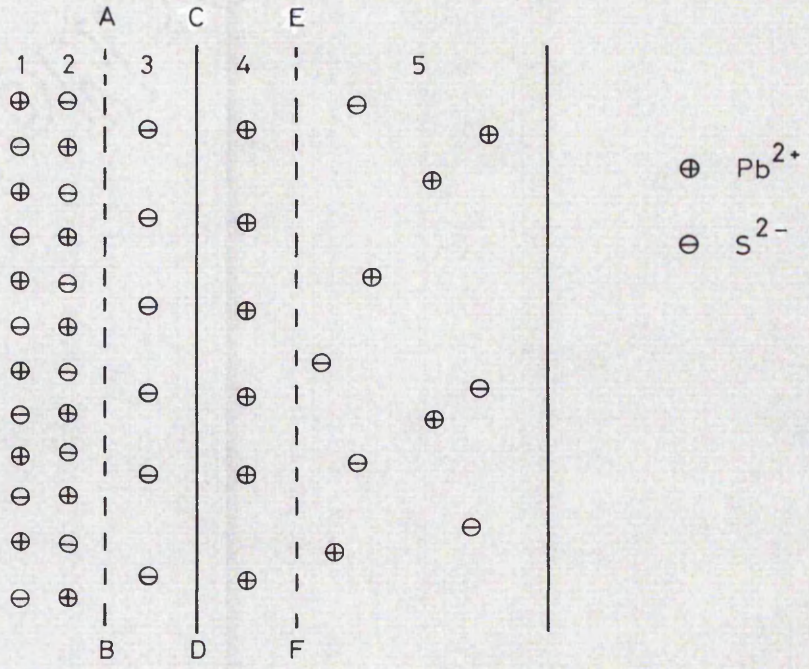
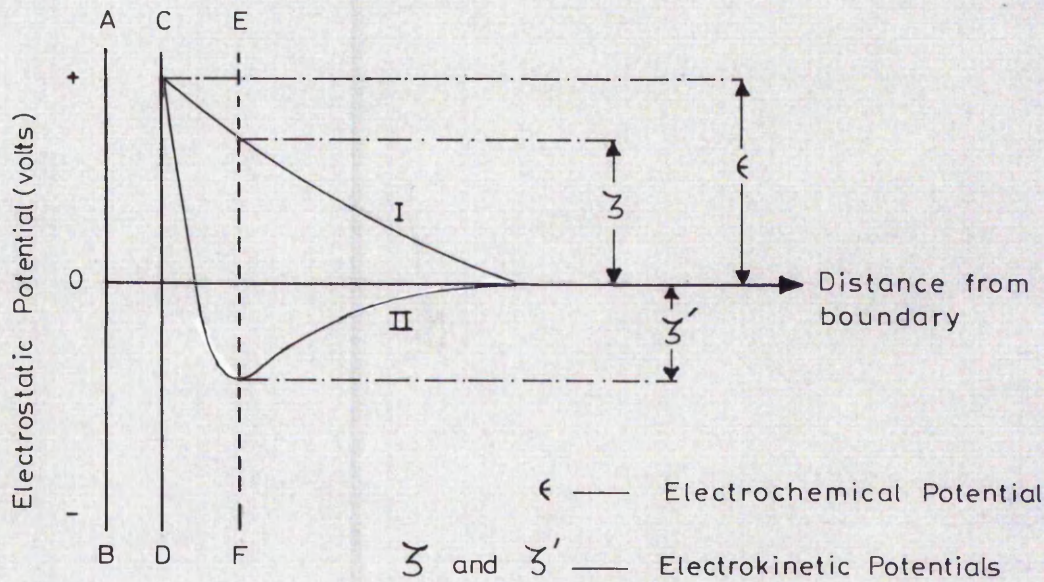


Figure 39. Variation of Electrical Potential in the Helmholtz Layer.



of the crystal. Row 4 represents those ions in the liquid phase which are bound to the surface and move together with the mineral particle. Zone 5 is the diffused layer of mobile ions. Zones 4 and 5 are sometimes collectively known as the Helmholtz layer. The difference in potential between the boundary of the fixed and diffuse layers (EF) and the bulk solution is the zeta potential. The electrochemical potential is the difference in potential between the solid-liquid interface (CD) and the bulk solution. The two potentials may be of the same or opposite sign as shown in figure 39, curves I and II respectively.

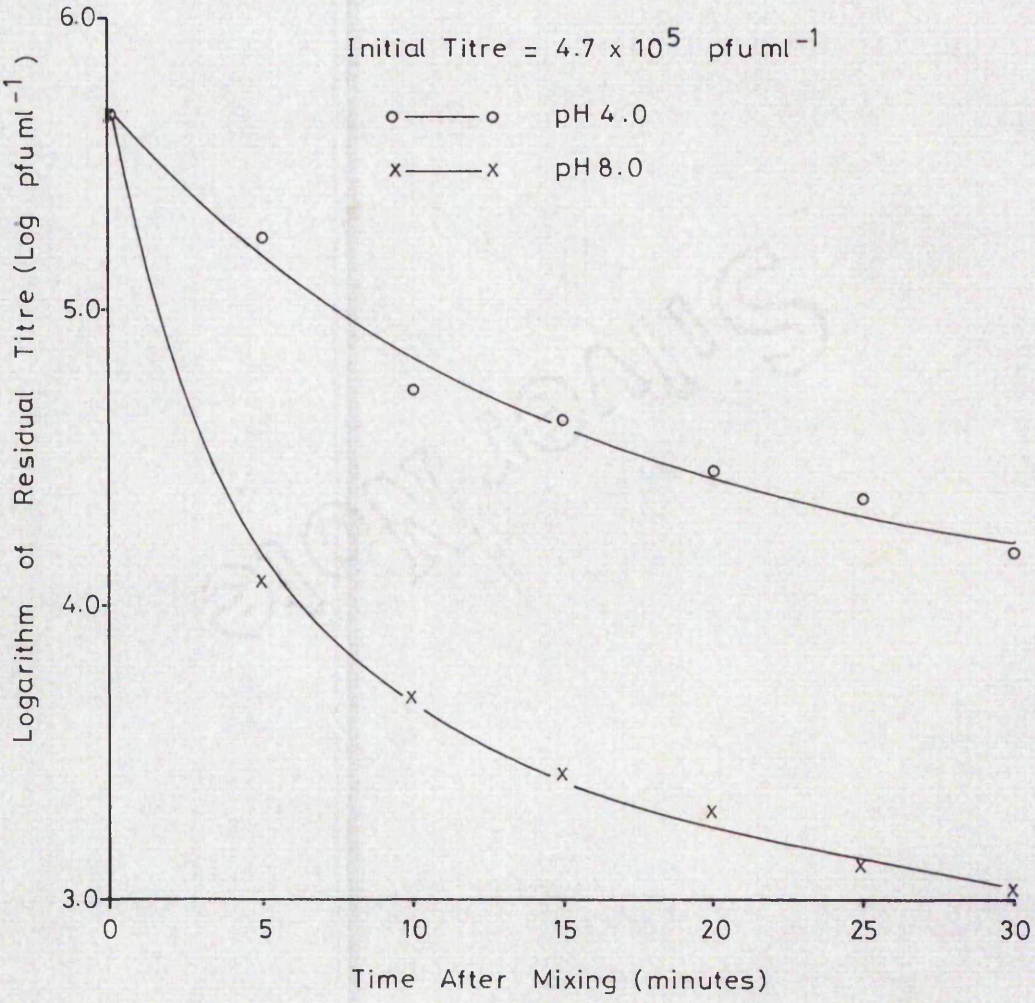
Ions of higher valency (Al^{3+}, Fe^{3+}, Th^{4+}, etc.), complex organic cations and surface active agents have a dramatic effect on the zeta potential and may even reverse its sign at very low solution concentrations. It is thought that surface active molecules intrude between the mineral surface and its associated double layer, forming an organic 'film' between the condensed and diffused layers which greatly affects ionic diffusion across this region.

Although the proteinaceous phage capsid has a very different surface structure from that of an inorganic crystalline salt, the migration of phage particles in an electrical field as demonstrated by the electrophoretic experiments, revealed that the capsid possessed ionically charged region(s). These would presumably have a Helmholtz layer associated with them and the resultant zeta potential could be seriously affected by adsorption of Arquad molecules to these region(s). Furthermore, adsorption of hydrocarbon chains would 'mask' the anionic groups of the capsid surface reducing their attraction for the quaternary ammonium groups of the surfactant molecules. This would reduce polar group adsorption and thus could explain the

reduction in the rate of phage inactivation with time, shown in figure 33. This hypothesis was consistent with the evidence that the proportion of the phage population inactivated during initial contact, increased as the Arquad concentration was increased. This would not be expected to occur if phage heterogeneity was responsible for the decreasing inactivation rate since the proportion of 'Arquad resistants' in a population would not be expected to vary with the Arquad concentration.

It is interesting to note that following the initial inactivation phase, the rates of inactivation in all concentrations of Arquad tended towards the same value. Thus it was assumed that the surfactant concentration at the surface of the capsids reached a saturation level at a solution concentration below 5 mgl^{-1}. This adsorbed surfactant caused a percentage loss in titre of $14.6 \pm 3.5\%$ (calculated from figure 33) during the period from 25 minutes to 30 minutes after mixing at all concentrations of Arquad tested. This closely corresponded to the earlier value of 15.0% inactivation after 5 minutes contact with Arquad at low pH which was attributed to the non-ionic adsorption of the hydrophobic part of the Arquad molecule. This therefore suggested that following the initial inactivation phase resulting from ionic interaction of the polar groups with the capsid, loss of infectivity was solely due to the effect of the non-polar hydrocarbon groups. If correct the inactivation rate after the initial phase would be independent of pH. This was verified by reacting phage with 30 mgl^{-1} Arquad at pH levels of 4.0 and 8.0 and as figure 40 shows, the inactivation rates did become virtually identical approximately 15 minutes after surfactant addition.

Figure 40. Effect of pH on the Rate of Inactivation During 30 Minutes Contact with Arquad.



To verify the proposed mechanism of inactivation resulting from non-ionic adsorption of the hydrophobic chains, it would be necessary to determine the effect of such adsorption on the electrokinetic potential of the phage capsid. Whilst such determinations are relatively straightforward for mineral particles they are extremely difficult for viruses owing to their small size and the problems of obtaining highly purified viral suspensions of suitable concentration and in sufficient quantity. Furthermore it is only possible to measure the overall zeta potential of a particle surface - localized variations being undetectable. However such variations especially in the region of the A-protein could be of great importance in the mechanism of inactivation. It is regrettable therefore that it was outside the scope of this work to pursue such investigations further.

Whilst not being able to prove the hypothesis it was proposed that adsorption of the non-polar hydrocarbon chains did cause an alteration in the zeta potential of the capsid surface by condensing the surrounding hydrated layers. This interfered with the adsorption of counterions in the region of the A-protein which resulted in a configurational alteration in the protein structure and subsequent RNA release. Some corroborative evidence for such an hypothesis has been provided by Riemersma (113) who found that cetylpyridinium chloride inactivated yeast cells by inducing a leakage of ions from the cell membrane.

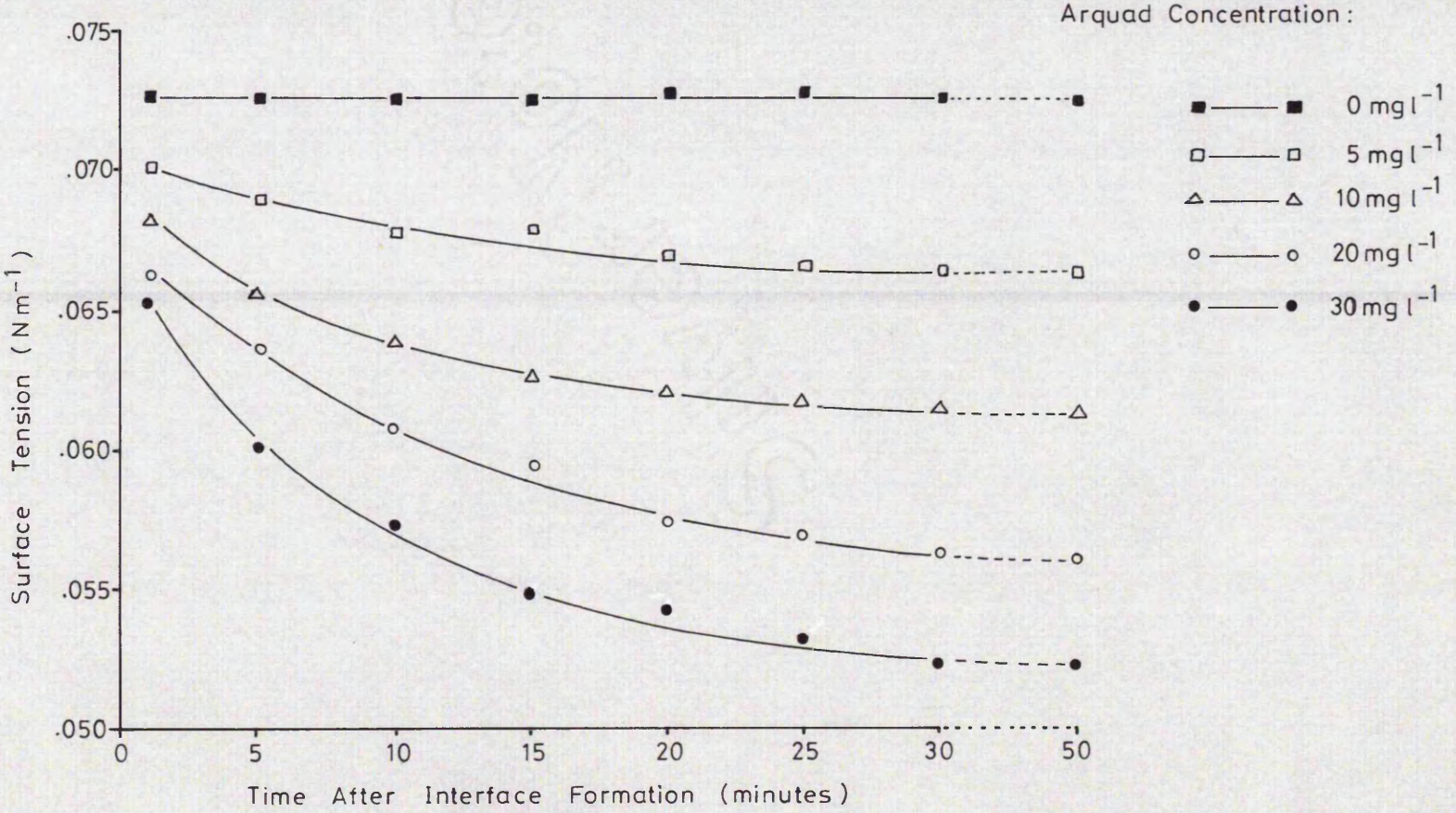
This proposal was also consistent with the suggestion that hydrophobic adsorption interfered with the considerably more toxic adsorption of the polar quaternary ammonium groups. However it was apparent that the time required for hydrophobic group adsorption to

completely overcome polar group toxicity was at least 15 minutes. This adsorption was analogous to the concentration of surfactant at a newly formed, air-liquid interface which could be monitored by measuring the decrease in interfacial surface tension with time. Accordingly the surface tensions of surfactant solutions at various concentrations were measured at intervals after pouring using a Du Nuöy tensiometer as described in section 2.14. As figure 41 shows, the adsorption of surfactant to the surface continued over several minutes until an equilibrium concentration was attained. Comparison of these curves with the inactivation curves (figure 33) showed that as the amount of adsorbed surfactant increased, so the rate of phage inactivation decreased, providing further evidence for the interference mechanism proposed earlier.

However it was recognized that the reduced rate of phage inactivation could have resulted from a lowering of the surfactant concentration in solution due to its adsorption to the interface and possibly to the glass walls of the flask. This alternative was rejected however on the evidence of standard solutions of Arquad prepared for surfactant determinations which showed no measurable reduction in concentration even after prolonged storage. Thus the amount of adsorbed surfactant was insignificant compared with the amount contained in solution.

During his toxicity studies, Riemersma noted that at a fixed concentration of surfactant, ion leakage from yeast cells was considerably increased by raising the solution temperature (113). From theoretical considerations, the Gibbs equation predicts that an increase in temperature will affect either the surface tension of an interface or the concentration of surfactant adsorbed to that

Figure 41. Decrease in the Surface Tension of Surfactant Solutions with Time.



interface:

$$\Gamma = - {}^{C}/_{RT} \cdot \frac{d\gamma}{dc}$$

Using solutions of 125 mgl^{-1} EHDA-Br, Grieves and Bhattacharyya (57) found a random variation in surface tension between 0.045 and 0.047 Nm^{-1} at temperatures between 8^{o} and 51^{o}. Thus according to the Gibbs equation, the amount of adsorbed surfactant was inversely proportional to the temperature. However Eigles, reported by Klassen and Mokrousov (84), found the amount of oleate adsorbed to the surface of fluorite particles in a flotation pulp increased from 193gtn^{-1} to 240gtn^{-1} when the temperature was increased from $16^{o}C$ to $50^{o}C$. Clearly the effect of temperature on the interfacial adsorption of surfactant was influenced by factors not included in the Gibbs equation such as surfactant solubility, the viscosity of the solution, etc.

Before assessing the effect of temperature on Arquad toxicity, its effect on the surface tension of an Arquad solution was determined. It was not possible to accurately control the temperature above ambient but by standing the sample dish in a beaker containing crushed ice, the surface tension at $0.5^{o}C$ could be determined. After allowing 30 minutes for equilibration, a reading of 0.0515 Nm^{-1} was obtained for a 30 mgl^{-1} solution equilibrated at an ambient temperature of $25^{o}C$. This was not considered to be a significant difference and therefore surface tension did not appear to be a temperature dependent function. Thus theoretically adsorption to the phage capsid and subsequent inactivation would not be expected to increase with temperature. However significantly greater amounts of surfactant than predicted might be adsorbed at higher temperatures for the reasons outlined above. Furthermore the interactions with adsorbed surfactant

which cause inactivation would be expected to have temperature dependent reaction rates, in common with other chemical reactions. Clearly therefore phage inactivation could be expected to be a temperature dependent function.

In order to experimentally verify the effect of temperature on surfactant toxicity, the method described in Section 2.9.1. was employed with each flask maintained at a constant temperature within a range between $1^{o}C$ and $50^{o}C$. Figure 42 shows the inactivation curves obtained over a 30 minute period following surfactant addition. Clearly elevating the temperature greatly enhanced the rate of inactivation presumably by increasing Arquad adsorption and/or the consequences of such adsorption.

Since the inactivation rate throughout the entire contact period was increased, the inactivation mechanisms resulting from both ionic and non-ionic adsorption must have been temperature dependent. This was as expected for the effect of temperature on the reaction kinetics of the proposed mechanisms and confirmed the findings of Riemersma (113).

Assuming the inactivation rates during the first 5 minutes (initial rate) and during the period 25-30 minutes (final rate) after mixing approximated to first order reactions, the inactivation rate constants could be plotted against the reciprocal of the absolute temperature as shown in figure 43. The gradient of each curve was measured and the energy of activation for each 'reaction' was calculated using the Arrhenius equation (72):

$$\log k = -\frac{E}{2.303RT + C}$$

where: k = rate constant $\left(2.303. \frac{\log N/No}{t}\right)$

Figure 42. Variation in Arquad Inactivation of MS-2 with Temperature.

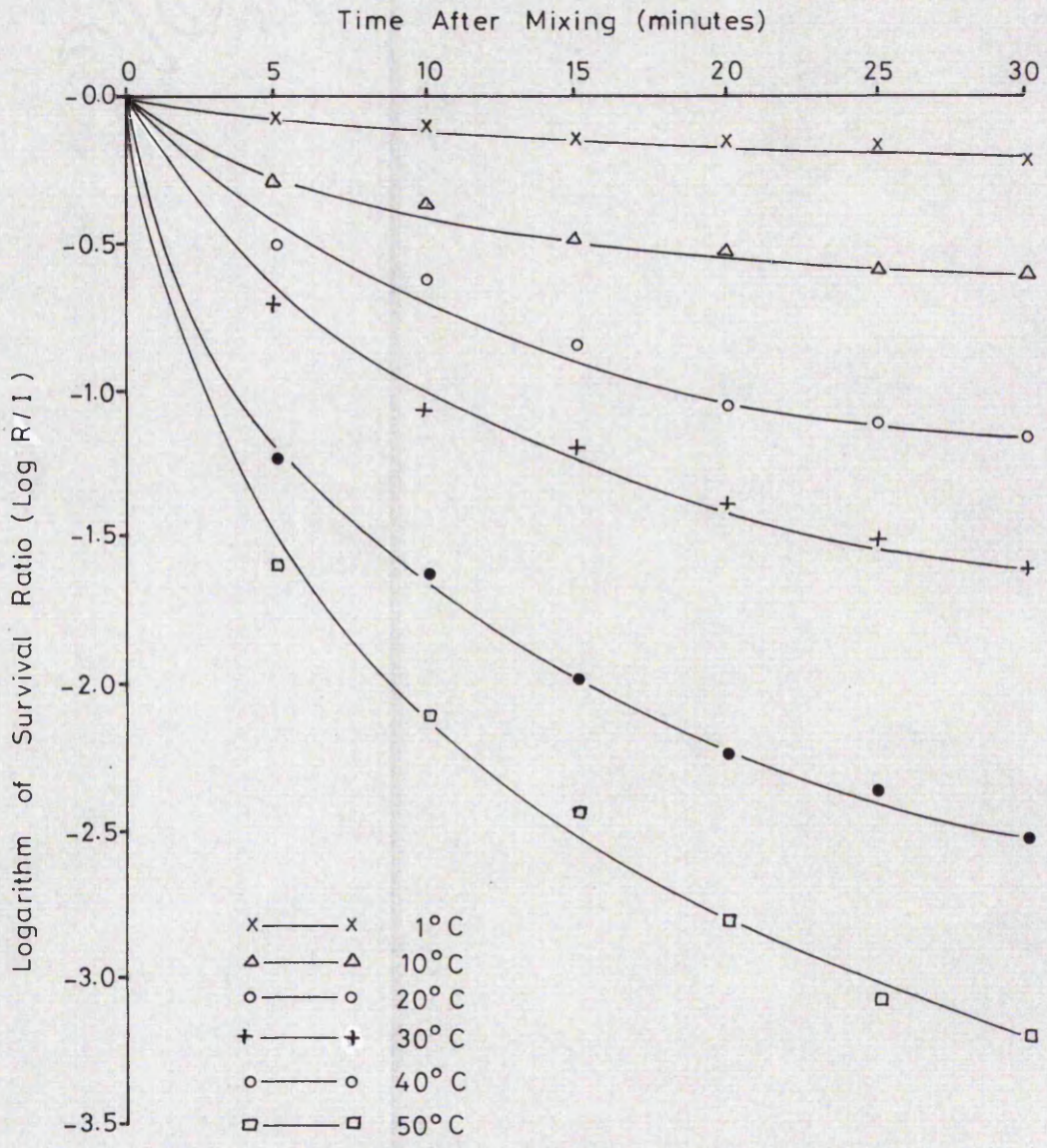
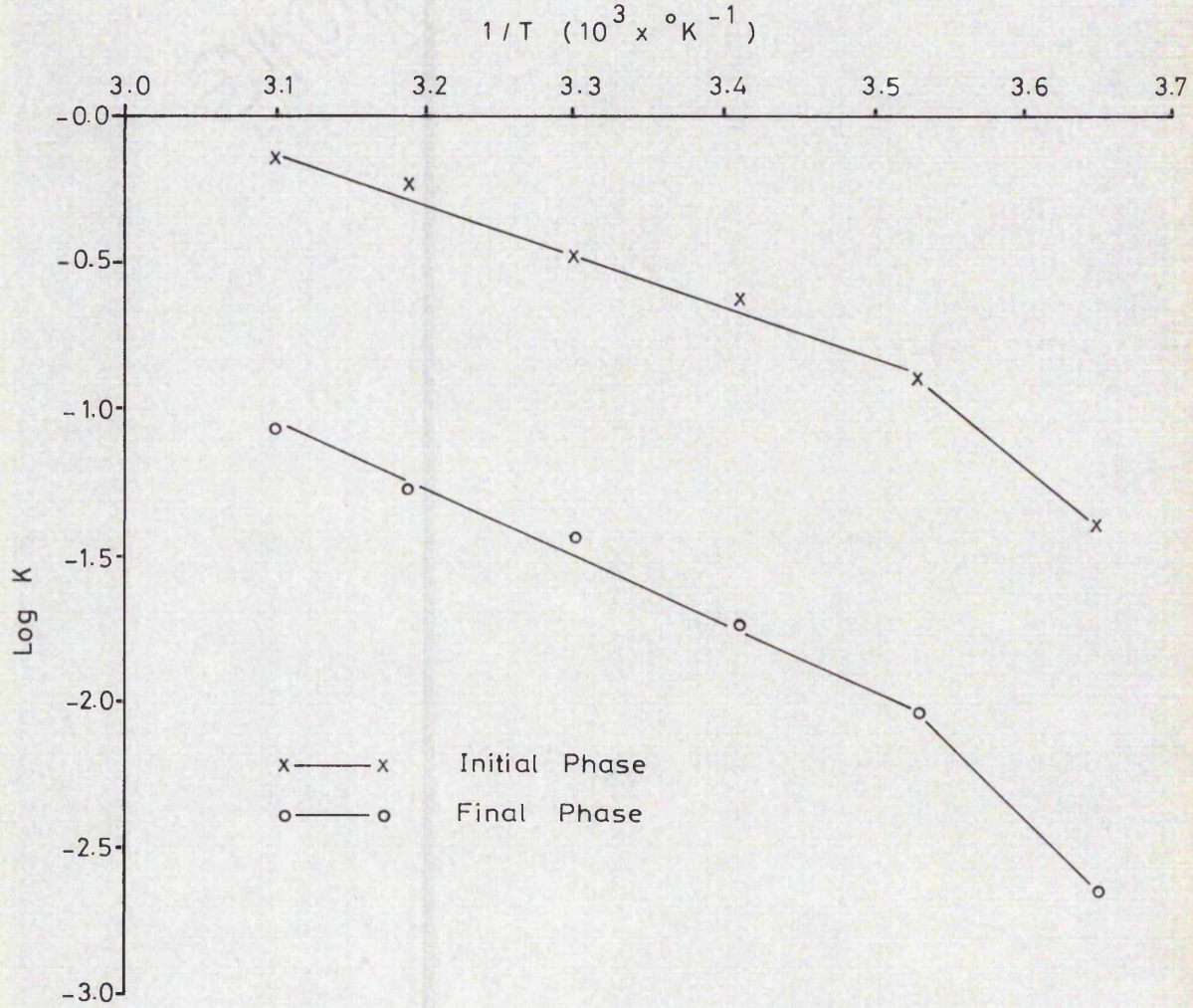


Figure 43. Arrhenius Plots of Inactivation Rate Constants versus Temperature.



R = Gas constant (8.314J $mole^{-1}K^{-1}$)

T = Absolute temperature (^{o}K)

E = Activation Energy ($Jmole^{-1}$)

C = Integration constant

This yielded values of 33.7 $kJmole^{-1}$ and 43.2 $kJmole^{-1}$ for the activation energies of the initial and final inactivation stages respectively. These stages were assumed to represent the toxicity of the polar quaternary ammonium group and of the non-polar hydrocarbon chain respectively. Unfortunately no comparative data is available for the activation energies of other chemical reactions with viruses. However Woese has reviewed the literature on thermal inactivation and quoted activation energies within the range 300-570 $kJmole^{-1}$ for bacteriophages, 71-665 $kJmole^{-1}$ for plant viruses and 75-1422 $kJmole^{-1}$ for animal viruses (146). Since all these values were significantly higher than those obtained for Arquad toxicity, it was assumed that the inactivation mechanisms were unrelated. This was contrary to the views of Woese who suggested that thermal and formaldehyde inactivation of Poliovirus proceeded by similar mechanisms.

The straight line Arrhenius plots were obtained by regression analysis of the rate constants for temperatures between $10^{o}C$ and $50^{o}C$ inclusive. It was found that for temperatures between $1^{o}C$ and $10^{o}C$, the activation energies of the initial and final inactivation mechanisms increased to 83 $kJmole^{-1}$ and 100 $kJmole^{-1}$ respectively. Evidently at low temperatures secondary factors influenced the inactivation mechanisms, indicating that they were not true first order reactions.

3.5. Ionic Regulation of Phage Inactivation and Flotation

The studies described in the previous section demonstrated that the toxicity of a surfactant was dependent upon its adsorption to the phage capsid, which had many similarities with adsorption to a mineral surface. Thus whilst pH affected the ionization of the phage surface, the influence of the hydrogen ion concentration on the Helmholtz layer and zeta potential was undoubtedly significant. Regulators of pH have been shown to affect the following parameters during mineral ore flotation (84):

1. The electrokinetic potential of mineral surfaces. Generally, a change from acid to alkaline pH decreases this parameter.
2. The degree of hydration of mineral surfaces.
3. The degree of dissociation of collector molecules.
4. H^+ and OH^- ions may adsorb in parallel or in competition with collector molecules and may even displace pre-adsorbed collector.
5. The desorption from the mineral surface of ions which hinder collector adsorption.

Factors 1 and 2 were probably at least partly responsible for the inactivation of MS-2 at pH extremes in distilled water (see for example figure 13). However all 5 interactions may have influenced to some extent the effect of pH on Arquad toxicity.

Apart from pH, other important regulators of mineral flotation are metal cations. These can alter the electrokinetic potential of a mineral surface and will compete with cationic collectors for active sites upon that surface. Cations lower the electrokinetic potential by 'condensing' the diffused region of the electrical double layer. In general, the higher their valency, the greater the electrostatic forces of attraction between them and the surface and the lower the

electrokinetic potential. Ions of similar valency decrease the electrokinetic potential in proportion to their specific volumes. The larger the ion, the greater its polarizability and the lower its degree of hydration; both factors enabling it to approach the surface more closely. For monovalent ions, the order of increasing effect is $Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$ and for divalent ions: $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$.

Assuming these ions similarly affected the proteinaceous phage capsid surface and its interaction with surfactants, then they might be expected to influence phage viability as well as Arquad toxicity. The extent to which these parameters were affected would be determined by the relative positions of the ions in the series given above. In order to verify the first of these hypotheses, the viability of bacteriophage in the presence of various cations was determined. Suspensions of MS-2 were incubated for 1 hour at $30^{\circ}C$ in 100 ml of various salt solutions using similar techniques to those described in Section 2.9.1. All the solutions were made to a concentration of 2.5 mM and the results were expressed as the percentage of the initial population (titre = 1.96×10^6 pfu ml^{-1}) inactivated during incubation (see table 15).

These results showed that none of the cations nor anions tested caused significant inactivation of MS-2. This was not unexpected since many of these ions are present at higher concentrations in growth and dilution media (e.g. nutrient broth, Ringer's solution).

Table 15 Showing Percentage Inactivation of MS-2 after Incubation for 1 Hour at 30°C in 2.5 mM Solutions of Various Salts

CATIONS	ANIONS	
	Cl^-	$SO_4^=$
Na^+	2.7	2.1
K^+	3.2	1.8
Mg^{2+}	3.6	2.9
Ca^{2+}	1.5	2.9
Ba^{2+}	2.8	NA
Cd^{2+}	2.6	NA
Ni^{2+}	4.0	NA
Distilled water	2.2	

NA = Not assessed

The average pH of the solutions was 6.4 and at this value the phage capsid would have had an overall net negative charge, as determined earlier. Thus the metal cations would have adsorbed to the capsid and could have been expected to lower the electrokinetic potential. Clearly, however, such a reduction was insufficient to affect phage viability.

Mineral flotation experience has shown that attachment of hydrophobic particles, such as coal dust, to air bubbles is greatly enhanced by the addition of low concentrations of inorganic salts, with divalent ions having a greater effect than monovalent. This is thought to result from the reduction in stability and thickness of the hydration layers surrounding the mineral particles and air bubbles which thus increases the speed and tenacity of attachment of the particles to the bubbles (84).

To determine whether similar effects occurred with virus particles, suspensions of MS-2 containing added electrolytes were aerated for one hour at 30°C. For this study the ions used were

limited to representative monovalent and divalent cations and anions which were readily soluble at the concentrations employed, i.e. Ca^{++}, Mg^{++}, Na^{+}, K^{+}, $SO_4^{=}$ and Cl^{-}. Regression analysis of the data (figures 44 and 45) showed that all the ions examined significantly increased phage inactivation during aeration. The results also showed that the metal sulphates caused greater inactivation than the metal chlorides and that divalent cations caused a greater reduction in titre than did monovalent cations. Arranging the cations in order of increasing inactivation was found to yield the same sequence as for their effect on the electrokinetic potential of a mineral surface noted earlier, namely: Na<K<Mg<Ca. Thus the greater the decrease in the electrokinetic potential caused by cation adsorption, the greater the phage inactivation. Since this did not occur in unaerated suspensions, inactivation must have involved enhanced surface adsorption as outlined above for mineral particles.

The increased inactivation with sulphate anions was an inexplicable phenomenon. It has been noted that sulphate increases the flotability of naturally hydrophobic minerals (84), although it is not known by what mechanism this is achieved.

It was noted that, at the concentrations used, none of the salts increased the 'foamability' of the phage suspensions. The surface area generated per unit volume of suspension was therefore unaltered and the increased inactivation could not be related to this factor as for the considerably more concentrated buffer solutions reported earlier. The degree of inactivation in the phosphate-citrate buffer at a concentration of 2.5 mM was 18% (figure 18). At this concentration the effect of the buffer upon the surface area to unit volume ratio was insignificant (figure 20) and the degree of inactivation was

Figure 44. Loss of Infective Titre upon Aeration of Phage Suspensions Containing Metal Sulphates.

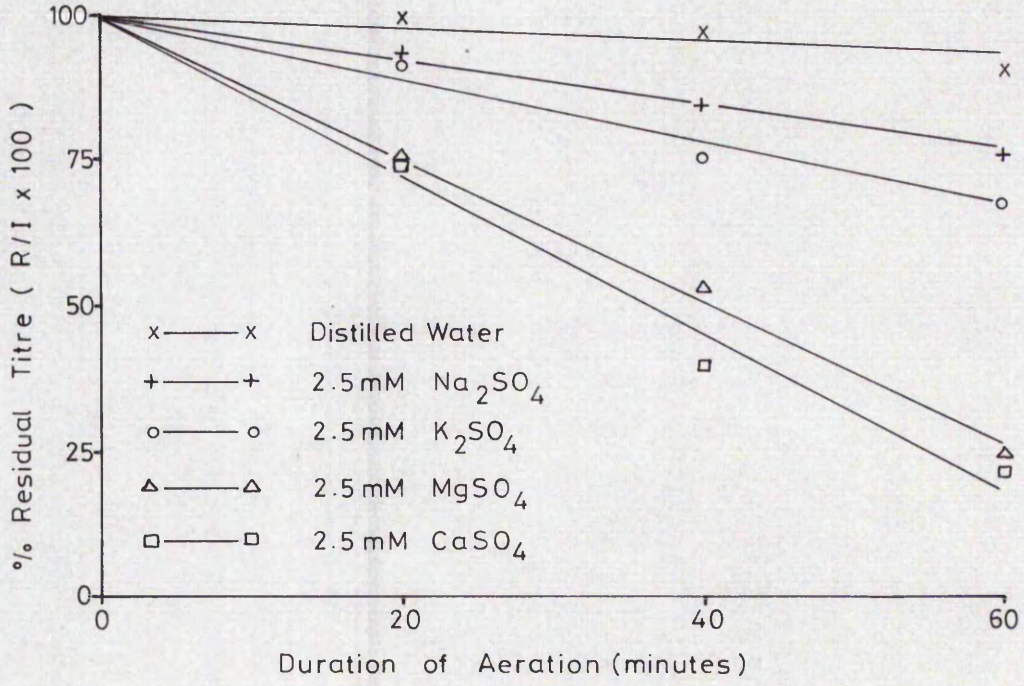
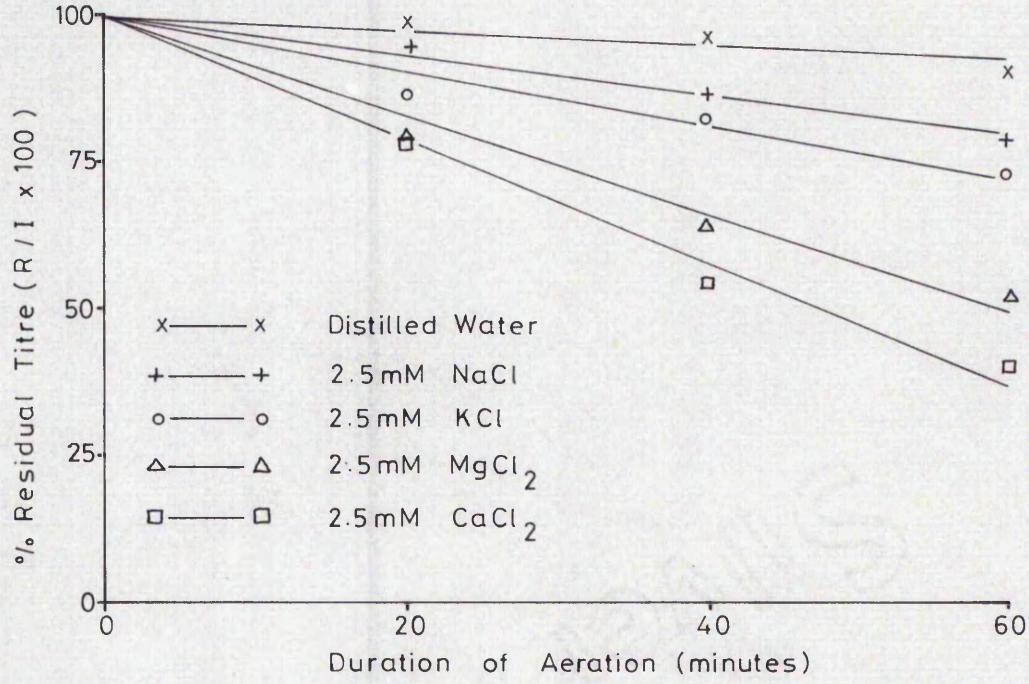


Figure 45. Loss of Infective Titre upon Aeration of Phage Suspensions Containing Metal Chlorides.



therefore comparable with that occurring in the monovalent salt solutions NaCl (20%) and KCl (27%). This relatively small degree of inactivation may have been due to inhibition of phage adsorption to the interfaces. The potassium and phosphate ions would have enhanced phage-bubble attachment, and therefore inactivation, but pre-adsorbed, negatively charged citrate ions would have inhibited such adsorption. This was feasible since the citrate ions were implicated in the reduction in surface tension of the buffer solutions by virtue of their surface activity. Further studies with aerated salt solutions were performed in the continuous flow flotation columns and will be reported later.

Having established the effect of ions on the viability of phage suspensions, the second hypothesis, namely their interference with surfactant toxicity, was investigated. From theoretical considerations it was anticipated that Arquad adsorption, and therefore toxicity, would be reduced and that divalent ions would be more effective than monovalent ions in this role. Figures 46 and 47 show the results obtained after 20 minutes contact, clearly demonstrating ionic interference with Arquad adsorption. Divalent cations reduced toxicity to a greater extent than the monovalent cations; the interference again being proportional to the size of the cation. For ions of the same valency, the greater their size the greater their effect on the electrokinetic potential of the capsid and the more they reduced adsorption of Arquad. It was also noted that sulphates reduced inactivation to a greater extent than the chlorides and this phenomenon was investigated in more detail and will be reported at a later stage.

It was also apparent from the graphs that interference with

Figure 46. Variation in Arquad Toxicity with Metal Chloride Concentration.

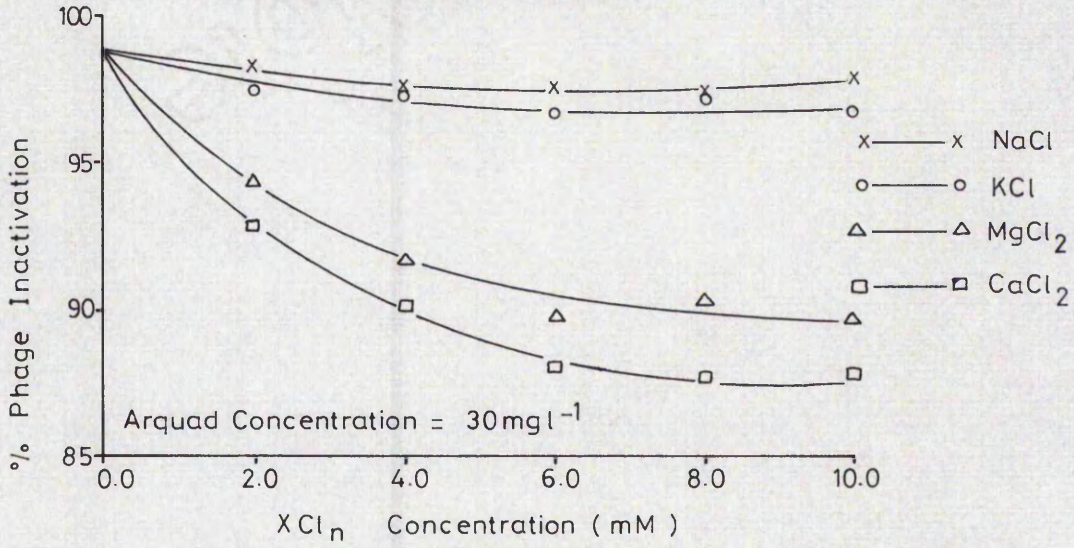
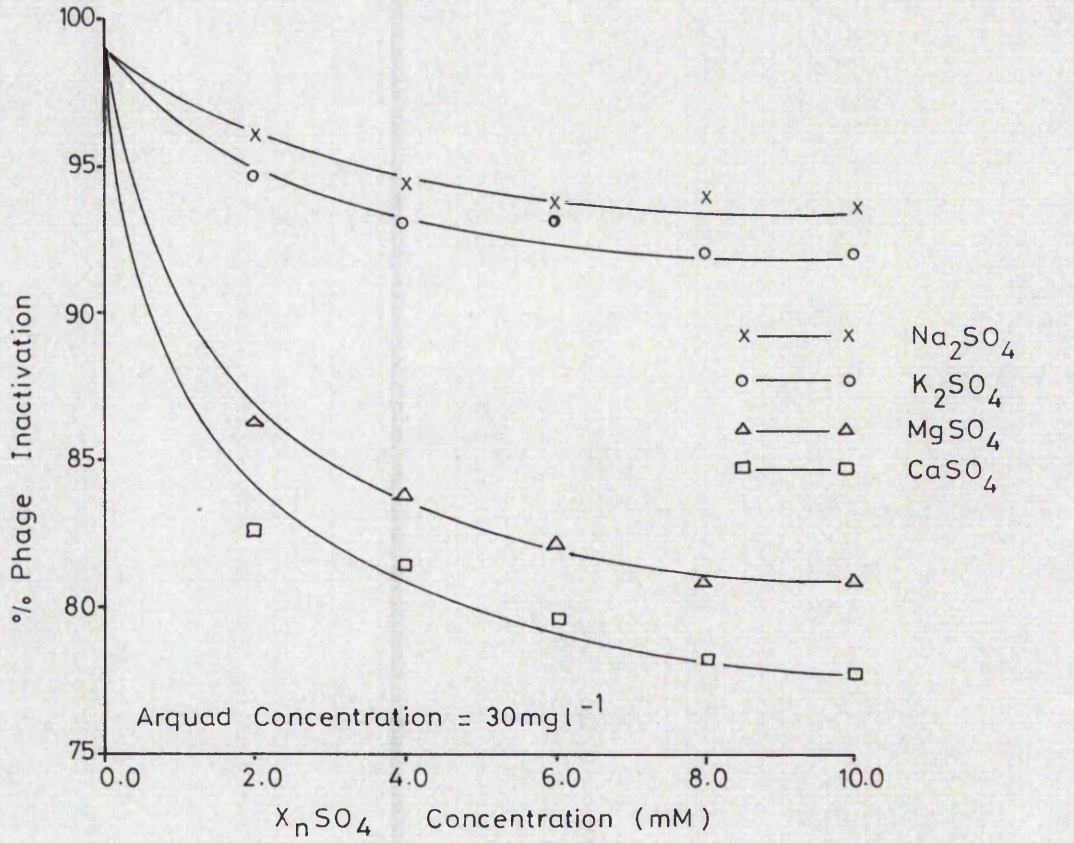


Figure 47. Variation in Arquad Toxicity with Metal Sulphate Concentration.



adsorption reached a maximum value at a concentration of approximately 10 mM for each of the salts examined. This was confirmed using 25 mM solutions of the metal chlorides in which the extent of the inactivation did not differ significantly from that found in the 10 mM solutions.

Preliminary studies were also undertaken using equimolar mixtures of the salts to assess any synergistic or antagonistic interactions between the ions. The results (figure 48) showed no evidence of such interactions and the reduction in toxicity apparently resulted from the combined effect of the activity and concentration of the ions comprising the mixture. Furthermore the maximum interference with toxicity was again reached at a total ionic concentration of 10 mM. Thus whilst the degree of protection afforded was dependent upon the specific ion(s) involved, the ionic strength of the suspension limited the overall protection.

At very low concentrations (less than 2 mM) it was noted that calcium and magnesium sulphates produced comparatively large reductions in phage inactivation. Further experimentation showed that calcium sulphate significantly reduced the rate of inactivation at concentrations as low as 0.025 mM. Figure 49 shows the rates of inactivation at various $CaSO_4$ concentrations compared with a distilled water control. Assuming an average molecular weight for Arquad T50 of 368.3 (as quoted by the manufacturers), the molar concentration of a 30 mgl^{-1} solution was 0.0814 mM. Thus calcium sulphate was capable of reducing Arquad toxicity at concentrations as low as one third that of the Arquad itself. This was particularly significant when one considers that the concentration of Arquad on the phage surface was considerably higher than its solution concentration, owing to its

Figure 48. Arquad Inactivation of MS-2 in Equimolar Mixtures of Various Salts.

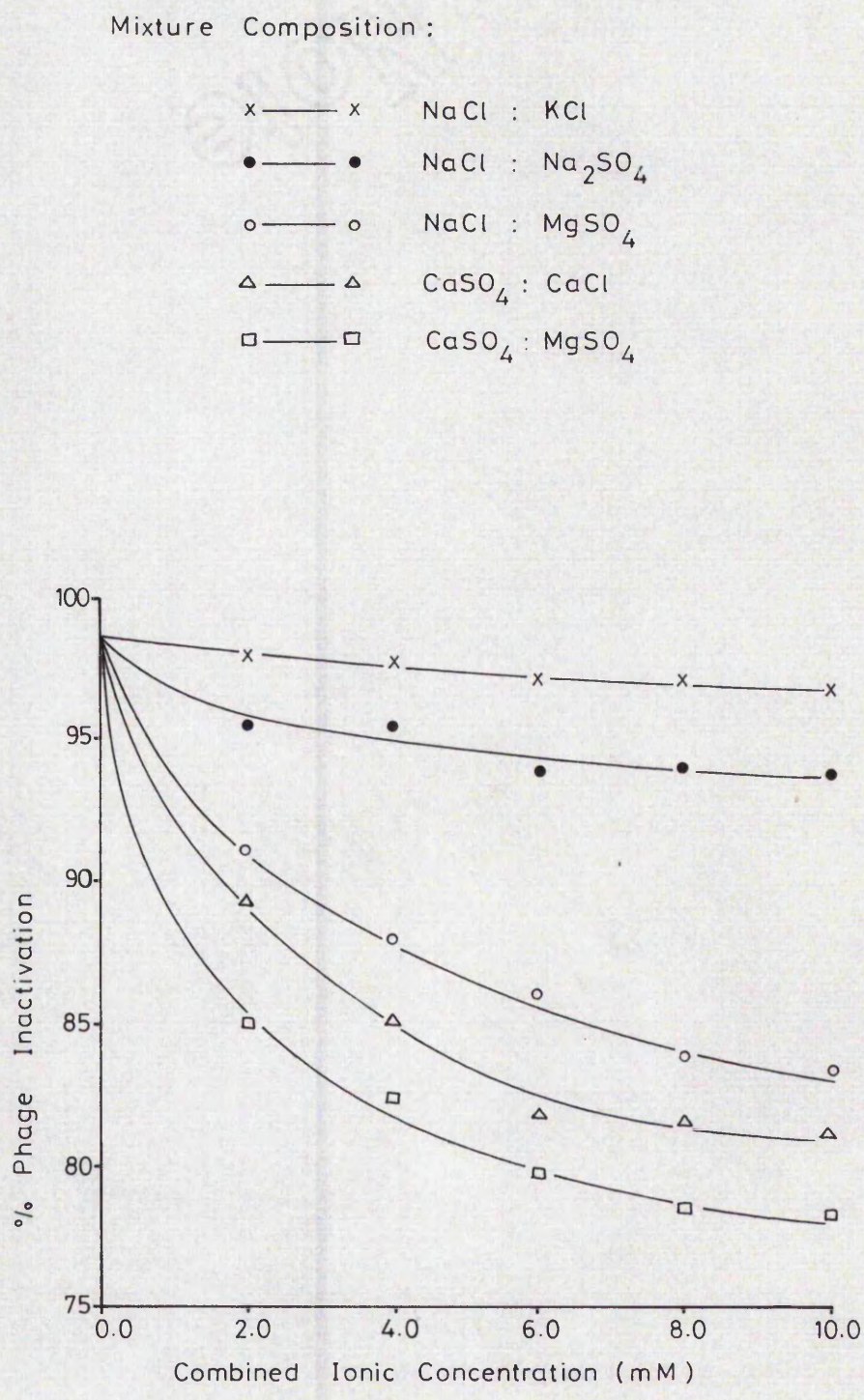
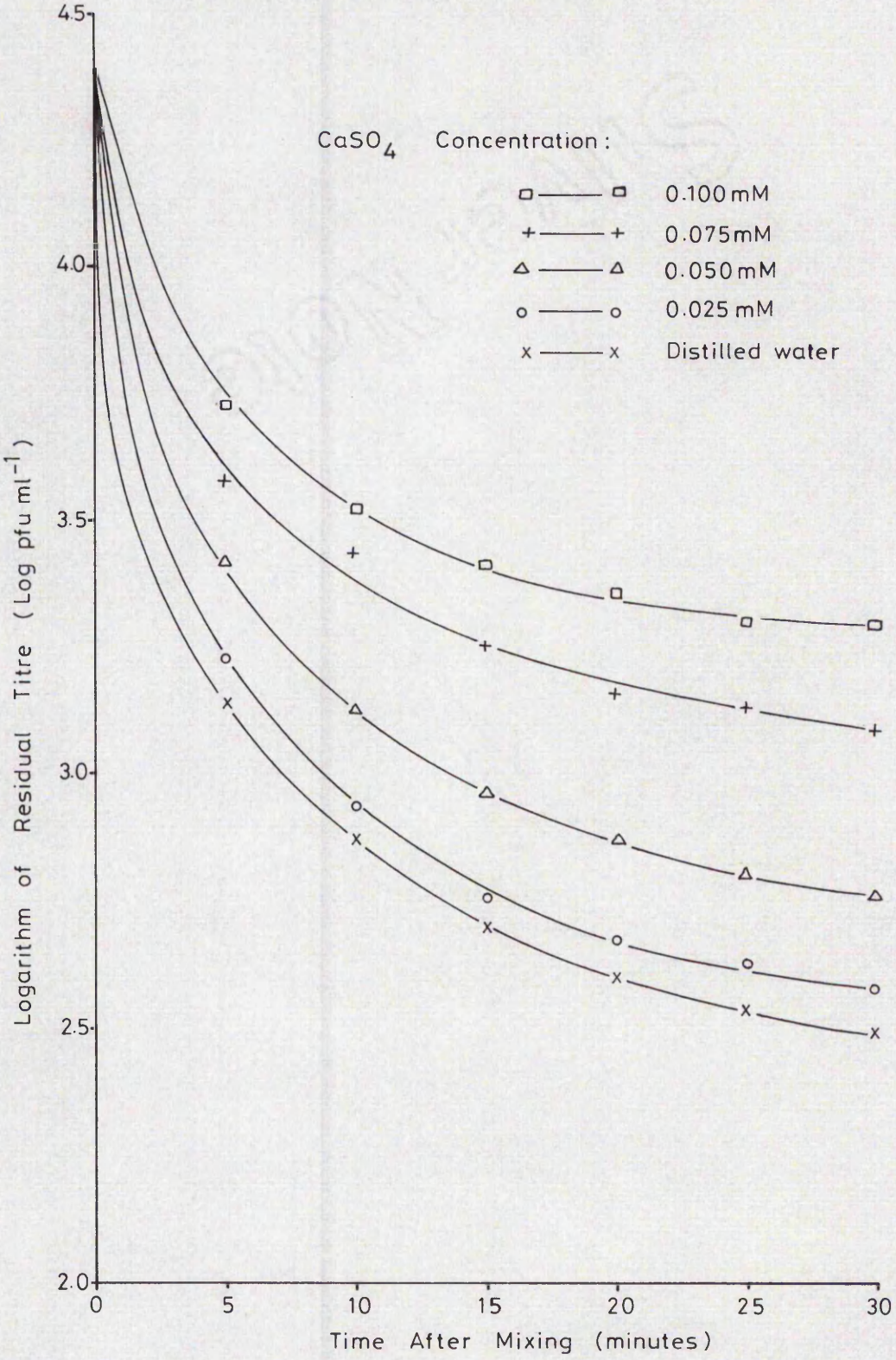


Figure 49. Rate of Inactivation in 30 mgl^{-1} Arquad Containing Calcium Sulphate.



surface activity.

It was also apparent from the graph that calcium sulphate only caused significant interference with the initial inactivation rate. This was to be expected since this phase resulted from ionic adsorption of the polar groups and was therefore susceptible to competitive adsorption by calcium ions.

The remainder of the experimental work to be presented was concerned with the inactivation and/or removal of phage during foam flotation and the influence of the parameters previously investigated on that process. The majority of these studies were undertaken using the continuous flow flotation columns described in Section 2.1.1. In all the subsequent experiments, the influent flow rates were adjusted to give a residence time in the columns of 20 minutes, and the air flow was maintained at 0.5 $lmin^{-1}$ to each column.

Initially a phage suspension in distilled water was delivered to the columns and aerated. The resultant percentage loss of titre was found to be 0.25% in contrast with the value of 1.5% calculated from the batch aeration studies reported earlier (see figures 44 and 45). The apparently increased phage survival was attributed to mixing within the flotation columns which resulted in fractions of the phage population receiving less than 20 minutes aeration.

Using 2.5 mM solutions of various salts, the inactivation in the flotation columns was again found to be less than in the batch aeration columns as shown in table 16.

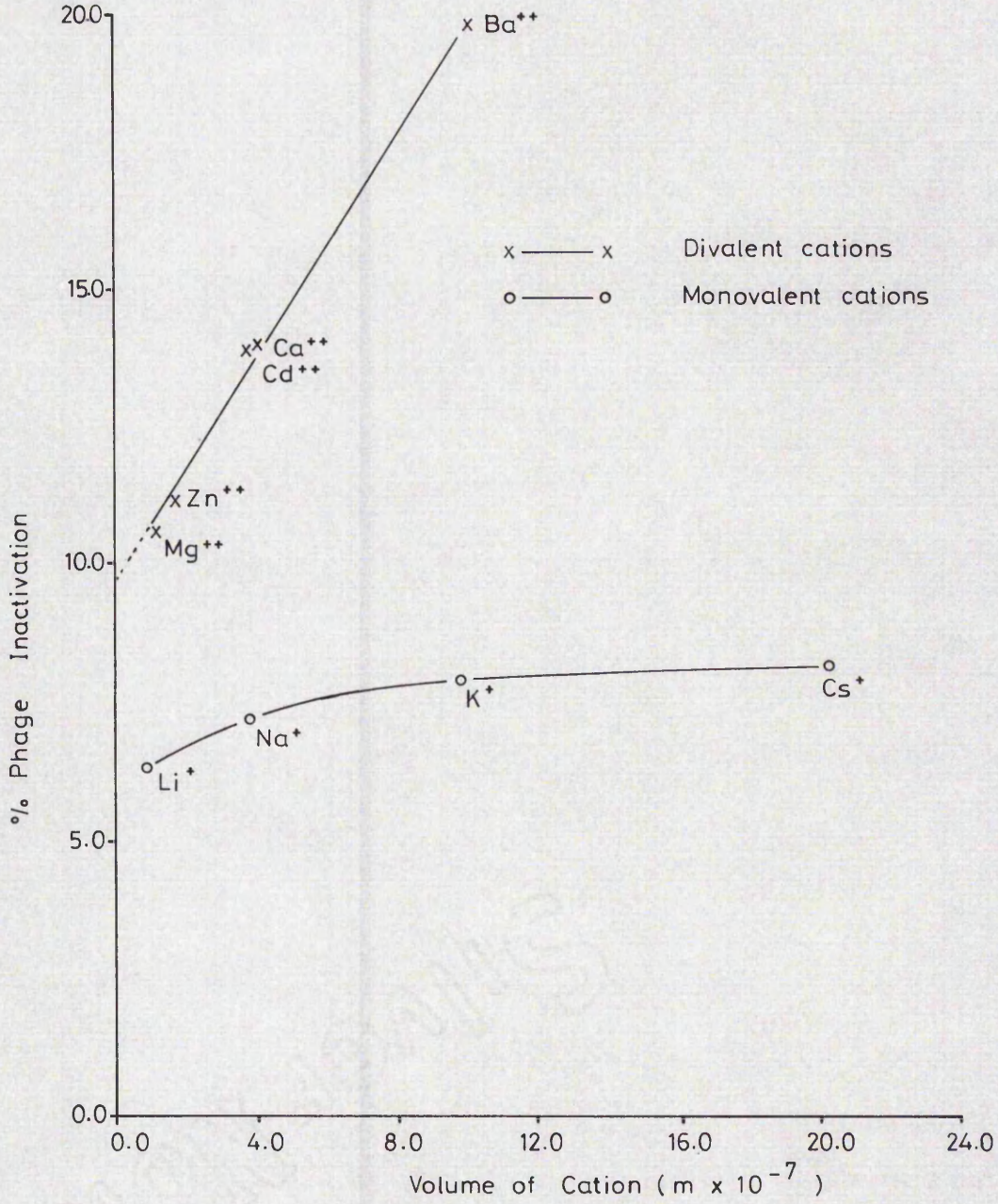
Table 16 Inactivation After 20 Minutes Aeration in Batch and Continuous Flow Columns in Various Solutions

Solution (2.5 mM)	Inactivation in Batch Columns (%)	Inactivation in Continuous Flow Columns (%)
Na_2SO_4	8.1	7.9
K_2SO_4	11.4	7.6
$MgSO_4$	25.0	14.2
$CaSO_4$	27.0	15.3
NaCl	7.2	7.2
KCl	8.2	7.9
$MgCl_2$	16.0	10.9
$CaCL_2$	20.4	13.5

The results also showed greater phage inactivation with divalent cations and anions and once again calcium was more toxic than magnesium. This was proposed earlier to be due to its greater size and consequently more pronounced effect on the electrokinetic potential of the capsid. If this was correct, then cations of increasing ionic size would be expected to show increasing toxicity towards MS-2. Accordingly phage suspensions containing 2.5 mM metal chlorides were aerated in the flotation columns and the degree of inactivation plotted against the volume of the cation (figure 50). Regression analysis of the data revealed that at zero ionic volume, i.e. no divalent cation present, aeration should theoretically cause a 9.6% loss of titre. This was higher than the 0.25% inactivation found earlier for distilled water and therefore the direct relationship between inactivation and ionic volume presumably could not be applied to ions below a certain size.

Similar experiments were undertaken using chlorides of the monovalent

Figure 50. Relationship Between Cation Volume and Inactivation in Aerated Salt Solutions.



cations Li^+ and Cs^+. From their ionic radii it was predicted that Li^+ (0.60A) would show less toxicity and Cs^+ (1.69A) would show more toxicity than either Na^+ (0.97A) or K^+ (1.33). Figure 50 shows this hypothesis was correct although the graph of ionic volume vs. inactivation was not linear as for divalent cations.

It had been proposed earlier and demonstrated in the foregoing experiments that the degree of inactivation increased with an increase in the valency of the cation. Thus trivalent cations would be expected to demonstrate greater toxicity than divalent cations. Accordingly lanthanum chloride ($La^{3+}Cl_3^-$) was added to a phage suspension to give a concentration of 2.5 mM and aerated in the flotation columns. This was found to increase inactivation to 94.4%, thus supporting the hypothesis.

Arranging all the cations examined in order of increasing effectiveness at enhancing surface inactivation, the following series was obtained:

$$Li^+ < Na^+ < K^+ < Cs^+ < Mg^{++} < Zn^{++} < Cd^{++} < Ca^{++} < Ba^{++} < La^{+++}$$

This exactly corresponded to the sequence of effectiveness at condensing hydrated layers surrounding mineral particles and air bubble interfaces, and in reducing the electrokinetic potential of mineral surfaces.

Although no comparable studies with viruses have been reported, Gaudin and his co-workers have studied the effect of several cations on the flotation of Escherichia coli from water (50). At a concentration of 0.67 M they found that the cations could be arranged in the following order of increasing enhancement of flotation:

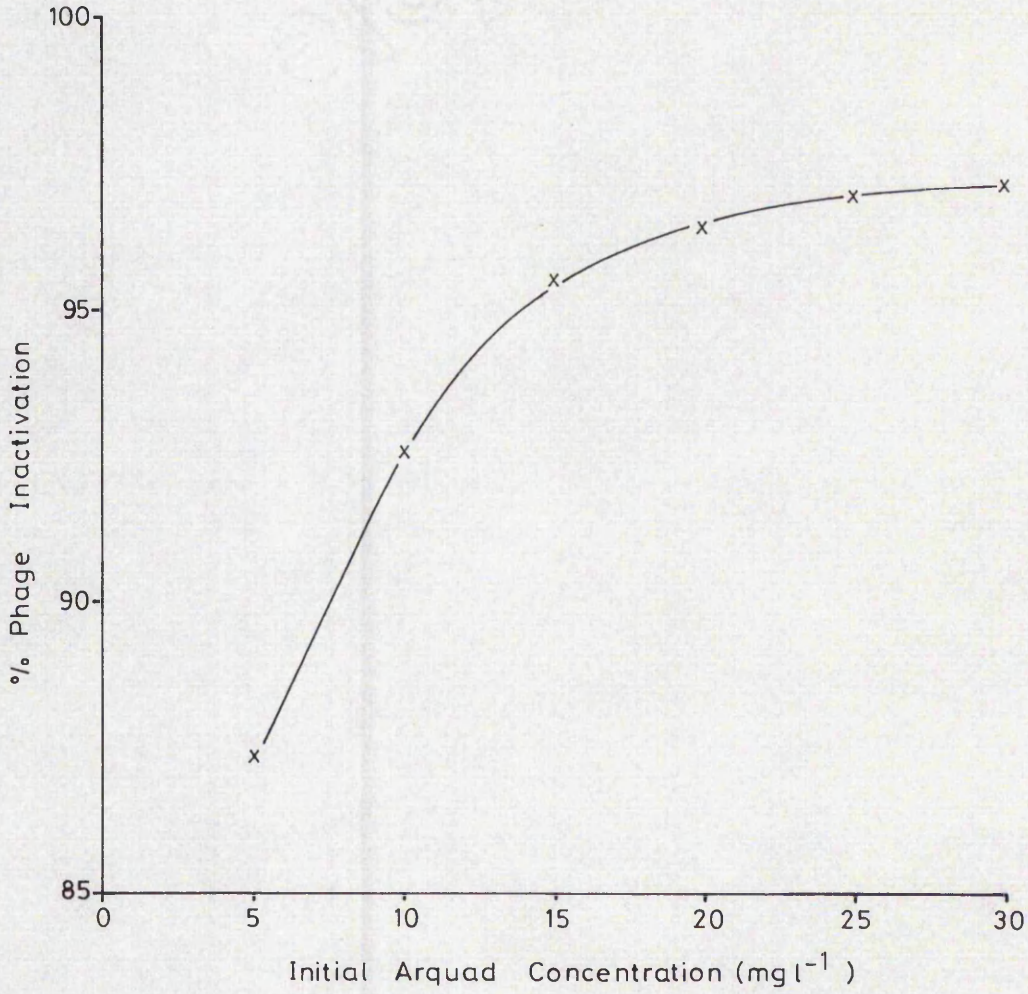
$$K^+ = Li^+ < Ca^{++} < Na^+ \leqslant Mg^{++} < Sr^{++}$$

Clearly this series did not coincide with that found for MS-2 inactivation. Gaudin noted however that the series was not inflexible and that factors other than the ionic size and charge might be involved such as the bacterial surface, pH of the cell environment, and changes in the surface configuration of the bacterium induced by adsorption of specific ions. Thus the mechanisms involved in the enhancement of bacterial flotation were not necessarily comparable with those of the surface inactivation of viruses.

Having established the effect of ions on surface inactivation during aeration and on Arquad toxicity, ionic interference with phage flotation could be assessed. Initially suspensions of MS-2 in distilled water were subjected to foam flotation with various concentrations of Arquad. The results in terms of the reduction in titre of the phage suspensions are shown in figure 51. As can be seen, the inactivation curve was asymptotic, tending towards a maximum value of 97.3% phage inactivation for the given operating conditions.

During these studies, it was noted that the foaming characteristics of the Arquad solutions altered as the concentration increased. At the lower concentrations, up to 15 mgl^{-1}, steady-state conditions were characterized by a lamina flow of bubbles to the surface of the liquid column and a steadily rising foam column whose collapsed volume was dependent upon Arquad feed concentration. At concentrations above 15 mgl^{-1} Arquad, no stable foam was produced initially and the bubble flow gradually became more turbulent as the surfactant concentration increased within the column. Steady-state conditions were characterized by a dense, unstable froth showing no distinction between liquid and foam phases and no discharge from the residual

Figure 51. Phage Inactivation During Continuous Flow Flotation.



overflow. Thus no removal of phage particles into a separated foam phase was possible and this was thought to account for the lack of further increase in phage inactivation.

This unstable froth formation may have been due to the extremely low ionic concentration of the distilled water. Grieves and Bhattacharyya (56) had noted that the fractionation of EHDA-Br increased as the electrolyte concentration increased. This was thought to result from the enhanced thinning of the inter-bubble lamellae which improved foam drainage and stability. Thus whilst a low ionic concentration prevented stable foam formation, the frothing ability of the Arquad solutions was unaffected, hence the excessive frothing observed.

Since the surfactant in the feed was not fractionated into a separate foam phase, its concentration was not reduced within the columns. Thus at the higher Arquad concentrations ($\geq$20 mgl^{-1}) phage inactivation might have been comparable with that occurring in unaerated suspensions. However adsorption of phage to the extensive interfacial surfaces generated in the flotation column was recognized as a possibly significant factor. This had already been shown to cause phage inactivation during earlier studies with aerated buffer solutions. Comparison of the data however showed that at the higher surfactant concentrations the degree of inactivation in the flotation columns was virtually identical with that in unaerated suspensions (see Table 17).

Table 17 Comparison Between Inactivation in Aerated and Unaerated Arquad Solutions

Arquad Concentration (mgl^{-1})	% age Inactivation in Unaerated Solutions	% age Inactivation in Aerated Flotation Columns
5	64.1	87.3
10	83.6	92.6
15	89.7	95.5
20	94.6	96.4
25	96.7	96.9
30	98.0	97.1

Thus at surfactant concentrations in excess of 20 mgl^{-1}, inactivation was not significantly enhanced by surface adsorption. This apparent contradiction with earlier results could have been due to the high concentration of Arquad adsorbed to the gas-liquid interfaces. This may have caused repulsion of the Arquad-coated phage particles thus effectively preventing them from adsorbing to the surface. Some evidence for protection against surface inactivation by competitive adsorption has been reported for bacteriophage T1 (139) and Foot-and-Mouth Disease Virus (12). In these studies, peptone and apolar amino acids (for T1) and inositol, dimethyl sulphoxide and glycerol (for FMDV) reduced inactivation in aerosols. Trouwbourst and Winkler (139) proposed that these surface active agents accumulated at the interface thus preventing virus adsorption, as was suggested above for Arquad.

At lower Arquad concentrations a stable foam was generated allowing fractionation of the surfactant and therefore a considerably reduced residual concentration (e.g. a 5.0 mgl^{-1} feed concentration reduced to 0.2 mgl^{-1} residual concentration). At these lower feed concentrations, competitive inhibition of phage adsorption was

negligible since the interfacial concentration of Arquad was considerably lower and Arquad-coated phage particles readily attached to the bubbles. This was reflected in the results (table 17) which showed greater inactivation during flotation than in unaerated suspensions. Thus at 5 mgl^{-1} feed concentration, inactivation was 36% higher during flotation than during storage in unaerated suspensions at the same concentration. This was despite a decrease in the bulk surfactant concentration due to fractionation and thus a greater proportion of the apparent inactivation must have resulted from phage removal in the foam rather than from surfactant toxicity.

Having determined the loss of phage titre during flotation with Arquad in distilled water, the influence of electrolytes on flotation could be studied. It has previously been shown that ions enhance bubble-particle adsorption but inhibit surfactant-particle adsorption. Flotation however involves other phenomena including surfactant-bubble adsorption and foam production. Grieves and Bhattacharyya (56) have reported that chloride and sulphate ions influenced the fractionation of EHDA-Br by enhancing surfactant ion adsorption to bubble interfaces and by affecting foam drainage and stability as previously noted. Using hydrochloric and sulphuric acids, the interplay between these two effects resulted in pronounced maxima in surfactant removals at specific anion concentrations. It was therefore expected that by reducing surfactant toxicity and enhancing surfactant removal from the flotation column, the overall effect of electrolyte addition would be to reduce phage inactivation during flotation.

Using the techniques described in section 2.1.1. various metal salts were assessed for their effectiveness at reducing inactivation during flotation with 10 mgl^{-1} Arquad. As figures 52 and 53 show,

Figure 52. Effect of Various Chlorides on Phage Inactivation During Flotation.

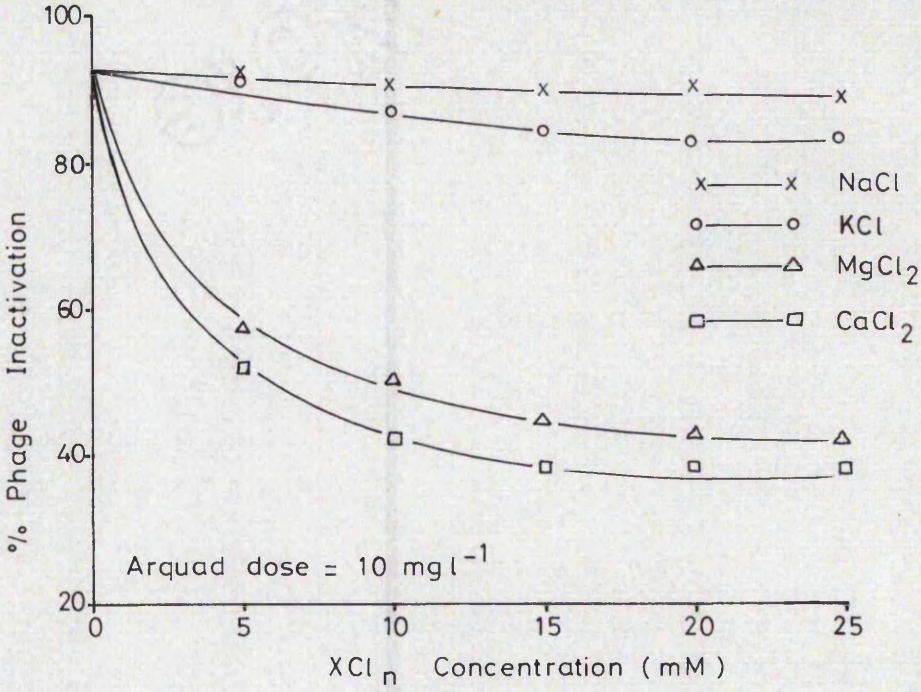
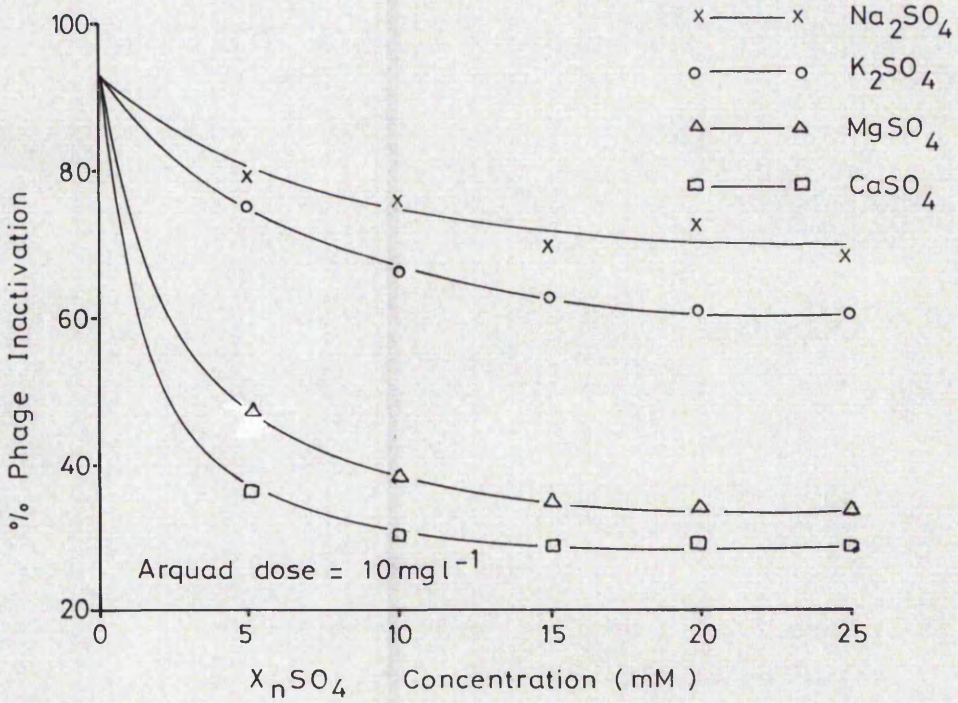


Figure 53. Effect of Various Sulphates on Phage Inactivation During Flotation.



the results were as expected with particularly marked reductions in phage removal using divalent cations.

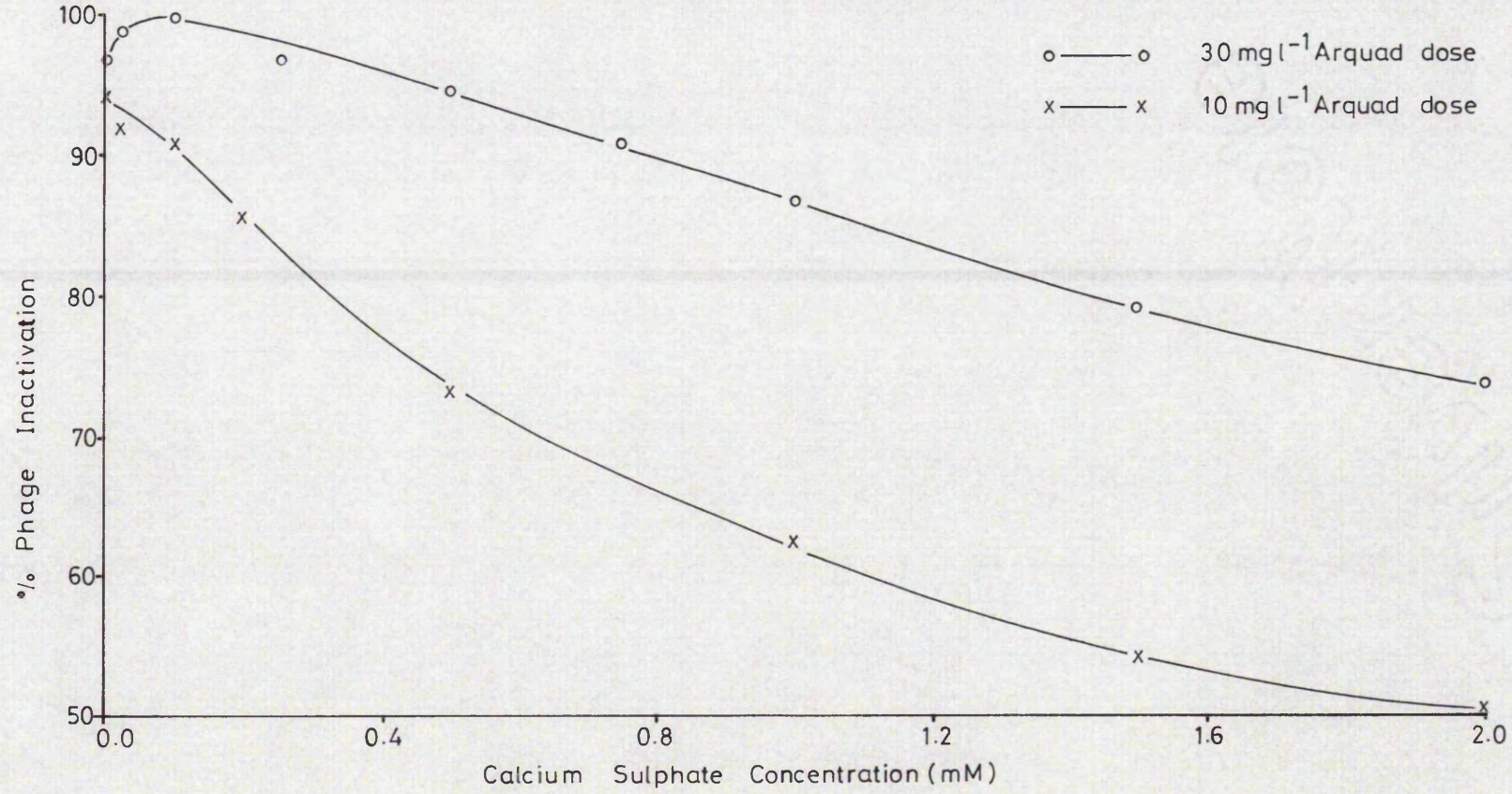
It was also apparent from the graphs that no further phage protection was achieved by increasing the salt concentration beyond approximately 20 mM. Figures 46 and 47 showed maximum reductions in the toxicity of 30 mgl^{-1} Arquad solutions at salt concentrations of 10 mM. Since the solution surfactant concentration during flotation was considerably less than the 10 mgl^{-1} feed concentration, maximum interference with toxicity would presumably have been achieved at a salt concentration considerably less than 10 mM. Clearly the increased phage protection at higher concentrations was due to the enhanced surfactant-bubble adsorption and improved foam stability.

Once again, interference was directly related to the size of the cation and its valency, and the sulphate anion was more effective than chloride. Interference with inactivation however was considerably greater than that found in unaerated suspensions. Thus figure 47 shows that 10 mM $CaSO_4$ reduced the inactivation in 30 mgl^{-1} Arquad from 99.1% to 77.7% whilst 10 mM $CaSO_4$ reduced the removal of phage during flotation with 10 mgl^{-1} Arquad from 92.8% to 30.0%.

It was also noted that with the divalent cations in particular, a considerable reduction in inactivation was achieved at very low concentrations ($<<5$ mM). This was further investigated using $CaSO_4$ concentrations ranging between 0.0 mM and 2.0 mM (see figure 54). The results showed significant interference with inactivation at concentrations as low as 0.02 mM.

It had been shown earlier (figure 51) that in distilled water, inactivation during flotation tended towards a maximum of 97.3% at approximately 30 mgl^{-1} Arquad with no significant increase at higher

Figure 54. Effect of Low Concentrations of Calcium Sulphate on Phage Flotation.



concentrations. It was postulated that the low ionic concentration of distilled water inhibited phage removal by preventing stable foam production and indirectly reducing phage adsorption to the interfaces. Addition of calcium sulphate to such a system would have stabilized the foam allowing surfactant and bacteriophage separation. However simultaneously the calcium sulphate would have inhibited surfactant toxicity, thus reducing inactivation. These conflicting mechanisms might therefore be expected to yield an optimum calcium sulphate concentration at which foam stabilization was maximal and inhibition of toxicity minimal. Accordingly a series of flotation experiments at an initial Arquad concentration of 30 mgl^{-1} (0.0814 mM) was performed to verify this hypothesis and the results are shown in figure 54. A maximum value for phage inactivation was found as predicted and was calculated to be 99.9% removal at 0.07 mM $CaSO_4$, i.e. at a $CaSO_4$ to Arquad ratio of 0.86 to 1.0.

A considerable difference was noticed between the performance of the flotation column with 0.02 mM $CaSO_4$ and with 0.1 mM $CaSO_4$. At the lower concentration excess frothing was evident as had occurred with distilled water, and no liquid-foam interface could be detected. At 0.1 mM concentration however, a stable, though wet, foam was produced and effluent was discharged from the residual overflow.

It has already been postulated that cations adsorb to the phage capsid and that many of the inactivation phenomena demonstrated may be attributed to the resultant lowering of the electrokinetic potential of the capsid. This, together with the repulsive effect of the positively-charged ions, interfered with the adsorption of the cationic Arquad molecules and partly accounted for the reduced inactivation in aerated and unaerated suspensions. It was therefore

postulated that sulphates reduced inactivation to a greater extent than chlorides as a result of their combination with the quaternary ammonium groups of the Arquad molecules in solution or at the gas-liquid interfaces. This was strongly indicated as a result of two unrelated experiments. The first was a study of the effect of calcium sulphate on flotation with different initial concentrations of Arquad. Using three Arquad concentrations, calcium sulphate was added in various amounts to form multiples of the Arquad concentration. Thus adding 553.5 mg $CaSO_4$ and 30 mg Arquad per litre yielded a suspension with a concentration of 4.07 mM $CaSO_4$ and 0.0814 mM Arquad. This was equivalent to a molar ratio of $CaSO_4$ to Arquad of 50:1. Plotting phage inactivation against molar ratio for the three Arquad concentrations yielded three almost parallel plots (see figure 55). This implied that the degree of inactivation was dependent upon the relative concentrations of Arquad and calcium sulphate and that therefore some interaction between them was involved.

The second piece of evidence for a sulphate-Arquad interaction was derived from surface tension measurements. It was found that the addition of very low concentrations of $CaSO_4$ to Arquad solutions significantly lowered their surface tension as shown in table 18.

Such lowering of the surface tension of surfactant solutions upon addition of salts has been described by Grieves and Bhattacharyya (56). They stated that quaternary ammonium salts were almost completely dissociated in solution and only weakly hydrolysed. Thus surfactant ions adsorbing to an interface created an ionic 'activation barrier', preventing further adsorption. This was reduced by the co-adsorption of anions.

Figure 55. Effect on Phage Flotation of Varying the Molar Ratio of Calcium Sulphate to Arquad in the Feed.

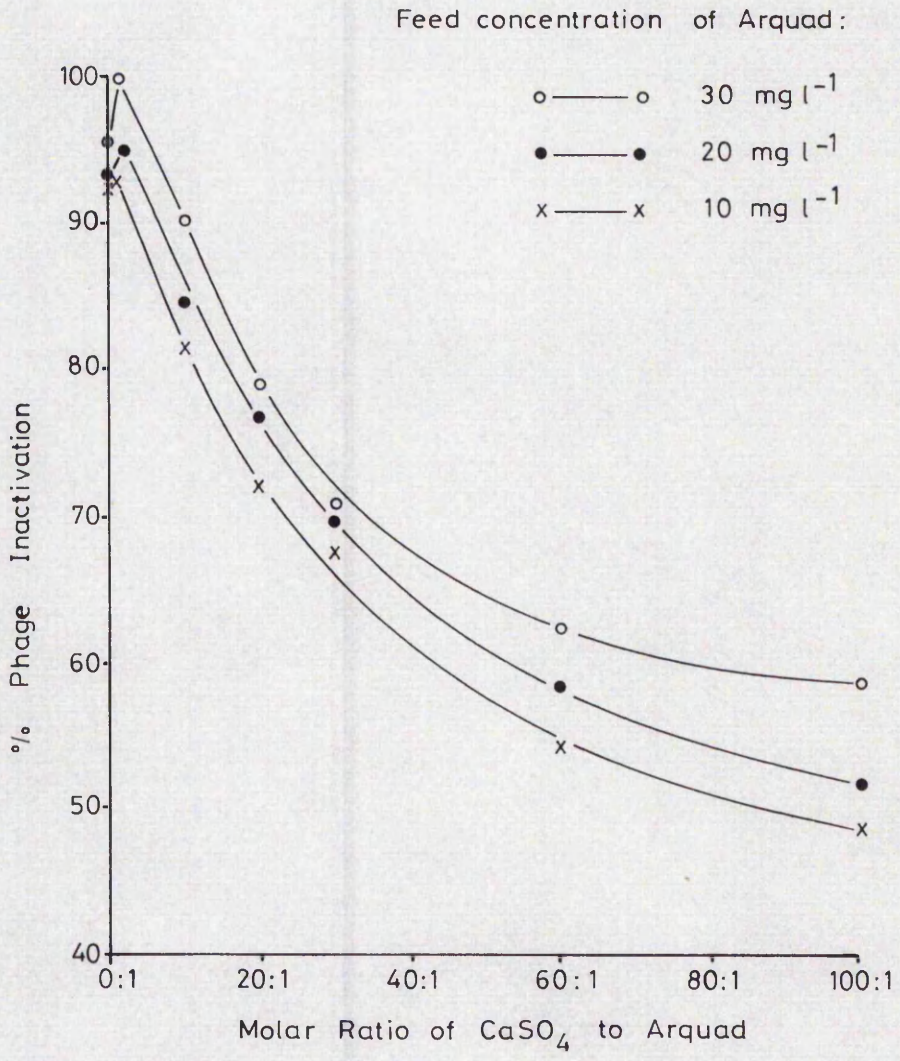


Table 18 Effect of Calcium Sulphate on the Surface Tension of 30 mgl^{-1} Arquad Solutions

Molar Ratio of $CaSO_4$ to Arquad	Concentration of $CaSO_4$ (mM)	Surface Tension Nm^{-1}
Distilled water	0.000	0.073
30 mgl^{-1} Arquad	0.000	0.051
0.25:1	0.020	0.046
0.33:1	0.027	0.042
0.50:1	0.040	0.041
1.00:1	0.081	0.044
2.00:1	0.163	0.044
50.00:1	4.070	0.046

In the table above, it is apparent that the greatest reduction in surface tension occurred at a molar ratio of $CaSO_4$ to Arquad of 0.5 to 1.0. At this ratio, the number of monovalent quaternary ammonium groups was twice the number of divalent sulphate anions, theoretically forming an ionic balance between these ions at the interface. If this were correct, it was postulated that the ratio of sulphate to Arquad in the foam phase should equal 0.5 to 1.0 and that the calcium concentration of the residual effluent would be correspondingly increased as a result of its exclusion from the foam phase.

In order to test these hypotheses, it was necessary to generate sufficient foam for the sulphate analysis by the gravimetric method described in section 2.3.2. The experiments were therefore performed in the larger flotation column described in section 2.1.2.

To determine possible interference from any contaminants in the Arquad, various concentrations of the surfactant were added to

solutions containing 10 mgl^{-1} Ca as calcium sulphate and the solutions analysed as described in section 2.3.1. The results (Table 19) showed no significant interference from the added surfactant.

Table 19 Optical Densities of Solutions Containing 10 mgl^{-1} Calcium and Various Arquad Concentrations

Concentration of Arquad in the Solution	Optical Density at 423 nM
0.0 mgl^{-1}	0.275
1.0 mgl^{-1}	0.280
10.0 mgl^{-1}	0.278
100.0 mgl^{-1}	0.272
1000.0 mgl^{-1}	0.278

After various preliminary experiments to determine suitable flow rates, concentrations, etc., samples were collected from three experimental runs performed under identical conditions as follows:

Air rate	7 $lmin^{-1}$
Arquad concentration	30 mgl^{-1}
Sulphate concentration	200 mgl^{-1} (as $CaSO_4$)
Inlet flow rate	105 ml min^{-1}

Calcium analyses were performed on the influent, effluent and after suitable dilution, the collapsed foam samples, and the means of the results are shown in Table 20. The influent Ca concentration compared favourably with the expected value of 83.3 mgl^{-1}. Also shown is a mass balance for the system which was within the limits of experimental error.

Table 20 Flow Rates, Concentrations and Mass Transfer Data Obtained During the Fractionation of Calcium Sulphate

Sample	Mean Flow Rate($ml min^{-1}$)	Mean Calcium Concentration($mg l^{-1}$)	Mass Transfer Rate Flow Rate x Concn($mg min^{-1}$)	
Influent	105.4 ± 0.9	84.1 ± 0.8	8.86	
Effluent	103.1 ± 1.3	85.0 ± 1.1	8.76	= 8.99
Collapsed Foam	2.9 ± 0.2	80.5 ± 0.6	0.23	

Clearly the calcium was at least partially excluded from the foam phase and concentrated in the residual effluent as predicted.

To determine whether sulphate was fractionated, samples were analysed using the method described in section 2.3.2. In addition, the surfactant concentrations of the residual effluent and the collapsed foam were measured by titration (section 2.2.1.) and by spectrophotometry (section 2.2.2.), respectively. Initially, Arquad concentrations of 10, 15, 20, 25 and 30 $mg l^{-1}$ were used at a constant sulphate concentration of 200 $mg l^{-1}$ as calcium sulphate. In these experiments the air rate was maintained at 7 $l min^{-1}$ and the influent flow rate held constant at approximately 110 ml min^{-1}. Table 21 shows the flow rates and concentrations obtained in these experiments together with the mass transfer rates for sulphate and Arquad derived from the product of these parameters.

The error in the mass transfer balance was calculated using the following formula:

$$A = \frac{\frac{I - (E+F) - I}{E+F} \times 100}{n}$$

where A = average percentage error

I = Influent mass transfer rate (mg min^{-1})

E = Effluent mass transfer rate (mg min^{-1})

F = Foam mass transfer rate (mg min^{-1})

n = Number of experimental runs (i.e. 5)

This yielded average percentage errors in this parameter for sulphate and Arquad of 0.45% and 14.38% respectively. The comparatively large error in the Arquad balance may have been due to adsorption of surfactant from the foam phase to the glass walls of the foam expansion chamber. This would have resulted in a low measured value for Arquad concentration in the foam phase.

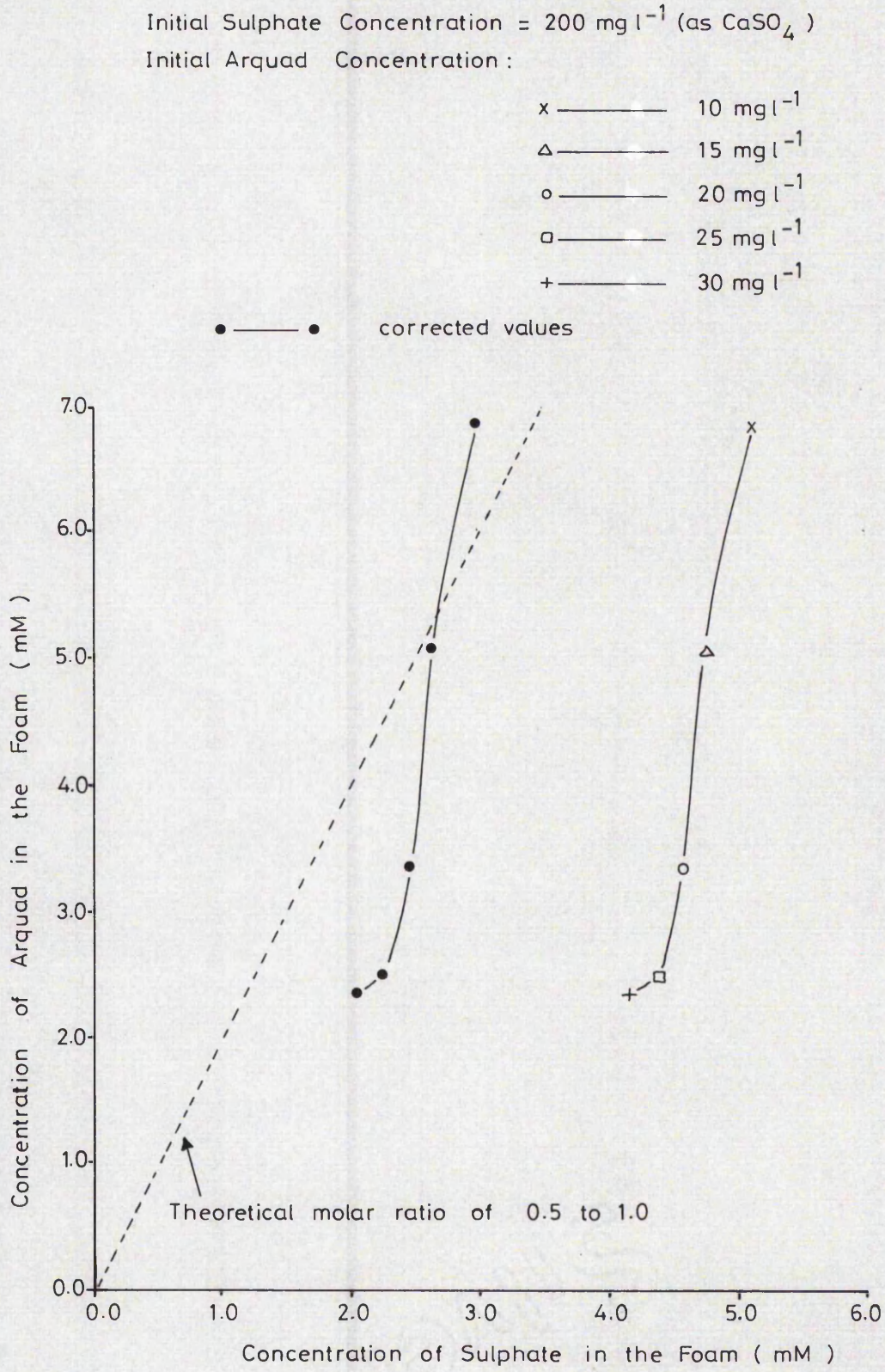
It was apparent from these results that sulphate was fractionated with the Arquad, in the foam phase as postulated. However when the Arquad and sulphate concentrations were converted to moles and plotted one against the other, the expected ratio of 0.5M sulphate to 1.0M Arquad was not found (see figure 56).

It was also noticed that despite a constant air rate of 7 $lmin^{-1}$ the volume of collapsed foam produced increased from 0.4 ml min^{-1} at 10 mgl^{-1} Arquad to 3.3 ml min^{-1} at 30 mgl^{-1} Arquad. Since the number of bubbles generated per unit time remained constant, the increased volume must have resulted from a reduction in foam breakage and/or a decrease in foam drainage. In the latter case, the extraneous liquid carried into the foam would be of the same composition as the residual effluent and would thus have distorted the measured sulphate to Arquad molar ratio. It was therefore assumed that the volume of interfacial liquid in the foam, where the molar ratio of sulphate to Arquad was believed to be 0.5 to 1.0, was negligible compared with the volume of entrained, residual effluent. Thus a correction factor

Table 21 Data Obtained During Fractionation of Sulphate with Various Initial Arquad Concentrations

INITIAL ARQUAD CONCN. (mgl^{-1})	SAMPLE	FLOW RATE ($mlmin^{-1}$)	SULPHATE CONCN. (mgl^{-1})	ARQUAD CONCN. (mgl^{-1})	SULPHATE MASS TRANSFER RATE ($mg\ min^{-1}$)	ERROR I - (E+F)	ARQUAD MASS TRANSFER RATE ($mg\ min^{-1}$)	ERROR I - (E+F)
10	Influent	109.3	208.64	10.0	22.80		1.09	
	Effluent	108.9	206.67	0.10	22.51	+0.10	0.01	+0.07
	Foam	0.4	489.04	2530.0	0.20		1.01	
15	Influent	109.9	206.57	15.0	22.70		1.65	
	Effluent	109.1	203.08	0.17	22.16	+0.18	0.02	+0.13
	Foam	0.8	452.30	1876.0	0.36		1.50	
20	Influent	111.2	209.09	20.0	23.25		2.22	
	Effluent	109.8	205.79	0.23	22.59	+0.04	0.03	+0.46
	Foam	1.4	438.13	1238.0	0.61		1.73	
25	Influent	110.5	208.33	25.0	23.02		2.76	
	Effluent	107.9	204.02	0.40	22.01	-0.08	0.04	+0.31
	Foam	2.6	419.27	927.0	1.09		2.41	
30	Influent	107.1	207.65	30.0	22.24		3.21	
	Effluent	103.8	202.18	0.52	20.99	-0.06	0.05	+0.25
	Foam	3.3	398.23	882.0	1.31		2.91	

Figure 56. Relationship Between the Concentrations of Arquad and Sulphate in the Foam.



could be applied by subtracting the effluent concentration from the foam concentration for sulphate and Arquad. These corrected values are shown as solid circles in figure 56. The plot of these corrected values bisected the predicted line at a point corresponding to an initial surfactant concentration of approximately 15 mgl^{-1}. The lack of correlation at other concentrations probably reflected the inadequacy of the correction factor. At low foam flow rates, the volume of the interfacial liquid probably became a significant proportion of the total collapsed foam volume thus causing an over-correction at initial concentrations below 15 mgl^{-1}. At concentrations above this, the wet foam produced was unstable and foam breakage increased the surfactant concentration in the entrained liquid, resulting in an under correction.

It was considered that these problems could be largely overcome by considerably reducing the initial concentration of calcium sulphate. The extremely efficient fractionation of the surfactant resulted in a very low residual concentration and the contribution of the entrained liquid to the amount of Arquad in the collapsed foam was therefore insignificant. Due to the high level of sulphate remaining in the effluent, however, the entrained liquid contributed approximately half of the total amount of sulphate in the foam.

Furthermore, phage inactivation was reduced by extremely low concentrations of calcium sulphate (see figure 54), and thus it was suspected that sulphate could be concentrated in the foam from extremely dilute solutions. Accordingly the experiments were repeated as before but with an initial calcium sulphate concentration maintained at twice the molar concentration of the Arquad. Thus at 10 mgl^{-1} Arquad the sulphate concentration was 0.054 mM and 0.162 mM at 30 mgl^{-1} Arquad.

The concentrations of Arquad and sulphate in the collapsed foam were determined as before and plotted against one another (see figure 57). A direct relationship was found and regression analysis of the data showed the actual concentration in the foam to be 0.46M sulphate to 1.00M Arquad. This was in reasonably good agreement with the predicted value of 0.5 to 1.0 which assumed a stoichiometric combination between sulphate and Arquad molecules.

Plotting the actual molar ratios obtained against the initial surfactant concentrations (see figure 58), showed that this ratio was virtually independent of feed surfactant concentration within the range 10 mgl^{-1} (0.027 mM) to 30 mgl^{-1} (0.081 mM). Earlier work using 200 mgl^{-1} (2.083 mM) sulphate and 30 mgl^{-1} Arquad (see figure 56) had demonstrated a fractionated sulphate:Arquad ratio of 0.43:1 after correction for the concentration of sulphate in the entrained liquid. This compared with a ratio of 0.45 obtained using the lower initial sulphate concentration of 0.162 mM (see figure 58), and thus suggested that the fractionated ratio was also independent of initial sulphate concentration. To verify this assumption, fractionation was carried out at an initial Arquad concentration of 30 mgl^{-1} and a range of sulphate concentrations between 0.02 mM and 1.00 mM. These corresponded to molar ratios of sulphate to Arquad between approximately 0.25 to 1.0 and 12.0 to 1.0. The results (figure 59) showed that, within the range examined, the molar ratio of sulphate to Arquad in the foam was independent of initial sulphate concentration as suspected.

The results of these experiments demonstrated that sulphate was concentrated in the foam in a manner dependent upon the Arquad concentration in the foam but relatively independent of either the sulphate or Arquad concentrations of the feed solution. This did not

Figure 57. Fractionation of Arquad and Sulphate from Solutions of Constant Molar Ratio.

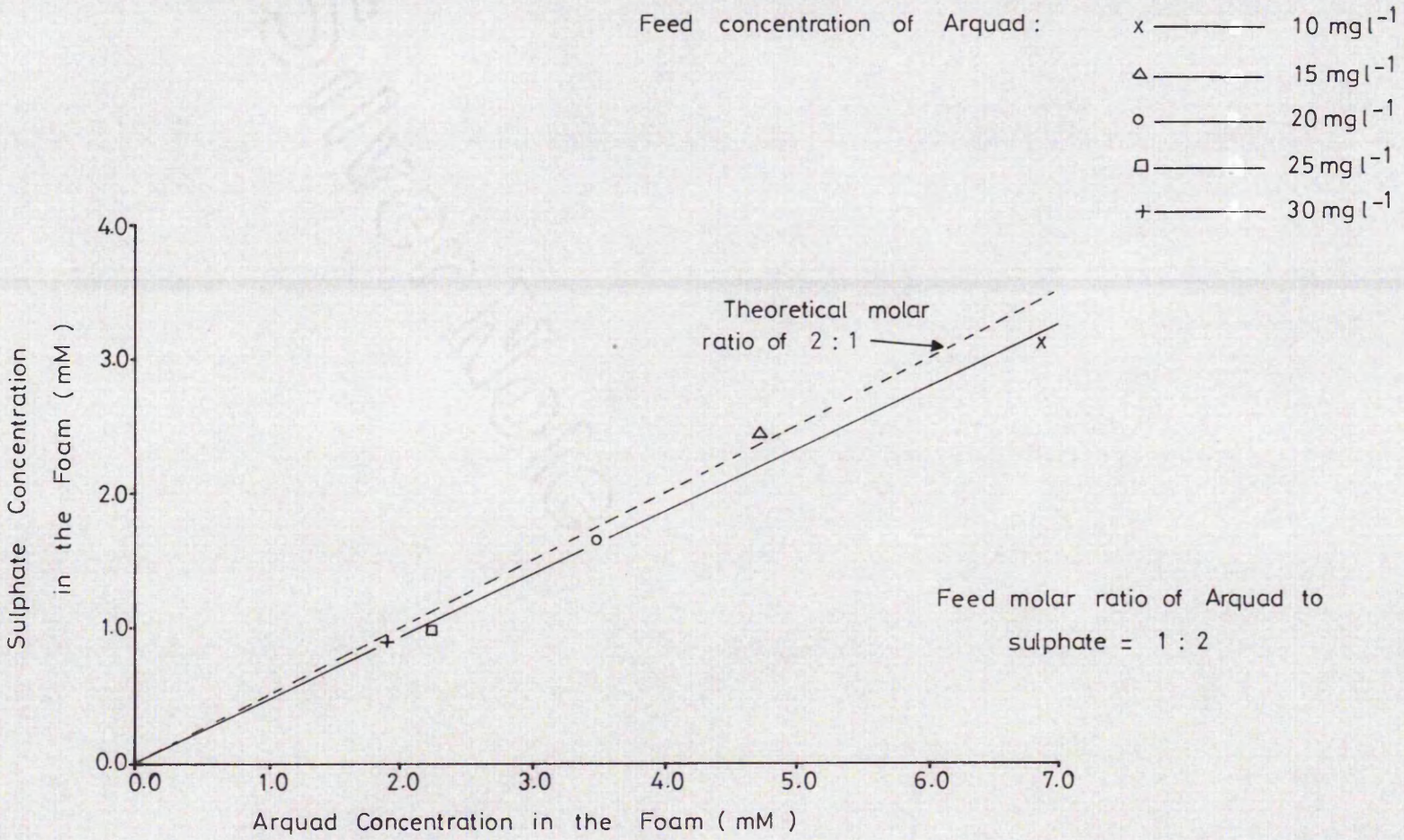


Figure 58. Relationship Between the Sulphate to Arquad Ratio in the Foam and the Initial Arquad Concentration.

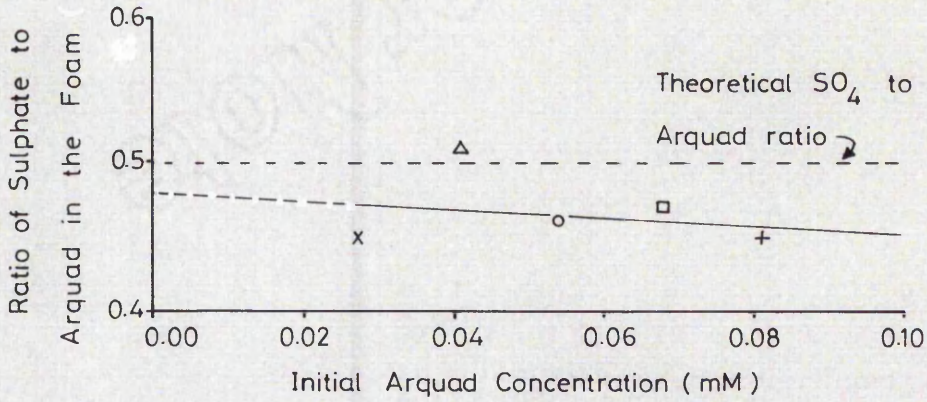
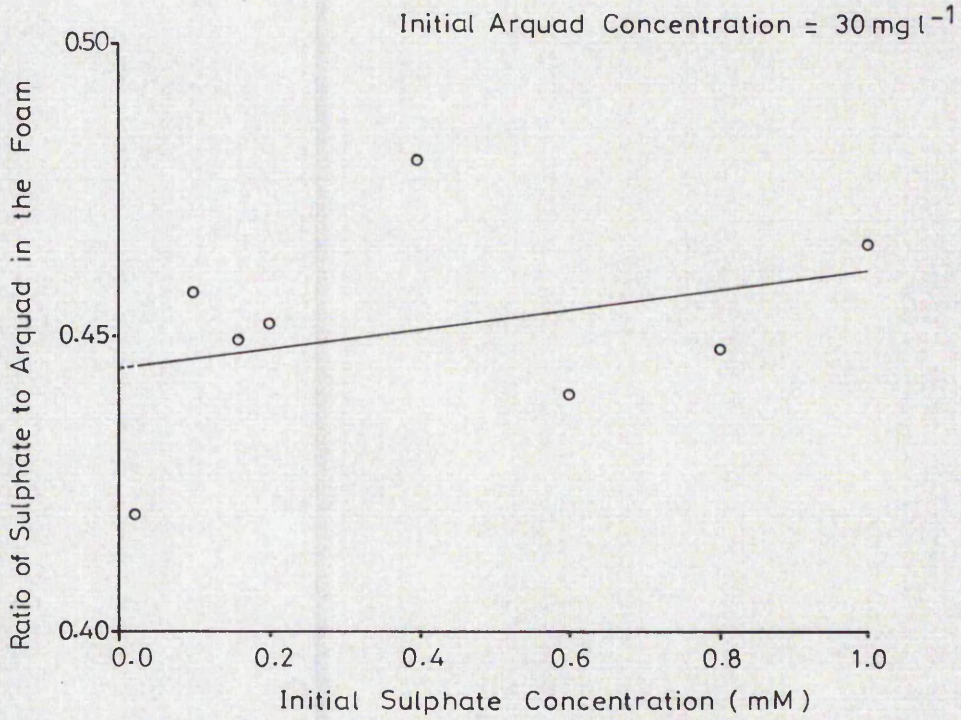


Figure 59. Relationship Between the Sulphate to Arquad Ratio in the Foam and the Initial Sulphate Concentration.



correspond with the data for phage inactivation which was shown to be critically dependent upon the Arquad to sulphate ratio in the feed solution (see figure 55). However it has been suggested by Grieves and The (64) that an ionic surfactant adsorbed to a gas-liquid interface acts as a soluble ion exchanger. This model was developed to explain the varying selectivity found for various monovalent anions during fractionation of EHDA-Br. In this case, the anions displaced bromide ions whereas in the experiments reported here, sulphate ions displaced associated chloride ions from the Arquad. Furthermore the actual molar ratio of 0.46:1.00 was very close to the theoretical stoichiometric ratio of 0.5:1.0 indicating that the chloride was virtually completely excluded from the exchanger. This was consistent with the greater affinity of the divalent sulphate ion for the quaternary ammonium ion. Whilst the exact nature and distribution of the charge on the capsid was unknown, it was reasonable to assume that the phage also had a high affinity for the adsorbed surfactant. If correct it was expected that an equilibrium would be established between the amounts of sulphate and bacteriophage adsorbed and that increasing the concentration of sulphate would therefore reduce the adsorption of phage by the exchanger. Since the graphs in figure 55 showed inactivation approached a minimum value at a ratio of 100M sulphate to 1M Arquad, it was proposed that this represented the minimum sulphate concentration required to completely prevent adsorption of the phage to the adsorbed surfactant ion exchanger.

In order to relate these findings to other anions, the experiments were repeated using phosphate. This ion was chosen for its importance in natural waters and sewage effluents and for its relative ease of assay. The orthophosphate concentration in the

samples was determined by the spectrophotometric method described in section 2.3.3. which had a detection limit of approximately 0.01 mgl^{-1} as PO_4^{3-}. Before proceeding with the experiments a solution containing 500 mgl^{-1} Arquad in distilled water was assayed for phosphate and yielded an optical density at 608 nm of 0.00, indicating that no orthophosphates or interfering ions were present in the Arquad.

Initially, solutions containing between 10 mgl^{-1} and 30 mgl^{-1} Arquad were prepared and potassium dihydrogen phosphate (KH_2PO_4) added to twice the molar concentration of the Arquad. These were fractionated in the large flotation column, foam samples were collected and analysed and the results expressed as for the experiments with calcium sulphate (see figures 60 to 62). Once again the phosphate was concentrated into the foam phase with the Arquad in a manner which was independent of the initial concentrations of either phosphate or Arquad. From figure 60, the molar ratio of phosphate to Arquad in the foam phase was found to be 0.72:1.

This result apparently contradicted the assumption made with sulphate since theoretically three Arquad molecules should combine with one phosphate ion (PO_4^{3-}) and thus the molar ratio should have been 0.33:1. However KH_2PO_4 dissociated in water to give three ionic species whose relative concentrations were dependent upon pH thus:

$$KH_2PO_4 \rightleftharpoons K^+ + H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+ \rightleftharpoons PO_4^{3-} + H^+$$

Each step was characterised by specific dissociation constants and pK values. From a knowledge of the pH and pK values, the molar ratio of proton donor to proton acceptor could be calculated approximately from the Henderson-Hasselbalch equation:

$$pH = pK + \log \frac{(A^-)}{(HA)}$$

Figure 60. Fractionation of Arquad and Phosphate from Solutions of Constant Molar Ratio.

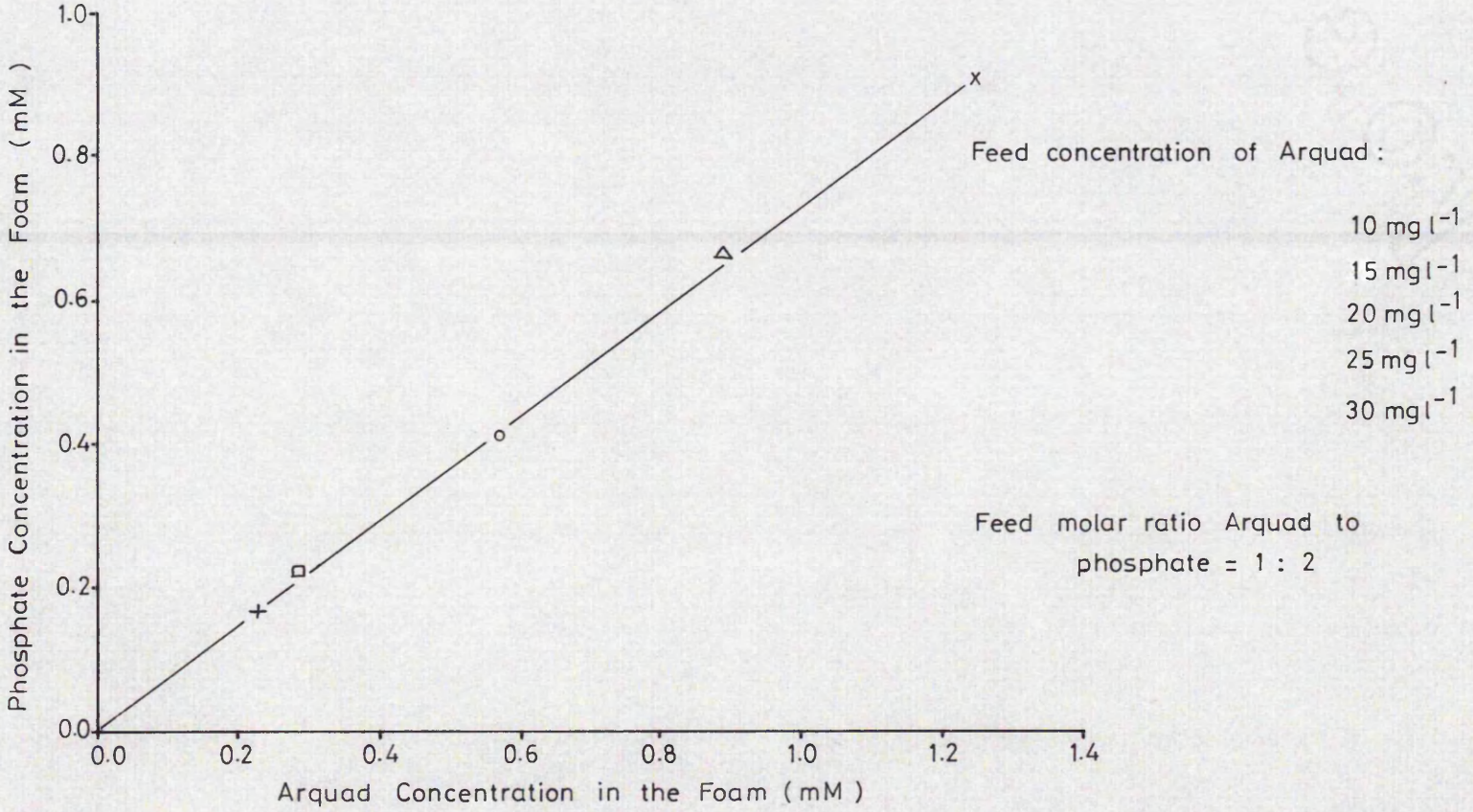


Figure 61. Relationship Between the Phosphate to Arquad Ratio in the Foam and the Initial Arquad Concentration.

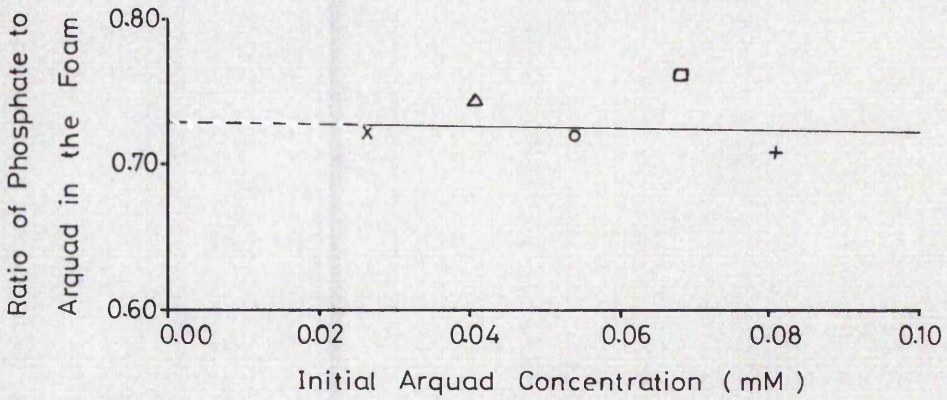
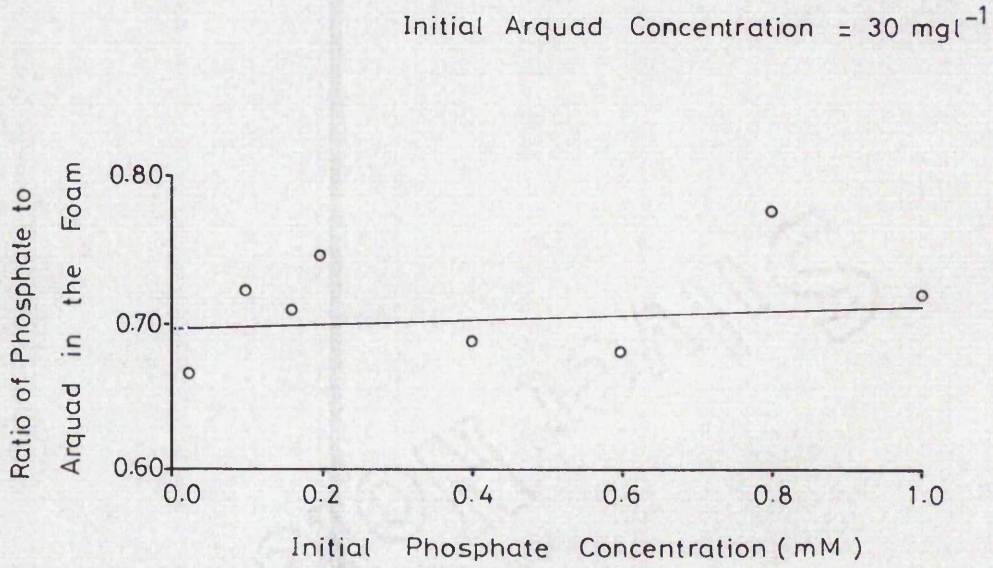


Figure 62. Relationship Between the Phosphate to Arquad Ratio in the Foam and the Initial Phosphate Concentration.



The pK values for H_3PO_4, $H_2PO_4^-$ and HPO_4^{2-} at 25°C were given as 2.12, 7.21 and 12.67 respectively (24) and the pH of the solutions during flotation was found to be pH 4.7. Using the above equation and assuming the pK value for KH_2PO_4 to be the same as that for H_3PO_4, the relative molar ratios of the species KH_2PO_4:$H_2PO_4^-$:HPO_4^{2-}:PO_4^{3-} were found to be:

$$KH_2PO_4 \rightleftharpoons K^+ + H_2PO_4^-$$

$$1 \quad : \quad 380$$

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$$

$$324 \quad : \quad 1$$

$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-}$$

$$1.1 \times 10^6 \quad : \quad 1$$

Clearly therefore the predominant ionic species in solution at pH 4.7 was $H_2PO_4^-$ and therefore the molar ratio of phosphate to Arquad should have been 1 to 1. The lower observed ratio of 0.72:1 again probably resulted from competitive adsorption by chloride ions as discussed earlier.

During this series of experiments it was noticed that the volume of foam produced decreased with increasing phosphate concentration in the feed. This phenomenon was also noted during the phage inactivation studies and the explanations proposed by Grieves and Bhattacharyya (56) have been reported earlier. From their data it can be calculated that the maximum removal of EHDA-Br was achieved at sulphate ion concentrations of approximately 0.1 mM and 0.4 mM for surfactant concentrations of 87.5 mgl^{-1} and 125 mgl^{-1} respectively. This resulted from an increase in surfactant-bubble adsorption and foam stabilization and drainage which would have reduced the volume of foam produced. It is therefore proposed that maximum surfactant

fractionation occurred at the minimum anion concentration producing the lowest foam flow rate.

Using 30 mgl^{-1} solutions of Arquad, the foam flow rates at various concentrations of added sulphate (as $CaSO_4$) and phosphate (as KH_2PO_4) were determined using the 200 ml flotation columns. The results showed minimum foam flow rates were achieved at sulphate and phosphate concentrations of 0.10 mM and 0.56 mM respectively (see figure 63). These values were comparable with the optimum concentrations found for EHDA-Br quoted above. It was also apparent from the data that sulphate was considerably more effective than phosphate at reducing the foam flow rate; as expected for a divalent anion. Furthermore the results showed that the maximum reduction achieved in the foam flow rate was the same for both sulphate and phosphate ions.

Referring to the earlier phage inactivation data (see figure 54), the sulphate concentration causing maximum enhancement of phage flotation was 0.07 mM. This corresponded closely with the minimum concentration of 0.10 mM required for minimum foam flow. It was therefore postulated that phosphate would similarly cause maximum enhancement of Arquad inactivation at a concentration of approximately 0.56 mM. This was verified by inactivation studies with various concentrations of added KH_2PO_4 which showed maximum enhancement of phage flotation at a phosphate concentration of approximately 0.75 mM (see figure 64). These results therefore confirmed the earlier hypothesis that the initial enhancement of phage flotation upon addition of salts was a result of improved adsorption of phage-surfactant complexes to bubble interfaces and increased foam stability and drainage.

Figure 63. Relationship Between Initial Anion Concentration and Foam Flow Rate.

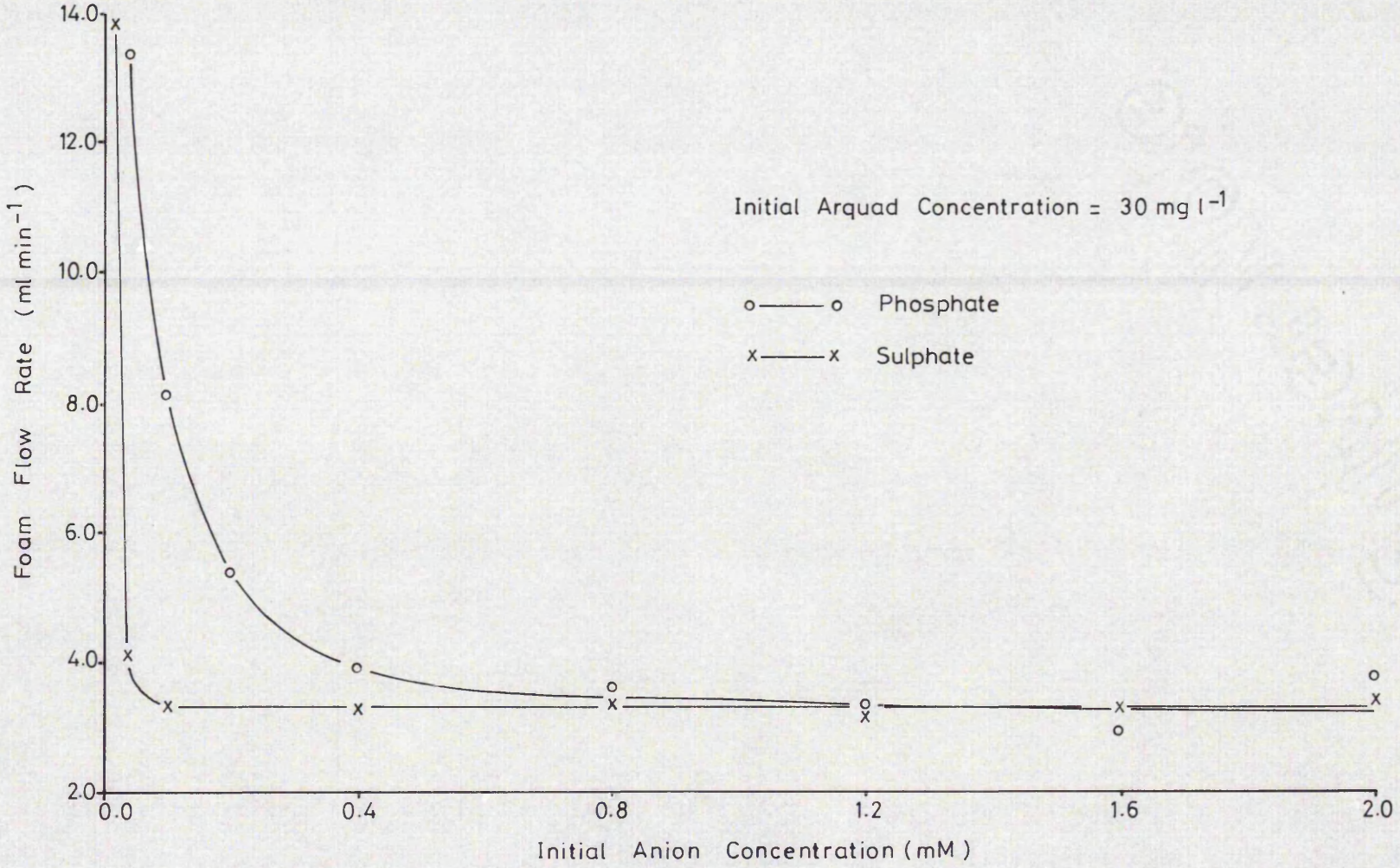
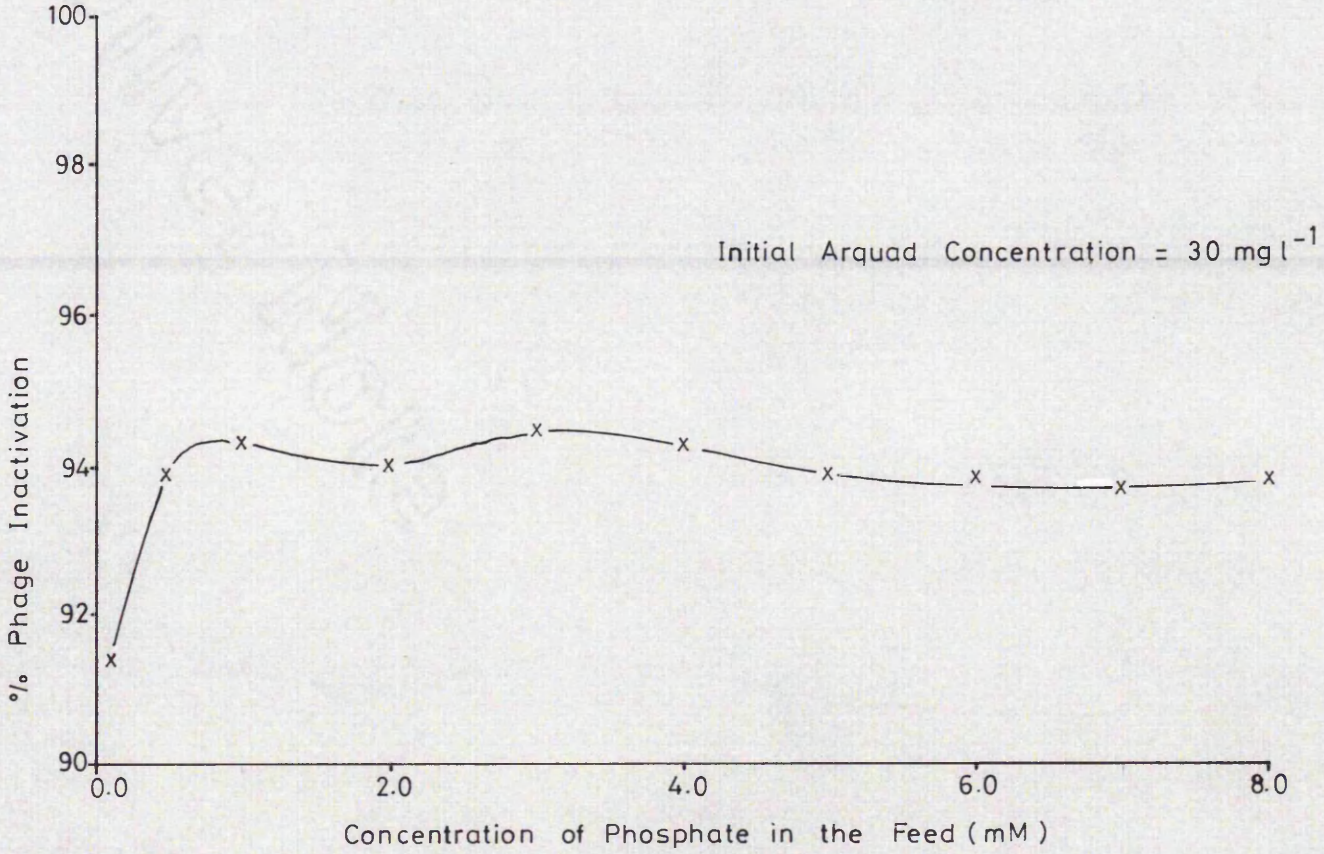


Figure 64. Effect of Phosphate Concentration on Phage Flotation.



It was also apparent from figure 64 that increasing the phosphate concentration beyond this value did not decrease phage inactivation as was found with sulphate. This was to be expected since the phosphate did not dissociate to form divalent anions as did the sulphate and was therefore far less effective in preventing phage inactivation by competitive association with the Arquad. The results also showed that the maximum degree of inactivation attained was lower than that achieved by the addition of $CaSO_4$ to a similar concentration of Arquad. In figure 54 sulphate increased inactivation of MS-2 to 99.9% compared with 94.5% for phosphate addition. This was probably due to the difference in pH of the suspensions; the phosphate solution having a pH of 4.7 compared with 6.1 for the sulphate solution. The lower pH would thus have reduced the toxicity of the Arquad, as demonstrated earlier in this thesis.

The mode of action proposed for ionic regulators of phage flotation can therefore be summarized thus:

The toxicity of the cationic surfactant towards MS-2 was reduced by competitive adsorption of cations to the phage capsid. The extent of this reduction was related to the size and charge of the specific ion; the larger and more highly charged ions being most effective. These ions could be arranged in a sequence of increasing effectiveness at reducing toxicity which exactly corresponded to their increasing effect on the electrokinetic potential of a surface. This suggested the involvement of this surface characteristic in the toxicity of the surfactant.

During flotation, low concentrations of ions enhanced phage removal. This resulted from stabilization of the foam phase, facilitated by a reduction in the hydrated layers surrounding the

bubbles, due to the presence of the ions. This enabled greater thinning of the inter-bubble lamellae and thus improved foam drainage. Reduction of the hydrated layers also increased the speed and tenacity of attachment of phage particles to the bubbles enabling more efficient phage removal.

At higher concentrations the anions competed with the phage particles for attachment to the cationic surfactant molecules causing a reduction in phage inactivation and removal. This phenomenon was again related to the valency of the competing anion.

4. CONCLUSIONS

The principle objective of this research project was to gain a more complete understanding of the interactions and mechanisms involved in the removal of viruses from a natural water or wastewater by foam flotation. A review of the literature revealed numerous examples of the application of foam flotation to water and wastewater treatment but these were largely concerned with the removal of suspended solids, BOD, turbidity, etc. with virtually no mention of viruses. Furthermore although these studies often attempted to quantify in mathematical terms the effect of various parameters upon the process, they failed to explain at a molecular level, the mechanisms by which such reactions took place. This was also true of the few viral studies, the most notable of which was that undertaken by M.D. Guy in this laboratory (67). He developed a mathematical model to predict the removal and inactivation of bacteriophage during foam flotation and it was this study which prompted the present project.

Flotation has been used for many years in the mining industry for the separation and concentration of mineral ores. Fundamental research into the process has established the nature of the interactions between a mineral particle surface and flotation reagents and between mineral particles and the gas bubble interfaces. Whilst recognizing the dissimilarities between mineral and viral particles, this investigation has sought to apply these principles to the flotation of viruses. This required an extensive literature search to combine available information on viral surface properties from studies as diverse as aerosolization and electrophoresis. However much of the data obtained was related to viruses other than MS-2 and

therefore some of this research was repeated using this bacteriophage.

From the outset it was recognized that in order to study inactivation, all the virus particles must be singly dispersed throughout the suspension. This was necessary because the assay procedure used to determine the infective titre of a bacteriophage suspension was incapable of distinguishing between single particles and aggregates. Thus extensive inactivation of aggregated virions could occur which would be largely undetected. This problem has frequently been overlooked in previous inactivation studies.

Our investigations showed that the degree of aggregation varied with the procedures used for phage preparation; deaggregation causing a maximum increase in titre of 57%. Of the disruptive procedures investigated the simplest and most effective was found to be one hundredfold dilution in distilled water. This was presumed to result from diffusion of ions from the aggregates which would cause increased hydration of the particles thus reducing their mutual attraction. Dilution in distilled water also had the advantage of causing no significant loss of titre even after prolonged storage. Survival however was maximal at a pH of 6.0 and was inversely proportional to the ionic concentration of the diluent.

During their extensive studies on the surface inactivation of viruses, Trouwborst and his co-workers (137) found considerable loss of titre during aeration of suspensions in concentrated (1.0M) sodium chloride solutions. They attributed this to the increased surface energy resulting from the added electrolytes which caused physical disruption of viruses adsorbed to the interfaces. Studies with aerated suspensions in phosphate-citrate buffer confirmed that inactivation increased with increasing buffer concentration and

furthermore was independent of pH. However measurements showed that the surface tension decreased with increasing buffer concentration, presumably as a result of the surface activity of the citrate ion. Thus inactivation was unlikely to be due to physical disruption by the increased surface tension of the interface as suggested.

An alternative mechanism of disruption by mechanical agitation has been proposed by a number of workers (25, 91, 100) although their procedures did not preclude the possiblity of surface inactivation. Consequently a method of agitation was devised which excluded gas-liquid interfaces and since no significant inactivation was found to occur, this proposition was rejected.

Further studies with aerated buffer solutions demonstrated that the surface area generated per unit volume of solution increased with the buffer concentration. The logarithm of the inactivation ratio ($\log_{10}$ Initial titre/Residual titre) was found to be directly proportional to this interfacial density which suggested that surface inactivation was unrelated to buffer concentration. This was confirmed by a technique developed for exposing phage particles to a constant interfacial area in a thin film of buffer in which inactivation was independent of the concentration. This finding was partly contradicted by later studies however, where the nature of the ions present and their concentration affected surface inactivation. Nevertheless the results showed that above a threshold value for surface area per unit volume of 140 $mm^2 ml^{-1}$, significant surface inactivation would occur, although its extent was dependent upon the type and concentration of ions present.

The lethal event in surface inactivation was shown by Trouwborst and others to be release of nucleic acid from the viral capsid (35, 134,

138) and it was therefore proposed that a similar mechanism was involved in MS-2 inactivation. This was thought to be analagous to the release and injection of RNA occurring after adsorption of the phage to the sex pilus of the host bacterium. Whilst not conclusive, some evidence for this was derived from electron microscopy of an inactivated phage suspension which appeared to contain a higher proportion of empty capsids than an untreated suspension.

In mineral flotation it is common practice to add surface active agents called collectors and frothers to the pulp to enhance the separation of mineral particles. Detergents are frequently present in wastewaters and may be added to natural waters to enhance flotation; some of these have the additional benefit of being toxic towards viruses. However virtually no information on their mode of action on picornaviruses was available.

Preliminary studies with the cationic quaternary ammonium salt Arquad T50, showed that toxicity towards MS-2 was directly proportional to pH and that within the range pH 3.0-8.0, the relationship could be expressed by the following equation:

$$\text{Log}_e \, {}^{I}/_{R} = 0.834 \, (\text{pH}) - 2.21$$

where I = Initial bacteriophage titre

R = Residual bacteriophage titre

A considerable increase in inactivation rate was noted between pH 8.0 and 8.1 but thereafter the rate decreased to that exhibited previously and expressed by the above equation.

From these results it was postulated that the inactivation increased with pH because of the greater adsorption of surfactant by the more negatively charged phage capsid. Thus the adsorption (and

subsequent toxicity) of an anionic surfactant would be expected to increase as the pH was lowered and the capsid became more positively charged. This was demonstrated using the anionic alkyl sulphate Teepol 610, which showed increased inactivation at values below pH 4.0.

The discontinuation in the inactivation curves at pH 8.0 and 4.0 was postulated to result from configurational alterations of region(s) of the capsid. These exposed more ionizable amino and carboxyl groups for reaction with the anionic and cationic detergents respectively and this hypothesis was confirmed by electrophoresis. A technique for isoelectric focussing of intact virus in a 2.8% polyacrylamide gel was devised and MS-2 was found to exist in either of two interchangeable states characterized by isoelectric points at pHs of 4.1 and 7.6. This result was supported by the findings of Mandel (99) who showed that Poliovirus Type 1 was isoelectric at pHs of 7.0 and 4.5. Clearly other viruses may be characterized by two or more isoelectric points and more detailed studies of this phenomenon could provide valuable information on the structure of viral capsids.

The extreme toxicity of oppositely charged surfactants at different pH levels has obvious implications for water purification. Selection of the appropriate detergent for the particular viruses to be removed may be critically dependent upon the pH of the system. Thus whilst Armstrong and Froelich (4) stated that picornaviruses were highly resistant to quaternary ammonium salts, they did not recognize the dependence of the interaction upon pH. However the two isoelectric points found for Poliovirus would suggest that, like MS-2, its susceptibility to detergents would also be pH dependent. Furthermore

both Arquad and Teepol exhibited toxicity which was apparently unrelated to their ionic groups.

At a pH of 2.65 it was calculated that Arquad would show no toxicity towards MS-2. However in practice a 13% reduction in titre was found at this pH during the 5 minute contact period. This corresponded well with the value of 15% for inactivation in Teepol at pH levels above pH 4.0 and was therefore considered to be due to non-ionic interactions between the hydrocarbon chains of the surfactants and the capsid. These were thought to 'insulate' the electrical double layer associated with the surface, interfering with the ionic environment necessary for stabilization of the configuration of the surface proteins.

Studies on the rate of inactivation in solutions of cationic Arquad showed that initially this followed first order reaction kinetics, as demonstrated by Guy (67). However extended contact periods (up to 30 minutes) revealed that the rate decreased with time in a regular manner. This was not due to heterogeneity within the phage population since resistance was not demonstrated to be a heritable factor. Furthermore fractions resistant to thermal inactivation and those separated by density gradient centrifugation did not show any variation in Arquad sensitivity from that of an untreated control population. It was therefore concluded that the shape of the inactivation curves resulted from complex phage-detergent interactions.

Similar curves had been found by Gard (45) for the inactivation of Poliovirus by formaldehyde which he attributed to the initial reversible adsorption of formaldehyde which temporarily prevented infectivity. This could be partly recovered by incubation in

formaldehyde acceptors although similar procedures with Arquad failed to demonstrate any reversibility in inactivation.

The toxicity of the quaternary ammonium group alone was assessed using solutions of tetramethylammonium chloride and inactivation was found to follow first order reaction kinetics. This was in accordance with the initial rate of inactivation in the alkyl trimethylammonium chloride, i.e. Arquad. The rate of inactivation after prolonged contact with Arquad however was virtually identical with that found in alky sulphates, i.e. Teepol, and was therefore attributed to adsorption of the non-ionic hydrocarbon chains. This evidence led to the following inactivation mechanism being proposed: Initially (approximately the first 15 minutes of contact) the rapid rate of inactivation resulted from adsorption of Arquad to the phage capsid and ionic interaction between the A-protein and quaternary ammonium group(s) causing RNA release. However a proportion of the phage population adsorbed surfactant without these specific ionic interactions taking place. As the reaction proceeded ($\geqslant$ 15 minutes after contact) sufficient non-ionically bound surfactant had been adsorbed by the capsid to effectively prevent further ionic adsorption. Thereafter inactivation was dependent upon the unstable ionic environment created by the hydrophobic 'sheath' of Arquad molecules surrounding the capsid.

The rate of inactivation in surfactant solutions has important implications for the use of flotation in water treatment. A period of mixing of feed water and surfactant prior to flotation would clearly be advantageous to allow surfactant adsorption to suspended material and inactivation of potentially harmful pathogens including viruses. However these results indicate that little benefit would be

gained in terms of virus destruction by allowing a contact time in excess of 15 minutes. Thus a 30 mgl^{-1} solution of Arquad inactivated approximately 97.7% of the phage within the first 15 minutes and only a further 1% were destroyed by doubling the contact time to 30 minutes. The considerably increased cost and size of installation required for the longer retention time would therefore clearly not be justified. Inactivation rate was also dependent upon pH and detergent type as already noted. Thus plant performance may be enhanced by pH control, the cost of which may be offset by a lower detergent concentration requirement or by the use of a cheaper detergent.

The inactivation rate was also found to be dependent upon temperature. Using the Arrhenius equation it was found that the Activation energies for the initial ($\leq$ 5 minutes) and final ($\geq$ 25 minutes) inactivation processes were 33.7 $kJmole^{-1}$ and 43.2 $kJmole^{-1}$ respectively for temperatures between 10^{o} and $50^{o}C$. These were considerably lower than the values quoted for thermal inactivation of other bacteriophages (300 - 570 $kJmole^{-1}$) (146) and this could not therefore occur by the same reaction mechanism. This apparently contradicted the findings of Woese (146) who postulated that inactivation by formaldehyde occurred by the same mechanism as thermal inactivation. At temperature between 1^{o} and $10^{o}C$ the Activation energies increased to 83 $kJmole^{-1}$ and 100 $kJmole^{-1}$ for the initial and final inactivation processes respectively. This demonstrated that neither of these two stages followed true first order reaction kinetics and that their rates were not therefore solely dependent upon the concentration of surfactant. This supported the views of Gard concerning chemical inactivation of viruses (45), but

contradicted the postulations of others (28, 120, 132).

Having established the toxicity of solutions of Arquad in distilled water, the influence of inorganic ions on the system was assessed. It was known from mineral flotation studies that metal cations adsorbed to the surface of minerals, condensing the diffuse layers of associated ions and thus lowering the zeta potential. This effect was proportional to the size of the ion and its valency.

The particular ions used and their concentrations were limited to those most commonly found in natural and wastewaters. Thus at a concentration of 2.5 mM, the toxicity of these ions was found to increase in the following order:

$$Li^{+} < Na^{+} < K^{+} < Cs^{+} < Mg^{2+} < Zn^{2+} < Cd^{2+} < Ca^{2+} < Ba^{2+} < La^{3+}$$

In all cases the metal sulphates were more toxic than the chlorides. However in unaerated suspensions these ions caused very little inactivation after contact for one hour (< 4%) whereas in aerated suspensions the degree of inactivation increased markedly (20-80%).

Clearly phage particles were capable of withstanding any changes induced at their surfaces by these ions in unaerated suspensions. This was to be expected in view of their known resistance to many harsh chemical environments. However in aerated systems, the ions enhanced inactivation although at the concentration used, they did not increase the surface area generated per unit volume. Evidently this was brought about by enhanced adsorption of the phage particles to the bubble surfaces. From mineral flotation theory it was thought that the ions stabilized and condensed the hydrated layers surrounding the phage and bubble surfaces allowing them to approach more closely and therefore increasing the likelihood of attachment. This has been proven by electrokinetic potential measurements to occur at mineral

surfaces and the ions could be arranged in the same order of increasing effect as that demonstrated for virus toxicity (84).

When the metal salts were added to solutions of Arquad however, it was found that they reduced its toxicity towards MS-2 and furthermore the ions could be arranged in the same series of increasing effectiveness as above. This was not unexpected since the larger or more highly charged the cation the greater would be its effectiveness at competing with the anionic sites on the capsid. As predicted, the ions only interfered with the initial rate of inactivation since only this phase was due to ionic adsorption of the quaternary ammonium group.

Competitive inhibition of toxicity was found to reach a maximum value at a metal salt concentration of approximately 10 mM for all the ions tested and for all Arquad concentrations up to 30 mgl^{-1}. This lack of dependence upon Arquad concentration provided further evidence that protection was brought about by interaction of the cations with the capsid surface. The apparent contradiction between ions causing and preventing inactivation should not be overemphasised since at a concentration of 2.5 mM, calcium sulphate caused only a 2.9% loss of infective titre in one hour. However the same concentration added to a 30 mgl^{-1} Arquad solution reduced the degree of inactivation during 20 minutes contact from approximately 99% to 83%.

Again this has important implications for water treatment. Many of the ions tested such as sodium potassium, magnesium, calcium, chloride and sulphate are present at relatively high concentrations in the natural waters used for potable water supply. The World Health Organisation International Standards for Drinking-Water, for example,

give a permissible level of sulphate as 200 mgl^{-1} (131) or approximately 2.1 mM. This could considerably reduce the effectiveness of any pre-flotation contact stage by reducing surfactant toxicity as shown above. Furthermore natural waters contain a wide range of inorganic ions, particularly if polluted with industrial effluents, and complex interactions between these may enhance or interfere with their ability to reduce surfactant toxicity. Preliminary studies however suggested that mixtures of ions did not display synergistic effects but that inhibition of toxicity was the cumulative result of the concentration and activity of the individual ions. Furthermore it appeared that interference from a mixture of ions again reached a maximum at a total ionic concentration of 10 mM. This was considered an area of research requiring further investigation to enable predictions to be made about the interference with toxicity of mixtures of inorganic salts in solution.

With no additional ions present (i.e. distilled water), the inactivation occurring during 20 minutes flotation with Arquad tended towards a maximum value of 97.3% reached at a surfactant dose of approximately 30 mgl^{-1}. In these solutions it was noticed that fractionation of Arquad into a foam phase ceased at concentrations above 15 mgl^{-1} whilst considerable frothing occurred within the flotation column. However despite an extremely high surface area to volume ratio, inactivation under these conditions was virtually identical to that in an unaerated solution of the same concentration. This suggested that at high Arquad concentrations, surface inactivation was being prevented, possibly by the competitive adsorption of Arquad to the interfaces which reduced bubble-phage particle attachment. A similar exclusion mechanism was known to account for the protection

of bacteriophage T1 (139) and FMDV (12) by other surface active substances such as peptone and glycerol.

At Arquad doses of less than 15 mgl^{-1}, phage inactivation during flotation was greater than that occurring in an unaerated solution of corresponding concentration. This was despite a rapid reduction (to less than 0.5 mgl^{-1}) in Arquad concentration due to its fractionation, and thus demonstrated the significance of surface adsorption and consequent removal in the foam phase in the reduction of bacteriophage titre during foam flotation.

From earlier results it appeared that cations added to a flotation system might have conflicting effects upon phage inactivation. Surfactant adsorption to the capsid would be reduced (therefore less toxicity) although phage-bubble attachment would be enhanced (therefore increased surface inactivation). However interfacial adsorption of surfactant would interfere with the latter phenomenon and in practice the results showed that inactivation was reduced. The extent of this reduction was determined by the specific volume and valency of the cation and the order of increasing effectiveness of the ions was found to be the same as for their effect on Arquad toxicity. Furthermore the reduction in inactivation was proportional to the ionic concentration up to a maximum value which in this case was reached at a concentration of 20 mM. In addition, the degree of protection afforded by a given salt concentration was greater during flotation than during contact with an unaerated surfactant solution. Thus 2.0 mM calcium sulphate reduced the inactivation in a 30 mgl^{-1} surfactant solution from 99% to 84% whilst the same concentration added to a flotation system with the same initial surfactant concentration, reduced inactivation from 97% to 74%. Ionic interference clearly was

not limited to reducing surfactant toxicity and it was thought that by promoting surfactant adsorption to the interfaces, less surfactant was available for adsorption to the phage particles.

Ions have been shown to increase surfactant adsorption to an interface by reducing the activation barrier set up as the surfactant ions are adsorbed (56). Furthermore electrolytes promote foam stabilization and drainage by counterion adsorption which reduces interfacial hydration allowing greater thinning of the bubble lamellae to occur. Thus at surfactant concentrations above 20 mgl^{-1}, electrolyte addition allowed foam fractionation to occur, preventing the excess foaming found in distilled water. Phage flotation was therefore enhanced as demonstrated by the initial increase in inactivation during flotation upon addition of calcium sulphate. Thus with a 30 mgl^{-1} Arquad dose, the reduction in phage titre reached a maximum of 99.9% at a concentration of 0.07 mM $CaSO_4$.

The phenomenon of enhanced surfactant adsorption was also followed by surface tension measurements. By comparing the surface tensions of solutions containing Arquad and calcium sulphate in various molar proportions it was found that the minimum surface tension, i.e. maximum surfactant adsorption, occurred at a molar ratio of 0.5:1 $CaSO_4$ to Arquad. This was to be expected assuming all the monovalent quaternary ammonium groups of the surfactant combined with divalent sulphate ions at the surface. It followed therefore that the ratio of sulphate to Arquad in the foam phase should also be 0.5 to 1 and that the calcium ions would therefore be excluded and concentrated in the residual stream. Both these hypotheses were demonstrated although the experimentally determined sulphate to Arquad ratio was found to be 0.46:1. This ratio was found

to be independent of the initial Arquad concentration up to at least 30 mgl^{-1}, and of the initial sulphate concentration up to 2.0 mM.

This was unexpected since it had been found earlier that inhibition of toxicity increased up to a maximum at 20 mM $CaSO_4$ and this was presumed to result from enhanced surfactant removal. However Grieves proposed that an ionic surfactant adsorbed to an interface would act as a soluble ion exchanger (64). Thus phage particles competed with sulphate ions for adsorption to the exchanger and a concentration of at least 20 mM sulphate was required to completely exclude the phage. This phenomenon probably accounted for the difference between the expected and observed ratios of sulphate to Arquad in the foam phase since some competition from the chloride ions originally associated with the Arquad probably would have occurred.

These experiments were repeated using potassium dihydrogen phosphate (KH_2PO_4) when a molar ratio of 0.72:1 phosphate to Arquad was found in the foam. This ratio was again found to be independent of the initial concentrations of Arquad (up to 30 mgl^{-1}) or phosphate (up to 1.0 mM).

The value of this ratio was considered to be determined by competition with chloride ions as before and also to the relative proportions of the dissociation products of KH_2PO_4 at the particular pH of the bulk solution (i.e. 4.7). These were calculated to be:

$$KH_2PO_4 \rightleftharpoons K^+ + H_2PO_4^-$$

1 : 380

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$$

324 : 1

$$HPO_4^{2-} \rightleftharpoons PO_3^{3-} + H^+$$

$1 : 9 \times 10^{-7}$

Thus since the predominant species was the $H_2PO_4^-$ ion, the expected ratio was 1:1 $H_2PO_4^-$:Arquad and the lower measured value was presumably again due to competition from the chloride ions.

Measurements of the foam flow rate during flotation with both sulphate and phosphate showed that this parameter reached a minimum value at concentrations of 0.10 mM and 0.56 mM respectively. These corresponded to the concentrations causing the maximum increase in bacteriophage inactivation during flotation; the difference in concentration between the ions reflecting their relative efficiencies in promoting foam stabilization and drainage.

From the evidence obtained it was apparent that inorganic salts regulated virus flotation by three distinct mechanisms. Firstly at low concentrations the added ions improved foam stability and drainage thus enhancing virus removal. Secondly at higher concentrations the cations competed with the cationic surfactant for the adsorption sites on the viral capsid, reducing surfactant adsorption and therefore decreasing the likelihood of particle-bubble attachment. Thirdly the anions competed with the particles for adsorption to surfactant bound at the interfaces thus reducing virus removal by flotation. These effects clearly demonstrated that flotation of virus particles was a similar process to mineral particle flotation and was influenced by the same parameters.

It should be noted that none of the three mechanisms summarized above depended upon the toxicity of the surfactant towards the virus. In the case of MS-2, Arquad was relatively toxic and was thought to cause inactivation principally by adsorption to the A-protein. However the group of viruses of particular importance in water treatment, namely the enteroviruses, do not possess a specific

adsorption site comparable with the A-protein of MS-2. There is much evidence for their resistance to surfactants (4) and preliminary experiments in this laboratory have demonstrated that Poliovirus was extremely resistant to concentrations of Arquad as high as 500 mgl^{-1} (M.D. Guy, unpublished data). However careful selection of appropriate surfactants together with a possible adjustment of the pH may considerably enhance toxicity. For example, some evidence has been obtained in this laboratory that anionic Teepol is toxic towards Poliovirus at the lower pH values (Marion Smith, personal communication). Furthermore many substances are utilized in mineral flotation to promote foam stabilization and drainage (frothers) and enhance particle-bubble attachment (collectors). Detergents, such as Arquad and Teepol, frequently combine these functions but more efficient virus flotation may be possible by careful selection of appropriate collectors and frothers as used in mineral flotation.

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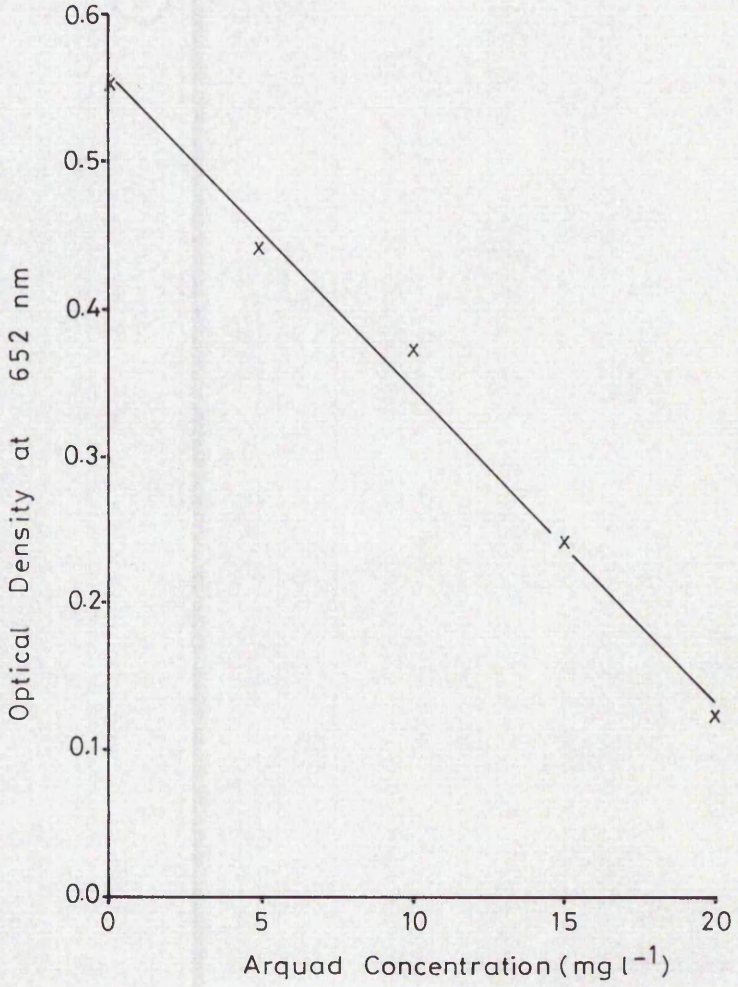
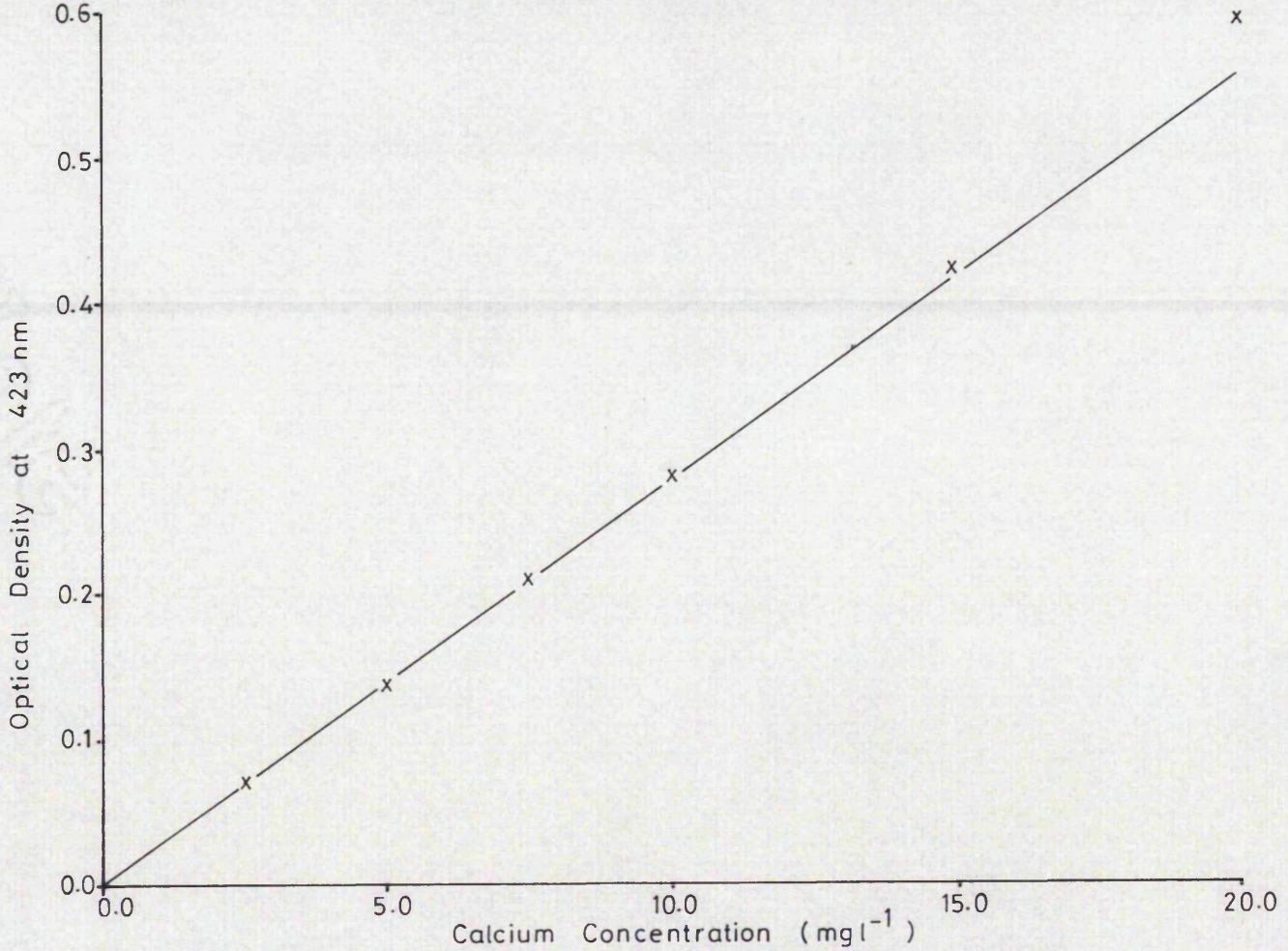
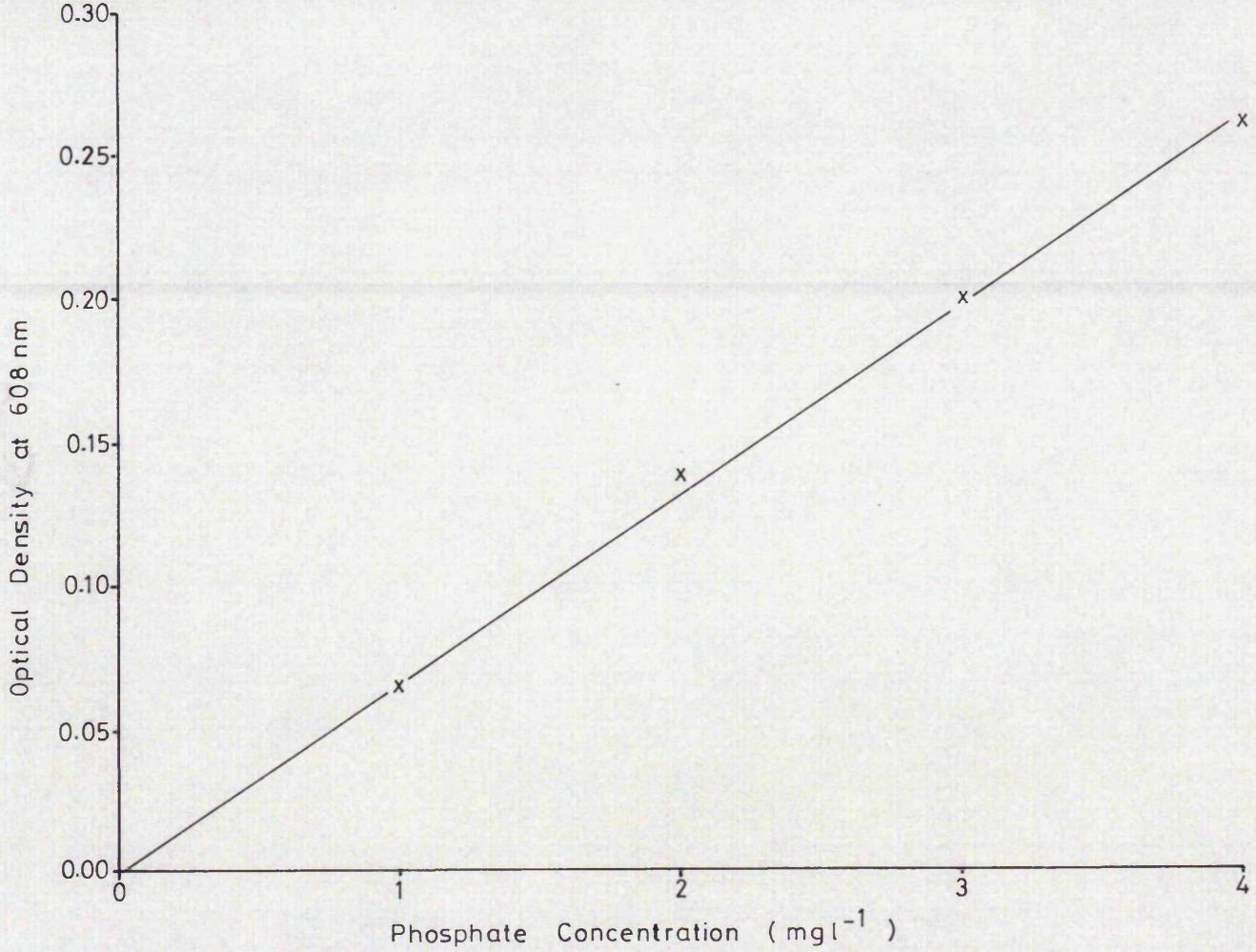
Appendix I. Standard Curve Relating Arquad Concentration to the Optical Density of a Methylene Blue Complex.



Appendix II. Standard Curve Relating Calcium Concentration to Atomic Absorption.



Appendix III. Standard Curve Relating Phosphate Concentration to the Optical Density of a Phosphomolybdic Complex.



APPENDIX IV

Composition of Reagent Solutions

Methylene Blue Indicator Solution

Dissolve 0.1g methylene blue in 100 ml distilled water. Transfer 30 ml to a 1ℓ volumetric flask and add 500 ml distilled water, 6.8 ml H_2SO_4 and 50g $Na_2HPO_4.H_2O$. Make to volume with distilled water.

Wash Solution

As for indicator solution omitting the methylene blue.

Sulphomolybdic Reagent

Solution A - 10g ammonium molybdate dissolved in approximately 70 ml distilled water and made up to 100 ml.

Solution B - 150 ml sulphuric acid poured into 150 ml distilled water and allowed to cool.

Solution A poured into Solution B and allowed to cool.

APPENDIX V

Suppliers and Manufacturers of Equipment and Materials

Chemical reagents	BDH Chemicals Ltd., Poole, Dorset.
QED detergent	Diversey Ltd., Cockfoster, Barnet, Herts.
Bacteriological media	Oxoid Ltd., Basingstoke, Hants.
Ampholine	LKB Produkter, AB Stockholme, Sweden.
General laboratory equipment and glassware	A. Gallenkamp & Co. Ltd., Technico House, London.
Disposable plastic petri dishes and pipettes	Sterilin Ltd., Richmond, Surrey.
Edwards EB3A pump	Analytical Supplies Ltd., Derby.
Peristaltic pumps	Watson Marlow Ltd., Falmouth, Cornwall.
Flow meters	Glass Precision Engineering Ltd., Hemel Hempstead, Herts.
QVF glassware and sintered glass filters	JA Jobling & Co. Ltd., Process Plant Division, Trentham, Stoke on Trent.
Ultra filters	Laboratory and Electrical Engineering Co. Ltd., Colwick, Nottingham.
Spectrophotometers	EEL Ltd., Halstead, Essex.
Centrifuges and Ultrasonic Disintegrator	MSE Scientific Instruments, Crawley, W. Sussex.
pH meter	EIL, Richmond, Surrey.

Electrophoresis apparatus	Shandon Southern Instruments Ltd., Camberley, Surrey.
Photographic equipment	The Photo Centre, Nottingham.