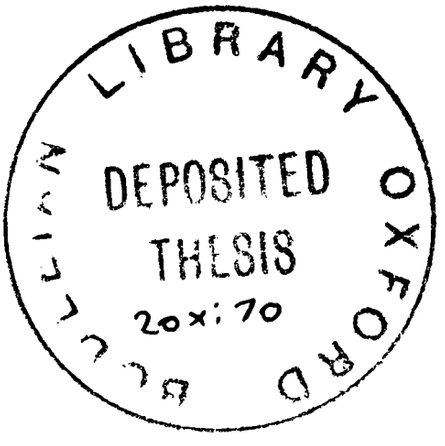
A STUDY OF THE PENETRATION OF HOST CELLS BY BACTERIOPHAGES

A thesis submitted for the degree of
Doctor of Philosophy
in the
University of Oxford

by
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CHEMICALS

Tris- (hydroxymethyl) amino methane (tris) supplied as Trizma Base, Sigma Co.

Ethylene diamino tetraacetic acid (EDTA) supplied by B.D.H.

ββ'-dimethyl glutaric acid (DMGA) supplied by Sigma Co.

2-(4'-tert.-butylphenyl)-5-(4"biphenylyl)-1,3,4-oxadiazole (Butyl PBD) supplied by Ciba.

Deoxyribonuclease (DNase) Worthington Biochemical Corporation Grade I

Ribonuclease (RNase) Sigma Chemical Co. Grade I

Lysozyme Sigma Chemical Co. Grade A.

Caesium chloride (CsCl) B.D.H. AR Grade

Carrier free ^{32}P-phosphate in dilute HCl, pH 2 - 3. The Radiochemical Centre, Amersham.

Carrier free ^{35}S-sulphate in isotonic saline. The Radiochemical Centre, Amersham.

Silicone M.S. Antifoam Emulsion Hopkins and Williams Ltd.

Bovine Serum Albumen (B.S.A.) Armour Pharmaceutical Co. Ltd.

ABBREVIATIONS

ØX 174, wild type	ØX
ØX 174, am3 mutant	ØX am3
ØX heated to 70° for 5 min	'70°-ØX'
multiplicity of infection	m.o.i.
plaque forming units	p.f.u.
deoxyribonucleic acid	DNA
ribonucleic acid	RNA

ABSTRACT

The object of the work described in this thesis was to investigate the attachment, and penetration of the host cell walls and membranes by the minute bacteriophage ØX 174. In an attempt to simplify the investigation, the interaction between viral particles and cell walls isolated from the bacterial host, Escherichia coli strain C, was examined. Conditions for maximum, irreversible adsorption to cell walls were shown to be in buffered solutions at pH 6-9, at 35-45°, in the presence of calcium or magnesium ions. It was established that these conditions gave efficient attachment to intact bacteria.

When phage particles labelled with ^{32}P-phosphate became attached to isolated bacterial cell walls, up to 30% of the viral ^{32}P was converted to a form accessible to the enzyme, pancreatic deoxyribonuclease (DNase). This indicated that some of the phage deoxyribonucleic acid (DNA) was released on attachment of virus to cell walls. At the same time, some of the adsorbed ØX 174 virus were converted to particles which could be eluted from the cell walls by a solution of sodium borate, containing ethylene diamine tetra acetate (EDTA). The particles were resolved into two components on fractionation by sedimentation through sucrose density gradients. One component consisted of particles with a sedimentation coefficient of 45-50S (50S particles). When viral preparations, doubly labelled with ^{32}P- and ^{35}S- were used, comparison of the value

of the $^{35}S/^{32}P$ ratio in the original phage and in the 50S particles, showed that the latter contained the same quantity of DNA as the original virus. In contrast to intact virus, however, the 50S particles were infectious towards bacterial spheroplasts, but not towards whole bacteria. Examination in the electron microscope of 50S particles, isolated by sedimentation through sucrose gradients, failed to reveal any gross contamination with cell wall or other material, which might have caused anomalous sedimentation behaviour. It was concluded that a conformational change in the arrangement of the viral protein sub-units occurred on attachment to the cell walls and was responsible for the changes in infectivity and sedimentation coefficient. Similar investigations on the second component, showed that it had a sedimentation coefficient of 70, was not infectious towards whole bacteria or spheroplasts, and had a reduced DNA complement. The particles appeared to be identical with the "top component" previously identified by others in preparations of ϕX 174 (e.g. Sinsheimer R.L., (1959) J. Mol. Biol. 1:37).

Attachment of ϕX 174 to intact, starved bacteria suspended in buffer also resulted in the formation of 50S and 70S particles, which were eluted from the bacteria by sodium borate and EDTA. After prolonged incubation (30 minutes) of virus with either cell walls, or intact bacteria, only the 70S component could be detected in eluates made with borate-EDTA

solution.

When the viral material eluted from cell walls was treated with DNase there was no loss in infectivity detectable in the bacterial spheroplast assay. It was concluded that all of the infectivity resided in the 50S component, and none in the viral DNA released on attachment of the phage particles.

The results show that changes occur in ϕX 174 particles when these attach to isolated cell walls or to non-metabolising bacteria. The possible role of the 50S component in the natural infectious process could not be determined from the experiments described here. Its properties suggest that it contains a hitherto unrecognised particle which can be formed by conformational changes in whole intact ϕX 174.

INTRODUCTION

All viruses are obligate, intracellular parasites, in which entry of the genetic material into the host cell is essential for replication. Successful infection therefore always involves penetration of the cell membrane and release of the viral nucleic acid from its protein coat.

When the virus can reach the membrane directly, as in animals, or plants which have damaged cell walls, the process is frequently achieved by host controlled ingestion of the whole virion, followed by intracellular removal of the coat. (Hoyle, 1962; Mandel, 1962).

In bacteria, however, the cell wall provides a more complex barrier, which is usually penetrated completely only by the viral nucleic acid, the protein remaining on the surface (Hershey and Chase, 1952). The process is achieved in one of two ways. Male strains of E. coli possess pili (Brinton, 1959; 1965) which pass through the cell wall. The filamentous, single strand DNA phage, fd, (Caro and Schnös, 1966), and RNA phage, such as R17 (Crawford and Gesteland, 1964) are believed to take advantage of this, because their nucleic acids pass into the host, through the hollow centres of the pili.

In other cases, it is a viral element which acts as "penetration organ". The T-phage strains attach to the host's surface by their tails. (T. F. Anderson, 1948a, 1948b; Lanni & Lanni, 1953). These are hollow, and permit the passage of DNA from the virus' head into the bacterium. (Brenner et al, 1959).

In the T-even phage, the DNA is "injected" into the host, when the tail sheath contracts, and pushes the hollow core through the cell wall. (Graham, 1953; Williams and Fraser, 1956). In strains with non-contractile tails, such as T_5 or λ, the method of achieving penetration is less clear. In T_5 infection, initially the phage releases only 8% of its DNA into the host (Lanni, 1960). This first-step-transfer material initiates protein synthesis without which the remaining DNA cannot enter (Lanni, McCorquodale and Wilson, 1964; McCorguodale and Lanni, 1964; Lanni, 1965). The success of entry depends, therefore, upon the phage using the host's metabolism to its own ends, but how the first-step-transfer material is released, or passes through the cell wall and membrane is unknown.

In no case of viral invasion, has the vital problem of how DNA passes across membranes been elucidated. Available evidence suggests, that even in the T-even phages, "injection" probably does not damage the membrane. (Simon and Anderson, 1967). The virus can only deliver the DNA to the membrane surface, and entry then depends upon host-controlled mechanisms. This suggests that the special features of bacteriophage entry are concerned with negotiating the cell wall. Once this has been achieved, entry is analogous in all viruses, being dependent upon host mechanisms.

Entry itself is, therefore, a non-specific process, and the high specificity of infection must be achieved before, or afterwards. The second alternative, is illustrated by T_5, since the

process is only successful if the virus can make full use of the host's machinery for replication. In penetration of the cell wall, the initial event probably carries the greatest specificity. There is no success without an efficient attachment to the cell surface. The viral and bacterial receptors, which are both genetically controlled, (Weidel & Kellenberger, 1955; Weidel, 1958; Kellenberger et al, 1965; Joys, 1965; Oram & Reiter, 1968) are responsible for this. The host range of a virus can be altered completely by a single mutation in either organism, for example, if it alters the structure of the bacterial surface receptors, or those on the virus' tail fibres. (Stent and Wollman, 1952; Garen and Kozloff, 1959). Individual phages also need different ionic environments and temperatures to achieve maximum adsorption (Hershey, Kalmanson and Bronfenbrenner, 1944; Puck, Garen and Cline, 1951; Tzagoloff and Pratt, 1964).

The pattern of penetration in bacterial viruses is, therefore, to make an efficient attachment to the bacterial surface, through specific recognition by a viral receptor protein, and then to negotiate the barrier afforded by the cell wall, using either a viral or a bacterial structure. The purpose of the present study was to investigate the details of penetration by the bacteriophage ØX 174. (Sertic and Boulgakov, 1935). The initial stage is typically specific, because the virus will adsorb only to receptors in the lipopolysaccharide layer, in cell walls of E. coli strain C, but not to E. coli B, or K12.

(Fujimura and Kaesberg, 1962; Sinsheimer, 1968). The structure of the viral coat protein subunits involved in adsorption has also been established. øX is a simple icosahedron, (Hall, Maclean and Tessman, 1959) in which the 12 coat-protein subunits are regularly arranged around the single strand of DNA (Sinsheimer, 1959a, 1959b; Freifelder, Kleinschmidt & Sinsheimer, 1964). Genetic analysis has shown that mutants of øX can be classified in 8 complementation groups
of which cistrons II, III and IV appear to be concerned with the formation of the viral coat proteins (Sinscheimer, 1968). The major unit of the capsid is believed to be coded for by cistron IV, but electron microscopy has shown that there are surface "spikes" at the 12 apeces of the icosahedron (Tromans and Horne, 1961). The spike polypeptides are encoded in cistrons II and III (Sinsheimer, 1968). Mutant viruses in which cistron II proteins are altered are not infectious, (Hutchinson, Edgell, Sinsheimer, 1967) suggesting that the spikes are responsible for attachment of øX to its host. Electron micrographs of adsorbed øX support this, because the virus are orientated so that one spike is in contact with the cell surface (Stouthamer, Daems, Eigner, 1963). Further details have also been obtained from acrylamide gel electrophoresis of whole øX particles (Hutchinson et al, 1967). In addition to showing different host ranges mutant particles often show different mobilities on gels. If sensitive bacteria are infected with two viral strains of different mobility, progeny

virus with all the intermediate mobilities are produced, suggesting phenotypic mixing of coat protein units within each particle. Hutchinson et al (1967) showed that the electrophoretic mobility of a virus particle can be used to calculate the relative proportions of the differently charged subunits, and hence the probability that the particle will have an altered host range. The relationship so obtained fits that expected if a single adsorption site contains five subunits. All five subunits in a given site must be of one kind for successful adsorption to a particular host strain. The figure is consistent with the five-fold symmetry of ϕX, and with suggestions that the spike is responsible for adsorption and consists of 5 subunits visible in the electron microscope (Sinsheimer, 1968).

The relatively simple structure of ϕX, in which the coat consists of a symmetrical arrangement of 12 identical subunits, each made up of five groups of three polypeptides, confers the advantage that it can attach through any of the 12 spikes. The unusual feature about ϕX, and closely related forms such as S13 (Burnet, 1927; Tessman, 1959) ϕR, (Kay, 1962) and α3 (Bradley, 1968) is, that although they have no tail with which to penetrate the cell wall barrier, they do not appear to use any special bacterial structure either, although they adsorb directly to the main surface of the bacteria. The question arises: is the DNA released from its viral coat, before passing, unprotected, into the host, or does the whole virus penetrate the wall, and enter

the cell, before being uncoated by a mechanism similar to that found in animal cells?

Investigation of penetration by ØX cannot be approached by the classical method for "tailed" bacteriophages, introduced by Hershey and Chase (1952). When they demonstrated, with T_2-phage, that only the viral DNA entered the host, they removed the empty protein coats from the infected bacteria by agitating the suspension in a Waring blendor. ØX is so small (diameter 248 $\pm$ 10 Å), or becomes so firmly embedded in the cell wall, that, once attached, it cannot be removed by shearing forces like those imposed upon T_2. Experiments have therefore involved less direct methods, for example, that developed from results of work by Fujimura and Kaesberg (1962). They investigated the details of adsorption in ØX, and showed that the virus will adsorb to isolated cell walls of E. coli C, under the same conditions as it does to intact cells of the bacterium. This suggests that all the bacterial components essential for attachment are present in the cell wall. The system, therefore, provides a means of separating the stages of adsorption and penetration of the cell wall, from entry of DNA into the cell. It provides a model system for the study of these initial steps of penetration, without the results being complicated by any of the subsequent events of infection.

Another useful system is relevant to the stage of viral entry into cells. Phage can reproduce successfully in host cells which

have been converted to spheroplasts (Fraser, Mahler, Shug and Thomas, 1957). It has also been demonstrated (Guthrie and Sinsheimer, 1960; Carusi and Sinsheimer, 1963) that isolated, single-stranded ØX-DNA, and DNA extruded from ØX particles which have been heated to 70° can infect spheroplasts of E. coli K12 strains. Spheroplasts can be used, therefore, to identify any particles, which, after adsorption to cell walls, are competent to permit infectious DNA to pass into cells. The system is also important because it is consistent with the theory that the cell membranes are ultimately responsible for the uptake of foreign nucleic acids. Although intact ØX cannot adsorb to, or infect spheroplasts, it does not preclude entirely the possibility that the bacterial membrane can also separate the viral protein from the nucleic acid. An alteration of particle configuration at adsorption, for example, might be the change needed to trigger such an uncoating.

A disadvantage of both the spheroplast and the cell wall systems is that they are subject to the limitations of all biological models and simplified systems. Any results obtained may be artefacts peculiar to the system, and not representative of what happens in vivo. Information obtained from such sources must therefore be corroborated by alternative approaches.

The rationale behind the following experiments was to use the systems afforded by cell walls and spheroplasts to elucidate the

sequence of events leading to successful penetration of the cell wall, and entry by ØX into the host cells. This conferred the advantage of simplicity, but the results could be checked by experiments involving intact bacteria.

MATERIALS & METHODS

MATERIALS

Bacteriophage

Wild type ØX, and the mutant ØX am3 were kindly provided by Prof. R. L. Sinsheimer, California Institute of Technology, Pasadena. ØX am3 contains an amber mutation in the lysis gene, cistron I (Hutchinson & Sinsheimer, 1966).

Bacteria

Escherichia coli C is the usual host strain for ØX. It is restrictive for ØX am 3 . The virus can penetrate and multiply in this strain, but there is no lysis.

Escherichia coli CR34/C416 thy$^-$ is the permissive host for ØX am 3 , and is derived from a cross between a K12 strain containing an amber suppressor, and a C strain, susceptible to ØX (Denhardt and Sinsheimer, 1965). Wild type ØX will also grow in it.

Escherichia coli strains K12W6; B; B/2 are resistant to ØX.

E. coli C; CR34/C416; K12W6 were kindly provided by Prof. R. L. Sinsheimer; E. coli B; B/2 by Prof. K. Burton.

Media

Hard Agar Sinsheimer (1959a)

Soft Agar Sinsheimer (1959a)

Fraser and Jerrell's Glycerol Medium. Fraser & Jerrell (1953)

TKB broth Sinsheimer (1959a)

Tris-EDTA buffer Tromans and Horne (1961)

Phage dilution fluid

NaCl	0.1	M
$MgCl_2$	0.01	M
gelatin	0.07%	

Adsorption media

(a) For measuring adsorption, and DNA release from øX.

NaCl	0.1	M	
$CaCl_2$	0.01	M	
or $MgCl_2$	0.1	M	
Tris-HCl	0.1	M	(pH 7.2 - 9.0)
or DMGA-NaOH	0.1	M	(pH 4.0 - 7.0)

(b) For use in experiments with spheroplasts.

KCl	0.01	M	
$MgSO_4$	0.01	M	
Tris-HCl	0.1	M	pH 7.2

(c) For use in experiments using sucrose density gradients.

KCl	0.01	M	
$CaCl_2$	0.01	M	
Tris-HCl	0.1	M	pH 7.2

Low phosphate TPA medium

Protoplast nutrient medium (PA medium) Guthrie and Sinsheimer (1960)

Protoplast nutrient medium + 0.2% $MgSO_4$ (PAM medium) Guthrie and Sinsheimer (1960)

Spheroplast stock medium (SS medium) Guthrie and Sinsheimer (1963)

0.35 ml	1.5 M	sucrose
0.17 ml	30%	BSA

0.02	ml	2 mg/ml lysozyme, in 0.25 M tris-HCl, pH 8.1
0.04	ml	4% EDTA
10.00	ml	PA medium

3 x D medium Fraser & Jerrell (1953)

Soft agar, for protoplasts Guthrie and Sunsheimer (1960)

Scintillation fluids

(a) Toluene based:

7 gm. Butyl PBD, per litre toluene

(b) Miscible with aqueous solvents (Ciba 4 scintillant)

7	gm per	litre	Butyl PBD
80	gm "	"	Naphthalene
400	ml "	"	Methoxyethanol
600	ml "	"	Toluene

METHODS

Assessment of Bacterial Numbers

Cell numbers were estimated in a Helber counting chamber of 5×10^{-5} mm^3 volume (Hawkesley).

Viable counts were made by spreading known volumes of bacterial suspensions on the surfaces of hard agar plates. Comparison of the two methods showed, that in growing cultures, 50% cells counted in the chamber were viable.

Estimation of Viral Numbers

Viable virus particles were assayed by the agar layer technique (Gratia, 1936; Adams, 1959). As øX forms plaques very quickly the plates were incubated at 37° for only 3 - 4 hours. The method was accurate to 100 ± 7 p.f.u.

/The total number of particles was estimated spectroscopically (Sinsheimer, 1959a). A value of $E_{260} = 8.14$ is given by a øX suspension at 1 mg/ml.

Preparation of øX

øX-wt were prepared by a modification of the method of Sinsheimer (1959a). Homogeneous, low titre lysates of $1\text{-}5 \times 10^8$ øX/ml. in 25 ml. volumes were made from single plaque inocula (Doermann and Hill, 1953). These stocks were used to prepare lysates for subsequent inoculation of larger volumes. E. coli C was grown in 250 ml. Fraser and Jerrell's glycerol medium to a titre of 5×10^8 cells/ml. The culture was aerated at 37°, by swirling in a one-litre Ehrlenmeyer flask. øX were added at an m.o.i. 10, and incubation continued until lysis occurred 45 - 60

mins. later. Foaming was controlled with Silicone MS Antifoam Emulsion. At lysis 12.5 ml of 0.01 M EDTA were added to prevent re-adsorption of virus particles to bacterial debris, and after a further 30 mins. aeration at 37°, a few grains of solid DNase, RNase and lysozyme were added to degrade extraneous nucleic acids and cell wall material. The lysate was swirled at 37° for 30 mins, and then shaken with $CHCl_3$ to complete lysis. The chloroform and cell debris were removed by centrifugation. Titres of such lysates were 1 - 5 x 10^{11} p.f.u./ml.

Purification of øX

øX was concentrated and purified by a modification of the method of Tromans and Horne (1961).

Solid ammonium sulphate was added to the crude lysate to give 50% saturation, and the suspension was left at 4° for 24 hours. The precipitate was removed by centrifugation at 30,000 g, for 30 mins. The pellet, which contained 80 - 100% of the total virus, was resuspended in 12 mls. Tris-EDTA buffer, and insoluble material removed by centrifugation at 5000 g, for 10 mins. This pellet was washed twice in 10 ml. of the same buffer and the combined washings and the first Tris-EDTA supernatant were then centrifuged at 100,000 g, for 2 hrs. The pellet of phage, so obtained, was resuspended in saturated $Na_2B_4O_7$ solution, pH 9.1. This suspension contained 20 - 30% of the total virus.

The virus were further concentrated, and 70S particles were

removed by density gradient equilibrium centrifugation (Sinsheimer, 1959a). Solid CsCl was added to the suspension of øX, to give a solution of density 1.44 (0.625 gm CsCl per gm. fluid). The suspension (13.5 ml.) was centrifuged at 0°, for 45 hrs., at 90,000 g, in the 40 angle rotor of the Spinco Model L centrifuge. Fractions of 10 drops each were collected from a hole made in the bottom of the tube. Samples with the greatest titres were combined and dialysed against saturated $Na_2B_4O_7$. The purified virus was stored at 4°. The final recovery was about 5% of the virus in the original lysate.

Estimation of the Purity of øX

The purity of øX preparations was checked using spectral criteria (Sinsheimer, 1959a). See Table 1.

Preparation of øX am3 Sedat and Sinsheimer (1964)

For small scale preparations, single plaque isolates of phage were made on the permissive host E. coli CR34/C416. These were used as inocula for larger preparations. E. coli C was grown in 100 ml. Fraser and Jerrell's glycerol medium to a titre of 3×10^9 cells/ml. øX am3 was added at an m.o.i. 1. Incubation was continued for 3 - 4 hrs., but there was no lysis. The bacteria were harvested by centrifuging at 12,000 g for 25 mins, and then resuspended in 5 ml. 0.25 M Tris, pH 8.1. Lysozyme solution (0.1 ml at 10 mg/ml in 0.25 M tris, pH 8.1) and EDTA (1.0 ml, 8% solution) were added, and the suspension left at 0°

for 15 min. It was kept at -20° overnight. After thawing, the suspension was taken through two cycles of alternate freezing and thawing, solid CO_2 and ethanol being used for the freezing mixture. A little solid RNase was added,

and the mixture was incubated at 37° for 15 min. DNase was added, with 2 ml. 0.2 M $MgSO_4$, and the mixture was incubated for a further 15 min. This reduced the viscosity. Debris was removed by low speed centrifugation. Titration on E. coli CR34/C416 indicated a total yield of 2×10^{14} p.f.u.

Titration on E. coli C showed between 0.1 - 0.5% virus were revertants to the wild type. To maintain a pure line of øX am3 it is necessary to keep the level of wild type below 0.01%, but this was not rigorously observed, because øX am3 was not used for a study of its mutant properties, but to provide high titre stocks of the virus. When the mutant is prepared in a restrictive host, the yield is 100-200 fold greater than that obtained with preparations of wild type phage.

Purification of øX am3

Since the lysate volume was less than 12 ml., concentration by $(NH_4)_2SO_4$ precipitation was omitted and preparations were purified just by CsCl density gradient centrifugation, as described for wild type preparations.

Preparation of ^{32}P-labelled øX am3

E. coli C was grown in 20 ml. of low phosphate TPA medium to

a concentration of 3 x 10^9 cells/ml. 20 m c carrier-free ^{32}P-phosphate was added in 2 ml dilute HCl, pH 2 - 3. ØX am3 was added at an m.o.i 5. Incubation was continued for 3 hrs. The cells were harvested by centrifuging, and then treated in the same way as the unlabelled ØX am3. The total number of virus recovered was 1.4 x 10^{12} p.f.u. Preparations of lower specific activity (see Table 2) were made by adding 2mC ^{32}P-phosphate to 20 ml. E. coli C.

Preparation of ^{35}S-labelled ØX am3

^{35}S-labelled ØX am3 was prepared in a similar way to ^{32}P-labelled ØX am3. 2mC ^{35}S-sulphate were used for 20 ml culture medium.

Preparation of ^{35}S- ^{32}P-labelled ØX am3

2mC ^{35}S-sulphate and 2mC-^{32}P phosphate were added simultaneously to a 20 ml culture of E. coli C, before the ØX am3 inoculum was added.

Purification of labelled ØX am3

The labelled ØX am3 were purified in the same way as unlabelled ØX am3.

Growth of E. coli C for Adsorption Experiments.

E. coli C was grown in 100 ml. Fraser and Jerrell's glycerol medium to 5 x 10^8 - 1 x 10^9 cells/ml. The cells were centrifuged at 5000 x g for 10 min, washed once in sterile distilled water,

Table 1. Spectral characteristics of ϕX preparations.

Virus	Preparation no.	λ max.	λ min.	E_{260}/E_{280}
ϕX-wt	(Sinsheimer, 1959)	260	240	1 : 1.53
ϕX-wt	1	260	240	1 : 1.50
	2	260	242	1 : 1.49
ϕX-am3	1	260	239	1 : 1.47
	2	260	241	1 : 1.48
	3	260	240	1 : 1.52
^{32}P-ϕX-am3	1	260	240	1 : 1.51
	2	260	240	1 : 1.52
	3	260	242	1 : 1.46
^{35}S-ϕX-am3	1	260	239	1 : 1.50
	2	260	240	1 : 1.49
^{35}S-^{32}P-ϕX-am3	1	260	241	1 : 1.48
	2	260	242	1 : 1.52

Measurements were made on a Unicam SP500 spectrophotometer.

Table 2. Initial Specific Activities of Labelled ØX-preparations.

Virus	Preparation no.	^{32}P cpm / viable particles	^{35}S cpm / viable particles
^{32}P-labelled ØX-am3	1	1.15×10^{-4}	-
	2	1.55×10^{-4}	-
	3	4.2×10^{-6}	-
^{35}S-labelled ØX-am3	1	-	1.7×10^{-6}
	2	-	2.1×10^{-6}
^{35}S-^{32}P-labelled ØX-am3	1	1.4×10^{-6}	5.0×10^{-6}
	2	2.0×10^{-6}	3.6×10^{-6}

Measurements were made in a Beckman Scintillation Counter.

and then resuspended in 100 ml sterile water (Fujimura and Kaesberg, 1962). They were stored at 0°, and used within several hours of harvesting.

For control experiments, suspensions of E. coli B and B/2 were prepared in the same way.

Preparation of Isolated Bacterial Cell Walls

For the preparation of cell walls, bacteria were grown in 15 litre batches. A 1 litre overnight culture of E. coli C at 1×10^9 cells/ml. in Fraser and Jerrell's glycerol medium was added to 14 litres of pre-warmed medium in a 20 litre flask. The culture was aerated at 37° until it reached a cell density of 1×10^9/ml. The bacteria were harvested by centrifuging at 2000 g, for 20 min., and resuspended in 300 ml water. Twenty-ml. batches of cells were mixed with 30 ml. of Grade 12 Ballotini beads and broken in a Braun disintegrator, at setting 1. (B. Braun, Apparatebau, Melsungen). The apparatus was cooled by a stream of CO_2 gas. The glass beads and unbroken cells were removed by passing the suspension through a coarse, sintered glass filter, and then centrifuging at 5000 g, for 10 min. The cell walls were harvested by centrifuging at 34,000 g, for 15 min, and re-suspended in 100 ml water. The unbroken cells were again treated in the disintegrator, and the resultant cell walls added to the main batch. All the walls were washed twice, and finally resuspended in water. The suspension was stored at 4°, over $CHCl_3$.

Cell walls from E. coli B were made in the same way, but

grown in TKB broth.

Morphology of Cell Walls

Cell wall preparations were examined by electron microscopy. See p.27 .

Plate 1 illustrates 2 typical fragments of cell wall, found in preparation from E. coli C.

Estimation of the Contents of Cell Wall Preparations

(a) Dry Weight and Amino Acids

To standardise different preparations of cell walls, samples were taken from each for estimations of the dry weight and hexosamine content (Table 3); 0.1 mg. dry weight was used as a unit of cell wall material.

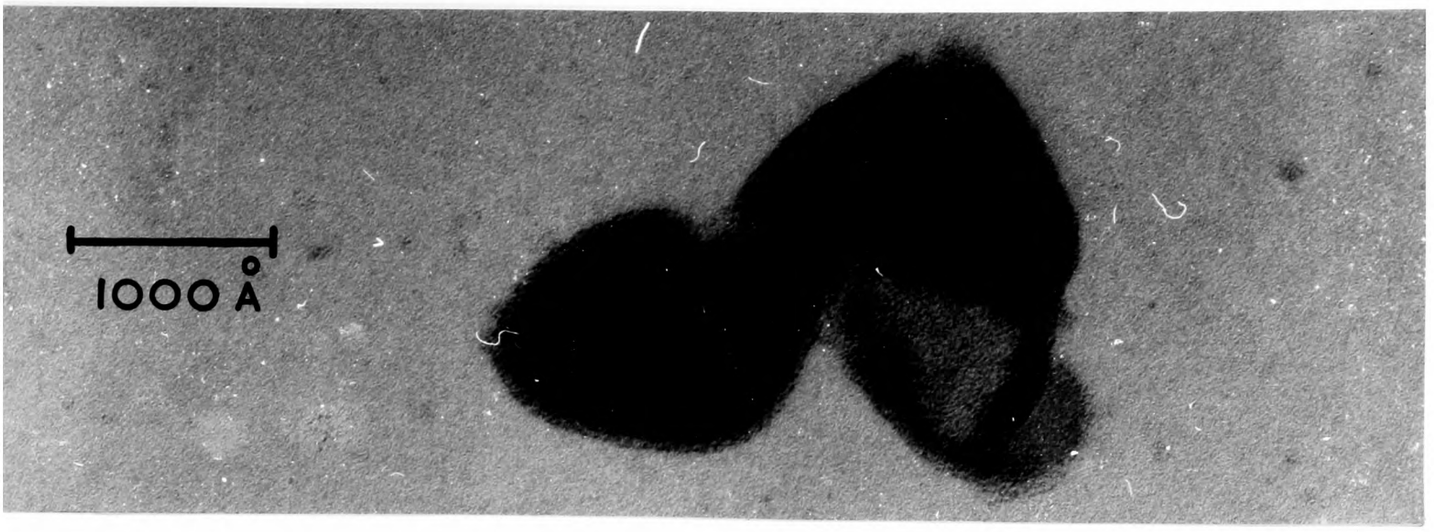
(b) Contaminating materials

Each millilitre of cell walls represented about 5×10^{10} - 5×10^{11} original bacteria. Since the maximum, final number of viable bacteria was 75/ml., it was assumed that very few unbroken cells remained in the suspension after treatment.

Colorimetric tests for RNA and DNA (Table 3) indicated that the cell wall preparation contained no detectable ribose and not more than 2 mμgm. of DNA deoxyribose per mg. of cell walls. This suggested that there was little contamination from cytoplasmic material.

Plate 1. Electron micrograph of fragments from isolated cell walls of E. coli C.

Cell walls were fixed in 0.25 M formaldehyde and 1.5×10^{-3} $MgCl_2$ for 5 mins. Uranyl acetate (1% solution) was used as a negative stain. Samples were examined in Phillips 200 Electron Microscope Magnification, x 222,000.


1000 Å

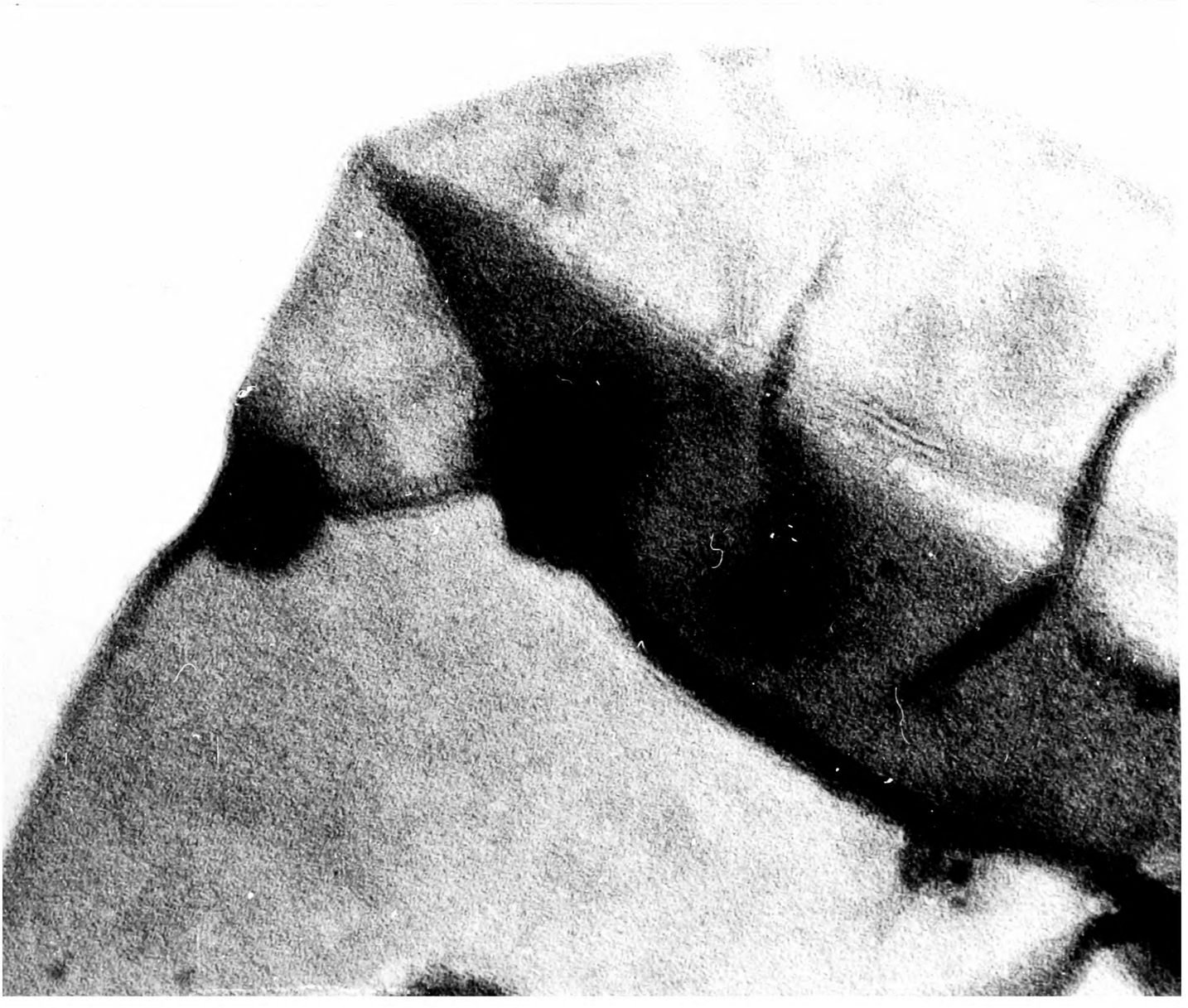


Table 3. Contents of Cell Wall Preparations

Preparation	No.	Dry weight (mg/ml)	Hexosamine (µg/mg.dry weight)(1)	Deoxyribose (µg/mg. dry weight (2)	Ribose (µg/mg.dry weight (3)	Whole cells (/ml)
E. coli C	1	1.31	3.82	0.02	0	10
	2	1.00	3.00	0.01	0	40
	3	15.15	0.99	0.01	0.001	75
	4	14.17	1.06	0	0	60
E. coli B	1	5.29	3.41			34
E. coli B/2	1	6.44	2.88	0.02	0.001	26

(1) Estimated by the method of Elson & Morgan (1933)

(2) Estimated by the method of Burton (1956)

(3) Estimated by the method of Ogur & Rosen (1950)

Preparation of spheroplasts from E. coli K12W6

Spheroplasts were prepared from E. coli K12W6 by the method described by Guthrie and Sinsheimer (1963). The stock remained active for several hours, at 0°.

Isolation of ϕX-DNA

DNA was extracted from a suspension of ϕX (1 ml. at 10^{12} - 10^{13} p.f.u.), with borate-saturated re-distilled phenol, at 70° (Huppert, Wahl & Emerique-Blum, 1962). The phenol was removed with ether at 0°, and the DNA dialysed against 0.05 M tris, pH 8.1.

Estimation of the Efficiency of Extraction

The relationship:
1 absorbancy unit at 260 mμ ≡ 1.22×10^{13} molecules DNA in 0.1 M tris, pH 8.1, 25° (Guthrie and Sinsheimer , 1963) indicated that 40 - 45% DNA molecules were recovered from ϕX treated with hot phenol.

Estimation of Purity of DNA

DNA was characterised by its ultra-violet absorption (Table 4)

Infectivity of Isolated ϕX DNA in spheroplasts

The infectivity of isolated ϕX DNA in spheroplasts is demonstrated by the increase in the number of phage after incubating the infected spheroplasts for 90 - 120 min. (Guthrie and Sinsheimer, 1960). Spheroplasts kept in complex media reconstitute

Table 4. Spectral criteria for the purity of ØX DNA.

DNA preparations	λ min.	λ max.	E_{260}/E_{280}
Sinsheimer (1959,b)	230	260	1.88
1	240	265	1.30
2	245	260	1.28
3	240	260	1.42
4	230	260	1.79
5	230	259	1.48
6	230	260	1.75

Measurements were made in a Unicam SP500 spectrophotometer.

some cell wall material, and if they are made from bacterial strains which are sensitive to ØX, both mature virus and free DNA can infect them. The effect of DNA alone is measured by using spheroplasts of E. coli K12W6, a strain resistant to infection by intact ØX particles.

When spheroplasts were treated with phenol-extracted ØX-DNA, results similar to those of Guthrie and Sinsheimer were obtained. The number of phage produced per spheroplast is directly proportional to the number of DNA molecules added over a range 10^6 - 10^{10} molecules/ml. (Figure 1).

ØX heated to 70° (10^{10} particles/ml. in 0.05 M tris, pH 8.1) were diluted into spheroplasts and incubated in the same way as the isolated DNA and spheroplasts. Control tubes contained ØX and spheroplasts. The results are shown in Table 5.

Preparation and Use of Sucrose Gradients

Linear gradients (5 ml volume) of 5 - 30% sucrose in 5 x 10^{-2} M tris, pH 8.1, and 3 x 10^{-3} M EDTA were made directly in Spinco centrifuge tubes. Samples (0.1 - 0.2 ml) containing ØX, and its derivatives were layered onto the surface, and the gradients were centrifuged at 0 - 4 ° for 2 hours, at 100,000 g (Spinco Model L SW39 or SW50 rotor). Two-drop fractions were collected through a hole made in the bottom of the tube.

Test for the linearity of the gradient

A 5 - 30% gradient containing no ØX samples was centrifuged

Figure 1. The number of progeny ØX produced by spheroplasts, per infecting molecule of ØX DNA.

Samples of ØX DNA (prepared as described on p. 22) containing from 5×10^5 to 5×10^{10} molecules per ml. in tris-HCl, 0.05 M, pH 8.1, were incubated at 37° with an equal volume of spheroplasts of E. coli K12W6 at 5×10^8/ml in PA medium for 15 min. 1 ml samples were diluted with 4 ml PAM medium and incubated at 37°, for 90 mins. The final number of p.f.u. produced by each concentration of DNA was estimated.

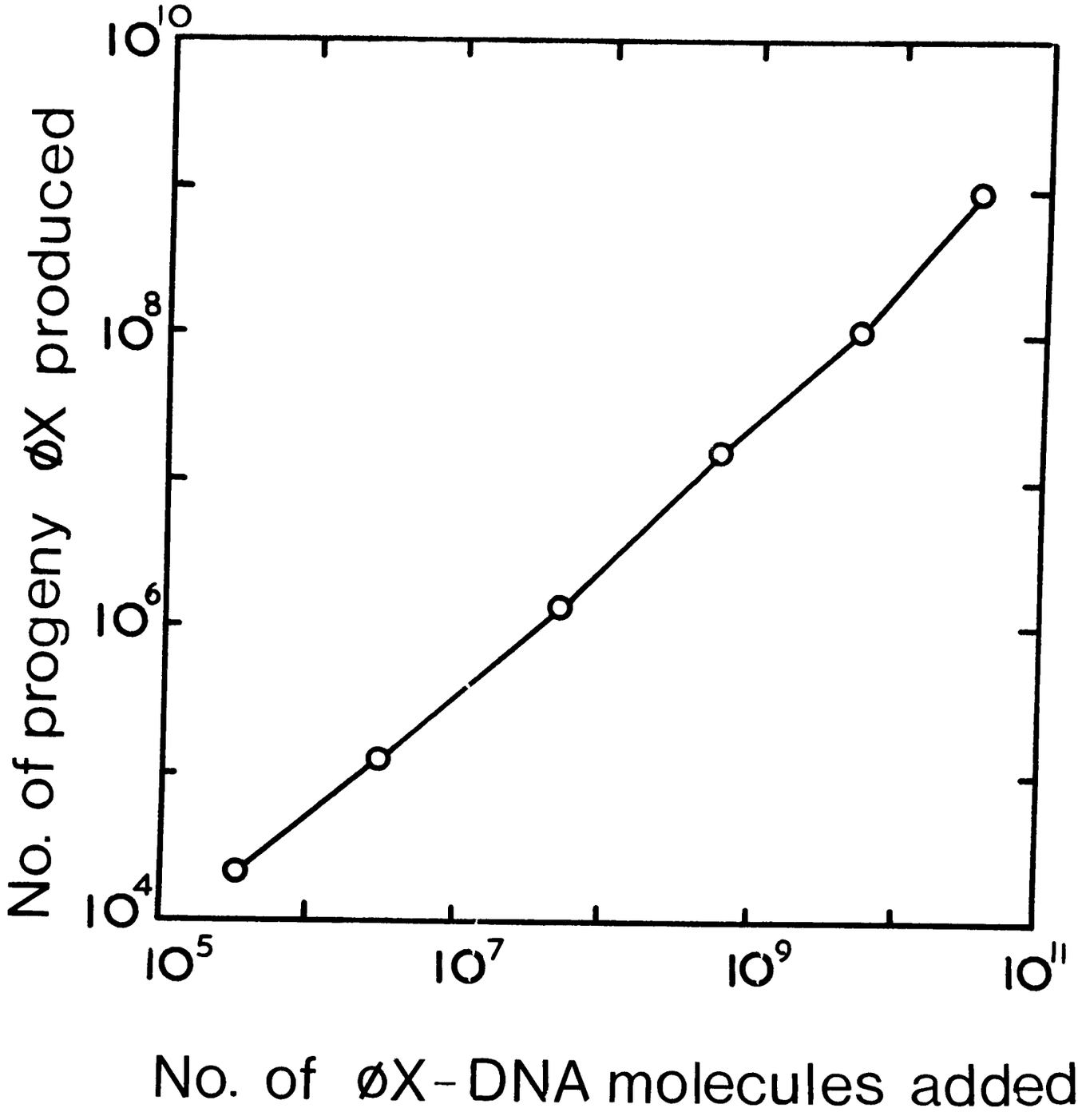

No. of progeny øX produced
10^10
10^8
10^6
10^4
10^5
10^7
10^9
10^11
No. of øX-DNA molecules added

Table 5. Incubation of '70°-øX' with spheroplasts.

øX (2.6×10^9 p.f.u./ml. in 5×10^{-2} M tris, pH 8.1) were heated to 70° for 10 min. They were incubated at 37° with spheroplasts of E. coli K12W6 (2×10^8 / ml. in SS medium) for 15 min. 1 ml. samples were diluted into 4 ml PAM medium and incubated for 90 min. at 37°. The number of p.f.u. was estimated, before and after incubation. øX kept at 37° for 10 min were used as controls.

Sample	p.f.u. /ml.
original conc. øX	2.6×10^9
viable virus after 10 min at 37° ('37°-øX')	2.6×10^9
viable virus, after incubating '37°-øX' with spheroplasts	2.3×10^9
viable virus after 10 min at 70° ('70°-øX')	10^2
viable virus, after incubating '70°-øX' with spheroplasts	7.0×10^6

as described and four-drop samples were collected. The refractivity of each sample was then measured (Abbé Refractometer, Bellingham and Stanley, London). The results are shown in Figure 2. It was concluded that the gradient was linear in the region corresponding to fractions 9 - 35, when two-drop fractions were collected.

Determination of S-values

As the sucrose gradients were not linear for the whole length, it was impossible to determine sedimentation constants using the relationship:-

$\frac{Sx}{Sy} = \frac{Dx}{Dy}$, where Sx = sedimentation coefficient of an unknown particle x.

Sy = sedimentation coefficient of a given marker y.

Dx = distance moved through the gradient, by the particle x.

Dy = distance moved through the gradient, by the marker y.

(Martin, R. G. and Ames, M. (1961) J. Biol. Chem. 236: 1372)

Approximate S-values were obtained by comparing the positions on the gradients of particles derived from ϕX, with those of known markers. (Figure 3). Intact ϕX have an S-value of 114 (Sinsheimer, 1959a), and were therefore used as one marker. A sample of ^{3}H-labelled ribosomal particles was kindly provided by Mr. M. R. Blundell. These have S-values of 70, 50 and 30S, and thus gave

Figure 2. Linearity of sucrose density gradients.

The sucrose gradient (5 - 30% (w/v) in 5 x 10^{-2} M tris-HCl, pH 8.1, and 3 x 10^{-3} M EDTA) was run for 2 hrs. at 4^{o}, and 100,000g, in the SW50 rotor of the Spinco Model L centrifuge. 4 drop samples were collected, and the refractivity of each was measured in an Abbe Refractometer.

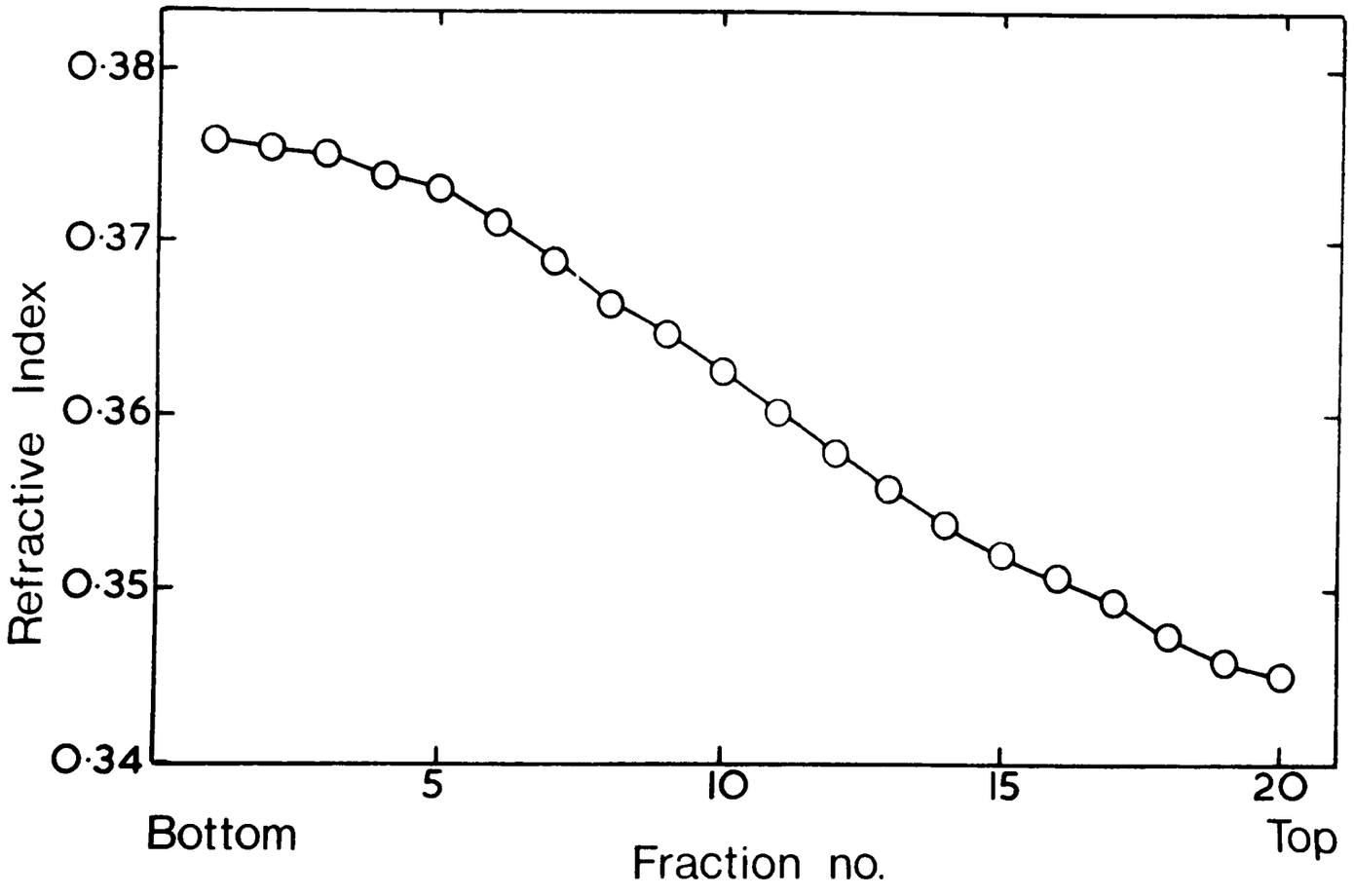
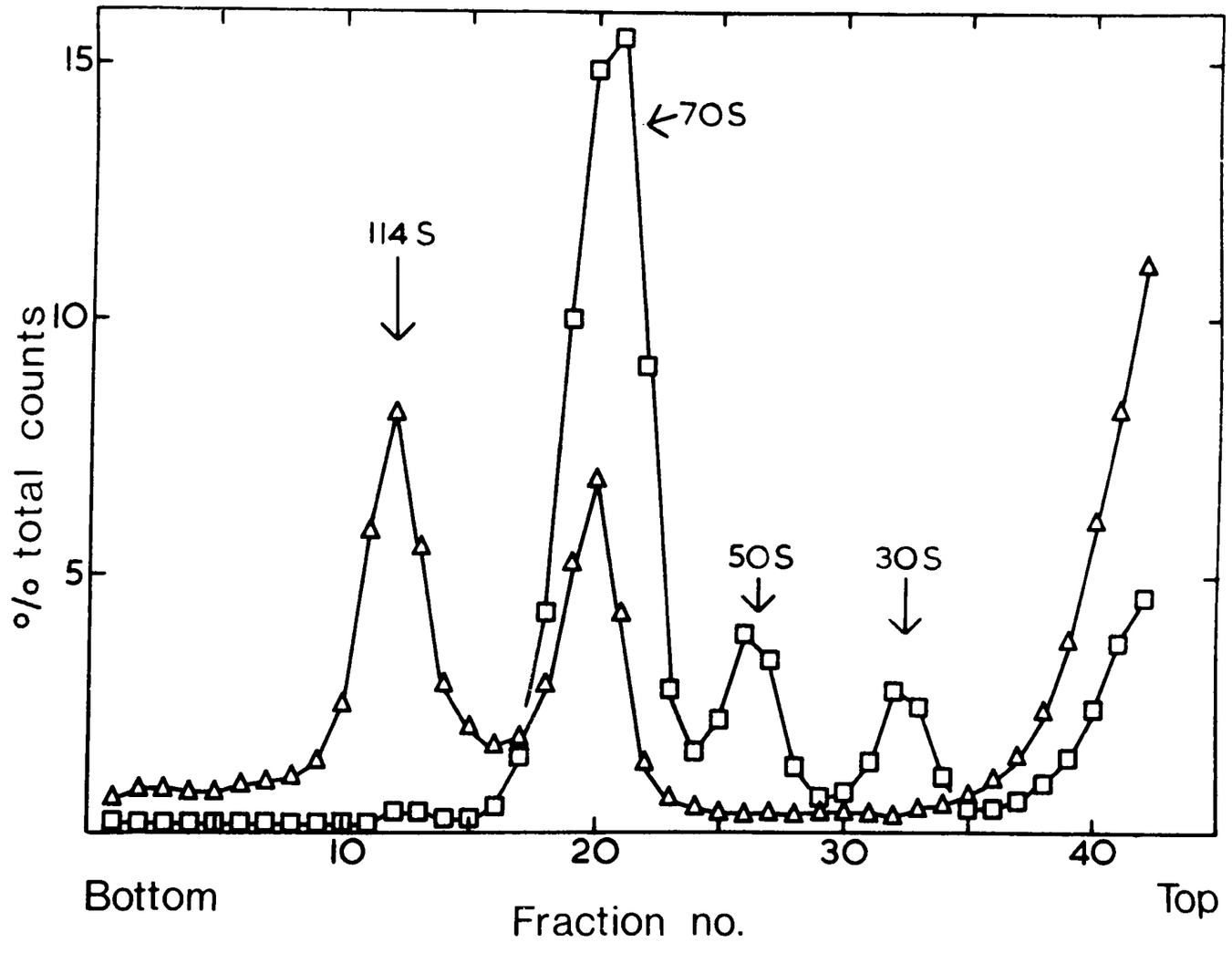

0·38
0·37
0·36
0·35
0·34
Refractive Index
5
10
15
20
Bottom
Top
Fraction no.

Figure 3. Sucrose density gradient analysis of ^{32}P-labelled øX, and ^{3}H-labelled ribosomal particles.

The sucrose gradients (5 - 30% (w/v) in 5 x 10^{-2} M tris-HCl, pH 8.1, and 3 x 10^{-3} M EDTA) was run for 2 hrs. at 4° and 100,000 g in the SW 50 rotor of the Spinco Model L centrifuge. 2 drop samples were collected for scintillation counting. The position of particles of known S-value are marked in the figure.

^{32}P-counts : —△——△—

^{3}H-counts : —□——□—


15
10
5
% total counts
114 S
70S
50S
30S
10
20
30
40
Bottom
Fraction no.
Top

three additional markers. Non-infectious particles, which were believed to correspond to Sinsheimer's "top component" of 70S (Sinsheimer, 1959a), were found at the same position as the 70S ribosomes.

The gradient collecting device used in all the experiments gave a consistent number of 2 drop fractions from 5 ml. gradients (42 $\pm$ 1 fraction), and, since there was close agreement between duplicate gradients containing the four markers, the results were used to provide an approximate calibration of S-values of particles obtained in subsequent sucrose velocity gradient experiments.

Preparation of samples for Electron Microscopy

Samples of øX particles and isolated cell walls were observed in a Phillips 200 electron microscope, by Dr. D. Kay (Sir William Dunn School of Pathology, Oxford University). They were fixed for 5 min. in 0.25 M formaldehyde, in the presence of 1.5×10^{-3} M $MgCl_2$. Uranyl acetate (1% solution) was used for negative staining (Hall, 1955; Kay, 1965).

Preparation of samples for counting radioactivity.

1. In a Lobetamat

In experiments measuring the proportion of acid soluble and acid insoluble material, ^{32}P-counts were determined in a Lobetamat (Isotope Developments Limited, Reading, England). Counts were proportional to the volume of solution added from 0.1 - 0.3 ml.

Samples (0.1 - 0.2 ml.) of solutions were placed on 2 cm aluminium planchettes. Those which contained NaOH were neutralised with N $HClO_4$. The samples were dried, before counting.

2. In a Scintillation counter.

The radioactivity in samples from sucrose gradients containing ^{32}P- and ^{35}S-labelled ØX was determined in a scintillation counter (Beckman Instruments, Inc.),using the ^{14}C-carbon; ^{32}P-phosphorus with ^{14}C-carbon; and ^{32}P-phosphorus isosets. Initially drops were collected directly into vials and dispersed in scintillant (a), but at high concentrations the sucrose formed insoluble crystals. Measuring ^{32}P- and ^{35}S-counts separately in the presence of increasing concentrations of sucrose showed that there was quenching, but that there was no concomitant alteration in the external standardisation values. Since this made it impossible to correct for quenching, it was found more satisfactory to collect the samples onto glass fibre strips (Whatman Paper Chromedia GF 82). When the samples had dried the filter paper was immersed in the toluene based scintillant (b). The ratio of ^{32}P-counts in Channels 1 and 2 was then 1.0 : 3.2 rather than 1.0 ; 2.2 for scintillant (a). The ratio was not altered by different concentrations of sucrose, ØX or cell wall material. Less than 0.1% ^{35}S-counts appeared in Channel 2 by either method. The material on the filter papers was not washed with TCA, because this reduced the counts from samples at the top of gradients. For

convenience the filter papers were put in scintillant in glass specimen tubes inside the counter vials. The counts (15,000 - 20,000 c.p.m. per sample in peak fractions) were corrected for background, and for overlap between the channels by the method of Bush (1964). For ease of comparison of different experiments the counts in each sample were presented as a percentage of the total counts of that isotope on the gradient.

Determination of the DNA content of ϕX particles.

In order to check differences in the ratio of DNA to protein in particles derived from ϕX, the ratio of $^{35}S/^{32}P$-counts was measured. For convenience in comparing particles obtained from different gradients the ratio for the 114S particles on each gradient was taken as 1.

EXPERIMENTAL PROCEDURE

and

RESULTS

Measurement of øX Adsorption

The initial work was to establish the optimum conditions for adsorption, and to confirm that these were the same for whole bacteria and isolated cell walls. It repeated work done by Fujimura and Kaesberg (1962) who incubated øX with E. coli C in solutions of $CaCl_2$ or $MgSO_4$ (10^{-3} - 10^{-1} M). They determined the rate of adsorption at 36° for different bacterial concentrations, and tested the effect of temperature (0 - 60°) and of pH (3 - 9) on the system. They altered the pH by adding either HCl or NaOH, but the solutions were not buffered.

In the present study, their work was repeated using øX at 5×10^4 p.f.u./ml. and E. coli C at 10^7 - 10^8 cells/ml., at 37°. Samples were taken at intervals and diluted through cold, $CHCl_3$-saturated dilution fluid to stop the reaction. Unadsorbed øX were measured by the plaque assay method. Control tubes contained no bacteria.

The high rate of inactivation in the absence of cell walls made it impossible to measure adsorption accurately (Figure 4). Addition of NaCl (0.1 M) and tris-HCl (0.1 M, pH 7.2) reduced the degree of inactivation. There was no fall in the numbers of p.f.u. when both were used, and the experiment was therefore repeated using adsorption medium (a). The controls contained E. coli B, no bacteria, or no divalent ions.

The results of the experiment are shown in Figure 5. There was no inactivation in the absence of either divalent ions, or E. coli C . The rate of adsorption remained constant at different

Figure 4. Inactivation of øX by different media.

øX (5×10^4 p.f.u./ml) were incubated at 37° in 0.1 M NaCl, 0.1 M $MgSO_4$, 0.1 M tris-HCl, pH 7.2, gelatin 0.07% solution (Adsorption medium (c)) —○———○— ;

0.1 M $MgSO_4$, 0.1 M tris-HCl, pH 7.2 —△———△— ;

0.1 M $MgSO_4$, 0.1 M NaCl —□———□— ;

0.1 M $MgSO_4$ —●———◎— .

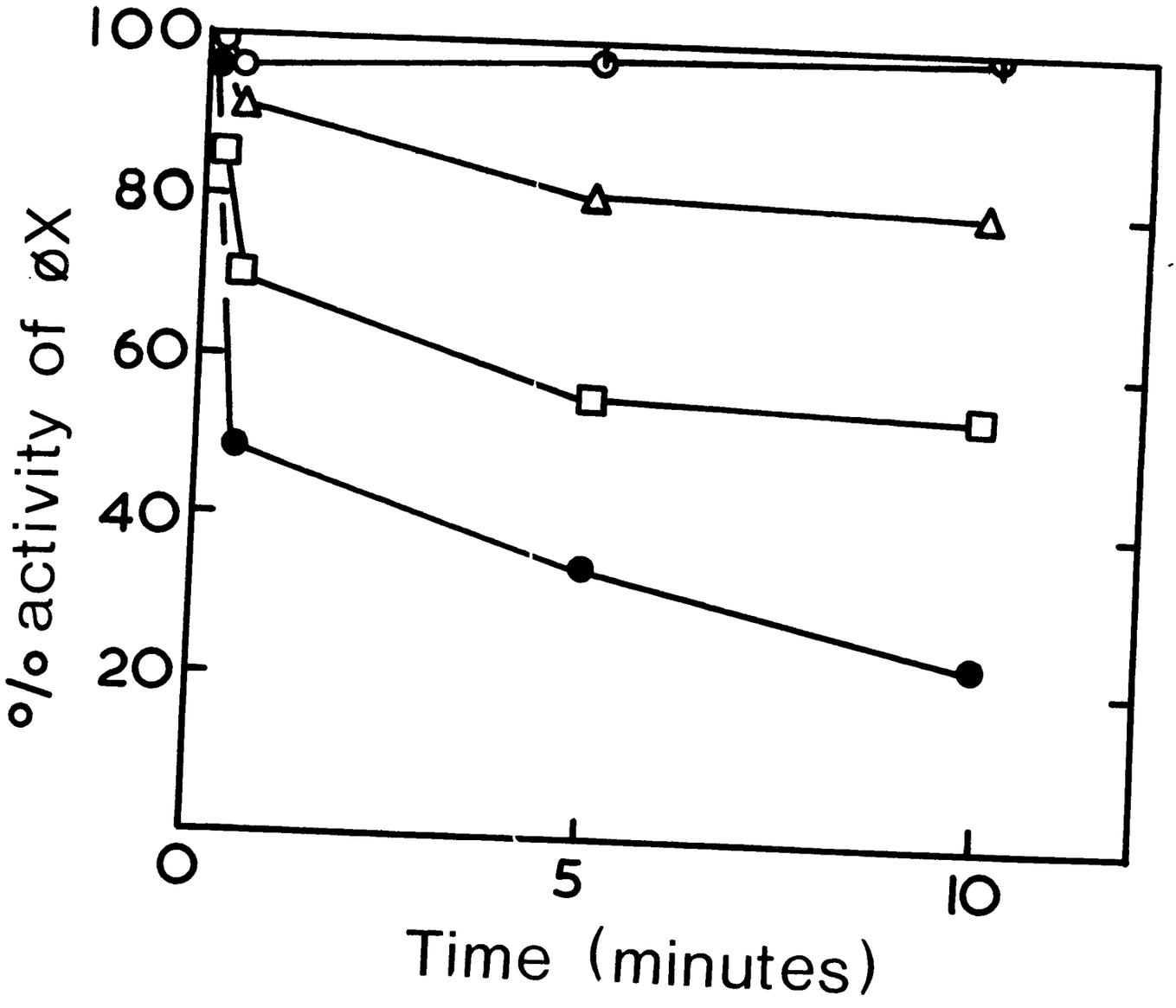
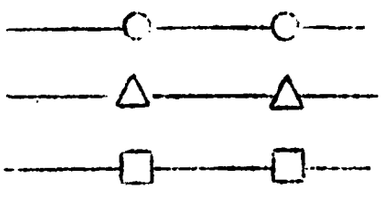

100
80
60
40
20
% activity of øX
0
5
10
Time (minutes)

Figure 5. Adsorption of ØX to E. coli C cells.

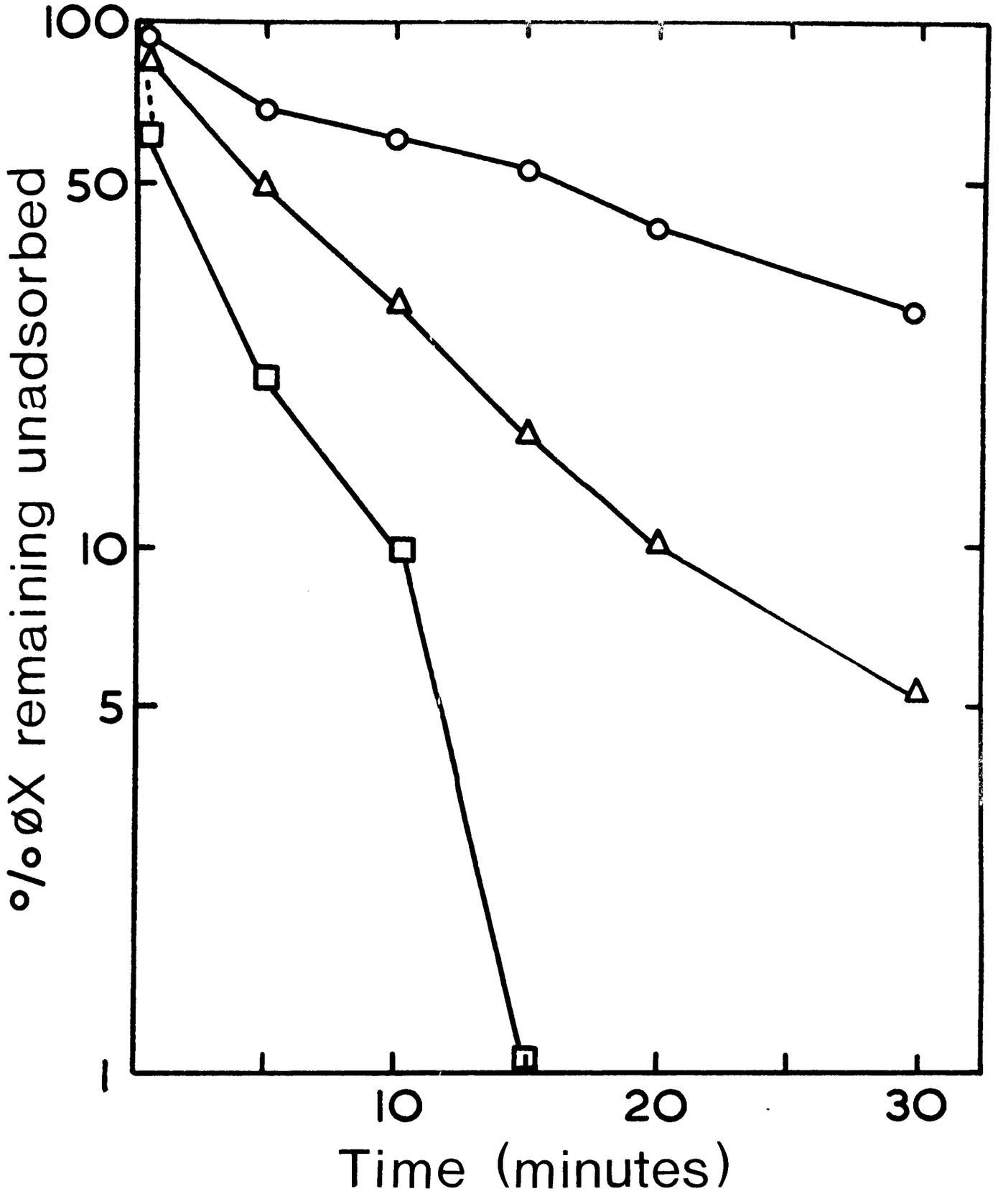
ØX (5×10^4 p.f.u./ml) were incubated at 37° in adsorption medium (a), with suspensions of E. coli C at the following concentrations:-



1×10^7/ml.

3×10^7/ml.

6×10^7/ml.


100
50
10
5
1
% ØX remaining unadsorbed
10
20
30
Time (minutes)

cell concentrations to over 90% adsorption. The rate constant K was determined from the equation

$$K = \frac{2.3}{Bt} \cdot \log \frac{Po}{Pt}$$

where B = bacterial concentration

t = time (sec)

Po = concentration of ØX added

P_t = concentration of ØX at time t

$K = 3.1 - 4.2 \times 10^{-9}$ ml. sec^{-1} $back^{-1}$

Fujimura and Kaesberg's value for $K = 1 \times 10^{-10}$ ml. sec^{-1} $bact^{-1}$

The results of altering the temperature are shown in Figure 6. The process is temperature-dependent, showing a maximum between 35 - 45°. The virus are inactivated at higher temperatures.

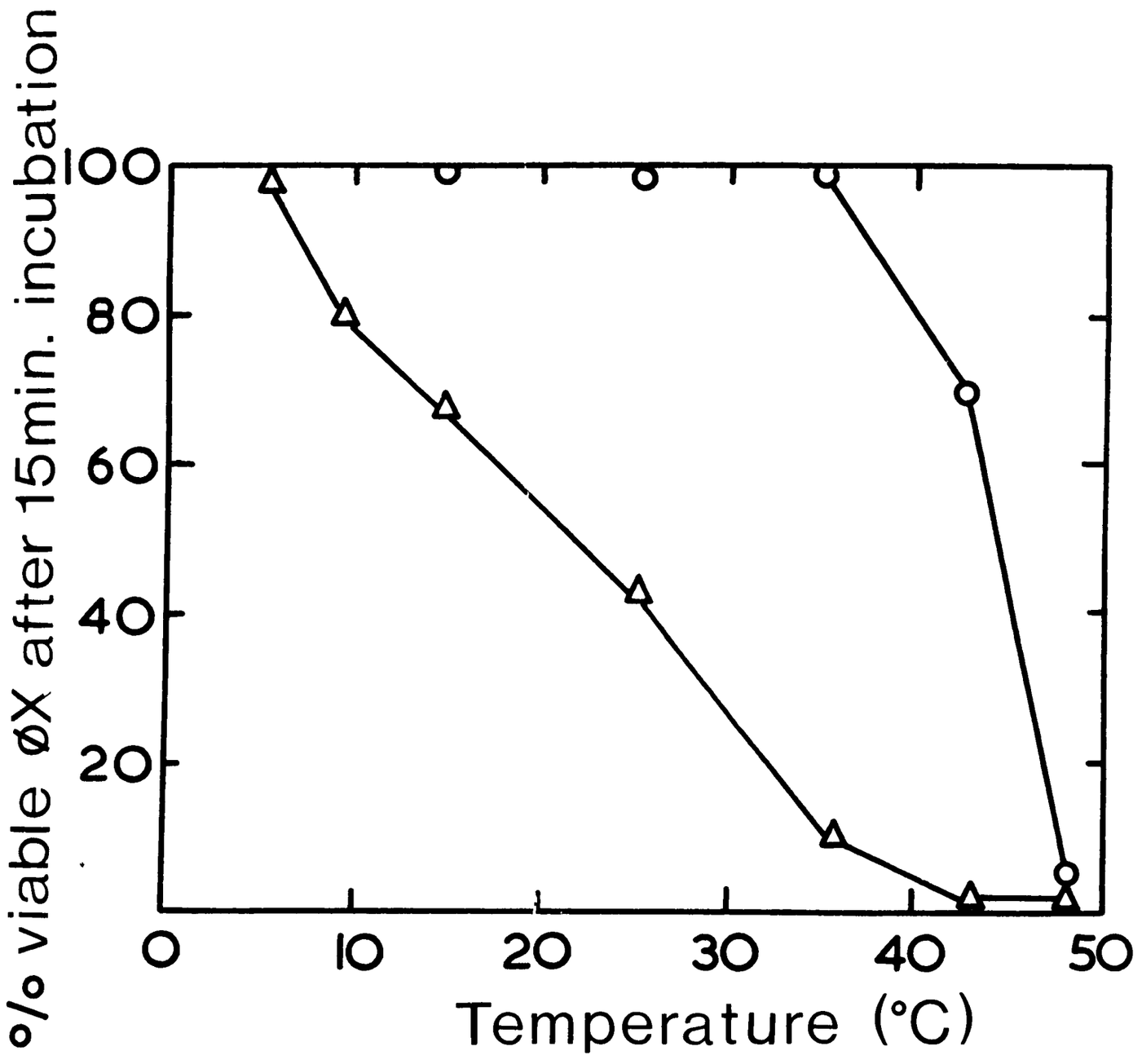
Variation in pH values was achieved by using buffered solutions. Tris-HCl was used for pH 7.2 - 9.0, and several buffers were tried for pH 5 - 7 (Figure 7). Since there was no inactivation with DMGA it was used for further experiments. The system was shown to be pH dependent, maximum adsorption occurring above pH 6.0 (Figure 8).

The reversibility of attachment was measured by washing cells after adsorption with either lysozyme-EDTA solution, pH 8.1, or $Na_2B_4O_7$-EDTA solution. The number of p.f.u. was estimated before and after washing (Figure 9).

Since no intact virus could be eluted, it was concluded that, under these conditions, adsorption was irreversible.

Figure 6. Adsorption of ØX to E. coli C cells at different temperatures.

ØX (5×10^4 p.f.u./ml) were incubated in adsorption medium (a) for 15 mins. with no cells ——o——o—— ; and with E. coli C at 1×10^8 cells/ml. ——△——△—— .


% viable ØX after 15min. incubation
100
80
60
40
20
0
10
20
30
40
50
Temperature (°C)

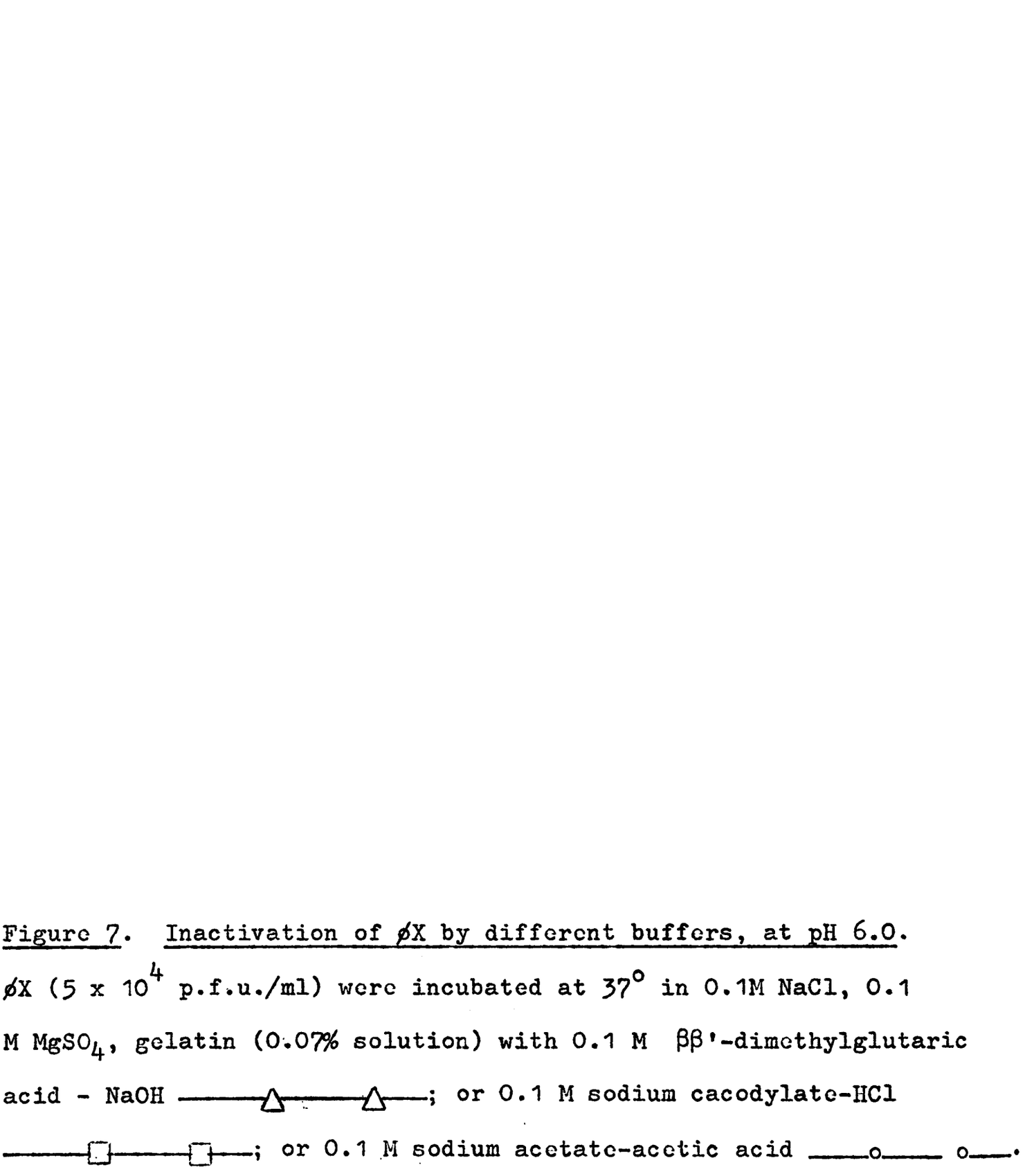


Figure 7. Inactivation of øX by different buffers, at pH 6.0.

øX (5×10^4 p.f.u./ml) were incubated at 37° in 0.1M NaCl, 0.1 M $MgSO_4$, gelatin (0.07% solution) with 0.1 M ββ'-dimethylglutaric acid - NaOH ——△——△——; or 0.1 M sodium cacodylate-HCl ——□——□——; or 0.1 M sodium acetate-acetic acid ——o—— o——.

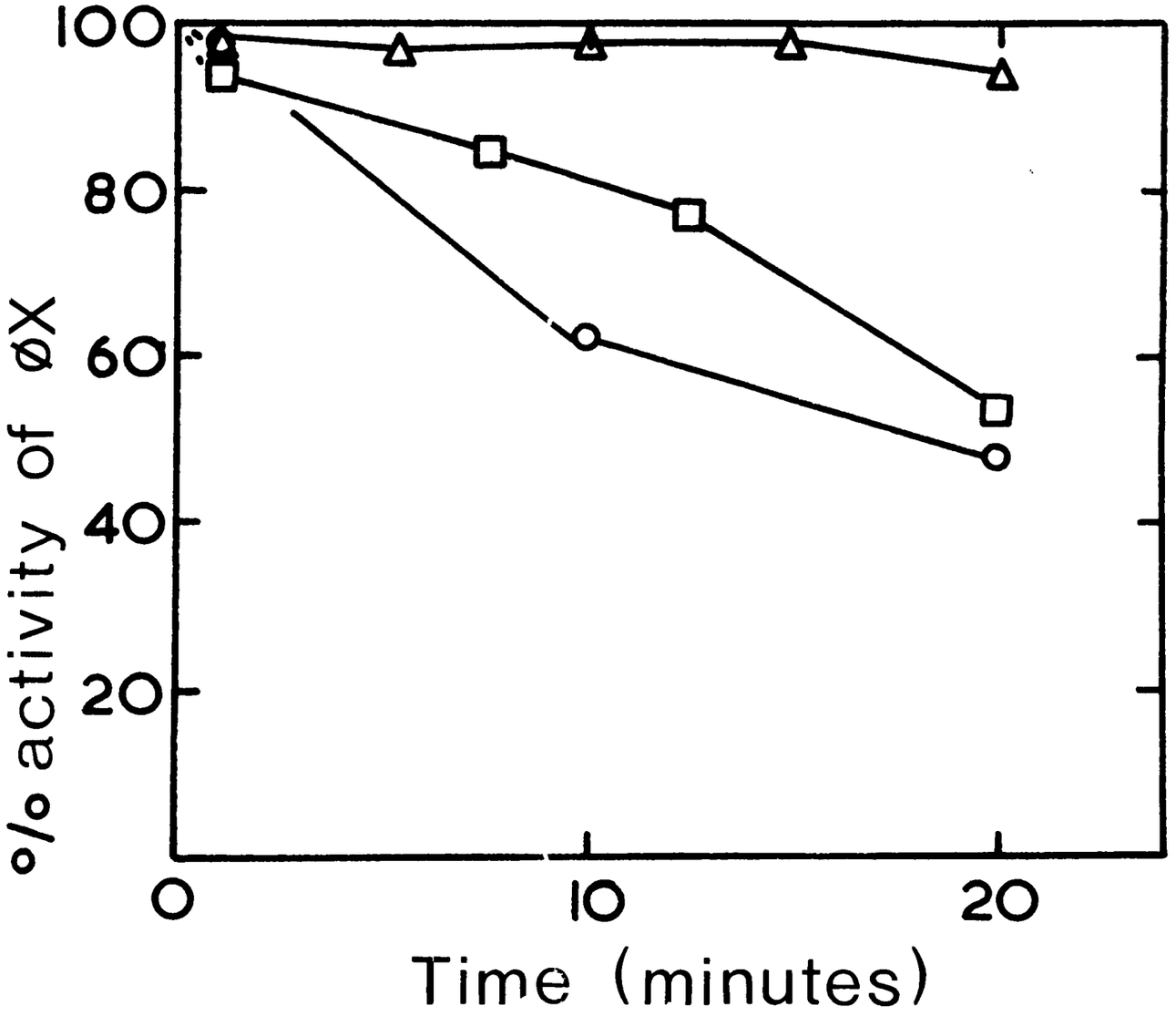
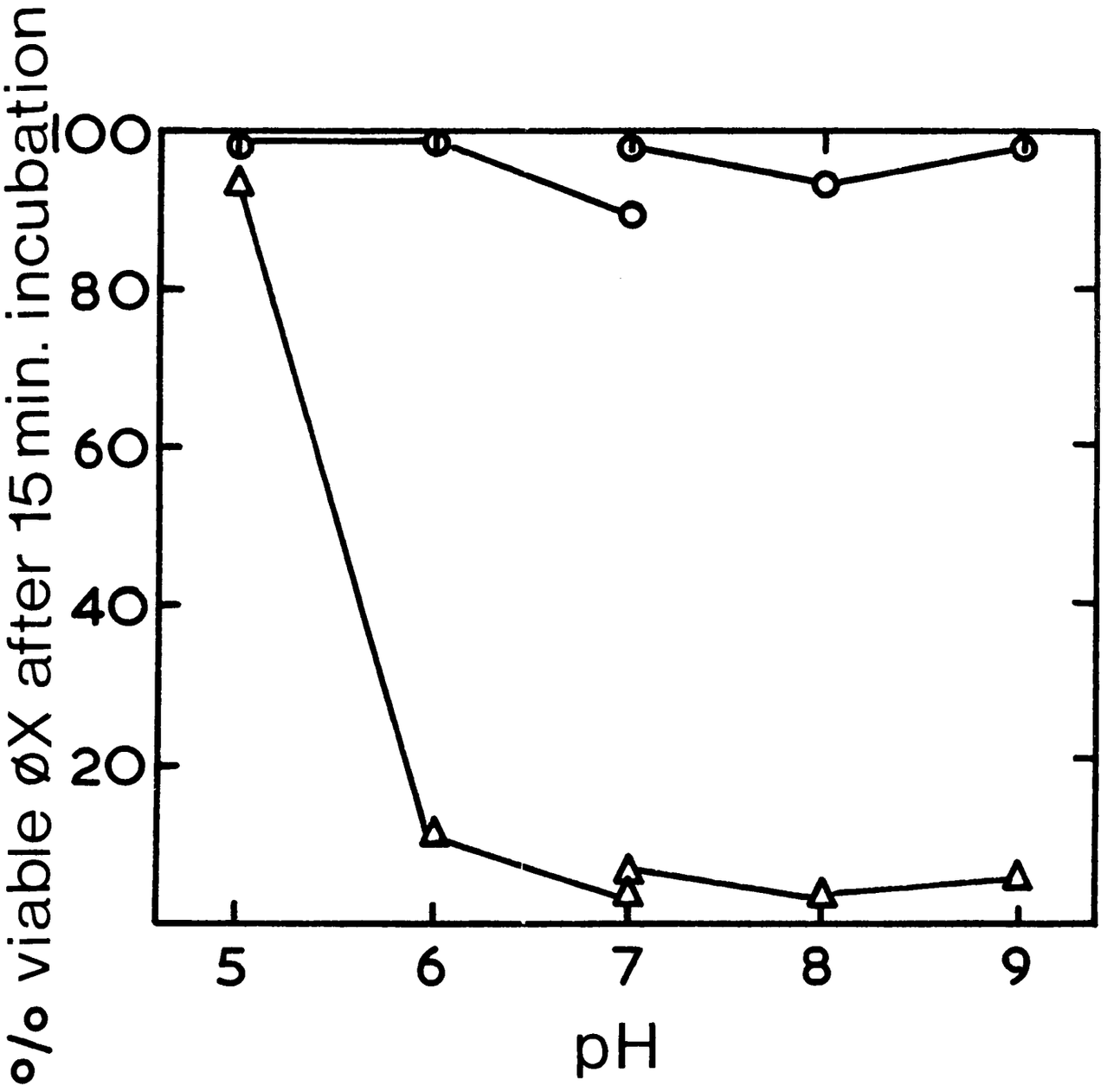

100
80
60
40
20
% activity of ØX
0
10
20
Time (minutes)

Figure 8. Adsorption of ØX to E. coli C cells at different pH values.

ØX (5×10^4 p.f.u./ml) were incubated in adsorption medium (a) (containing 0.1 M DMGA - NaOH, pH 4-7; or, 0.1 M tris-HCl, pH 7-9) for 15 min, with no cells ——o——o—— ; and with E. coli C at 1×10^8 cells/ml; ——△——△——.


% viable øX after 15 min. incubation
100
80
60
40
20
5
6
7
8
9
pH

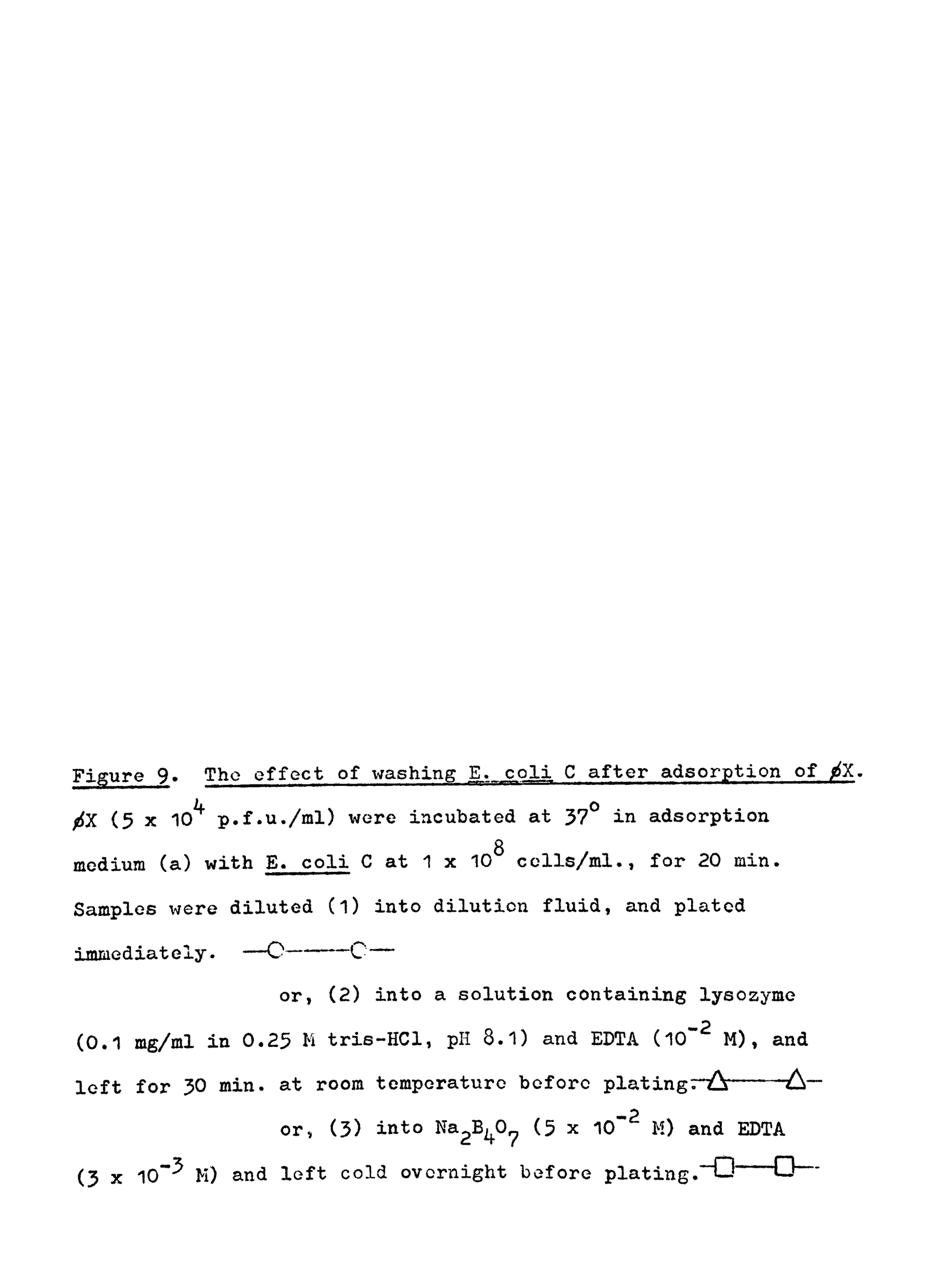
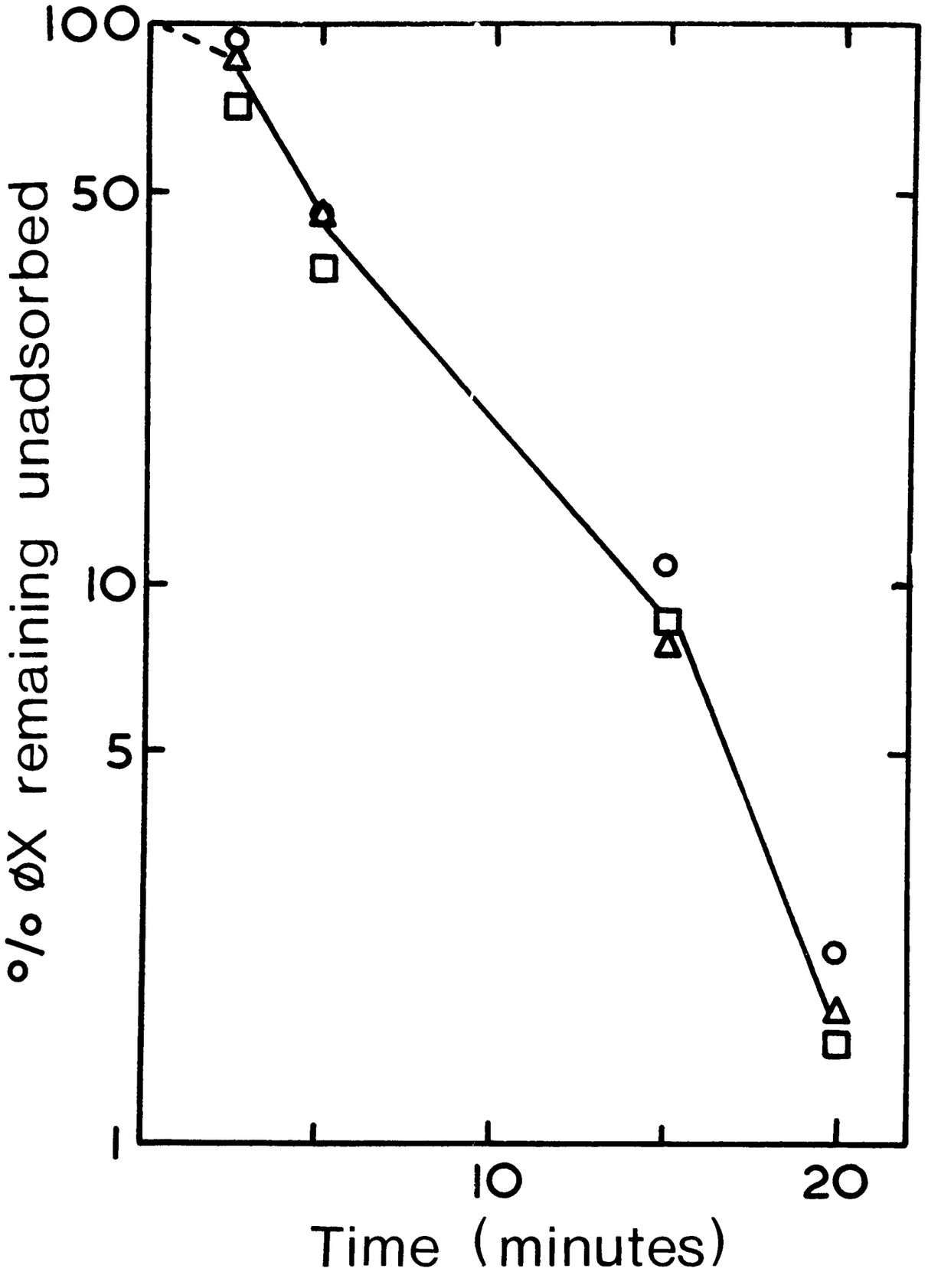


Figure 9. The effect of washing E. coli C after adsorption of ØX. ØX (5×10^4 p.f.u./ml) were incubated at 37° in adsorption medium (a) with E. coli C at 1×10^8 cells/ml., for 20 min. Samples were diluted (1) into dilution fluid, and plated immediately. —○——○—

or, (2) into a solution containing lysozyme (0.1 mg/ml in 0.25 M tris-HCl, pH 8.1) and EDTA (10^{-2} M), and left for 30 min. at room temperature before plating. —△——△—

or, (3) into $Na_2B_4O_7$ (5×10^{-2} M) and EDTA (3×10^{-3} M) and left cold overnight before plating. —□——□—


100
50
10
5
1
% ØX remaining unadsorbed
10
20
Time (minutes)

The experiments were all repeated, using the same conditions, but replacing E. coli C with cell walls. The results (Figures 10 - 13) show that ϕX will adsorb to cell walls in the same way as to intact bacteria. The pH and temperature ranges agreed with those found by Fujimura and Kaesberg, the optima being at pH 6 - 9, and 35 - 45°.

Figure 10. Adsorption of ϕX to E. coli C cell walls

ϕX (5×10^4 p.f.u./ml) were incubated at 37° in adsorption medium (a) with E. coli C cell walls at concentrations of 0.01 units/ml ——o——o——; 0.05 units/ml ——△——△——; 0.08 units/ml ——●——●——. The controls which contained E. coli B cell walls, or no cell walls showed no drop in phage titre (100 ± 7%).

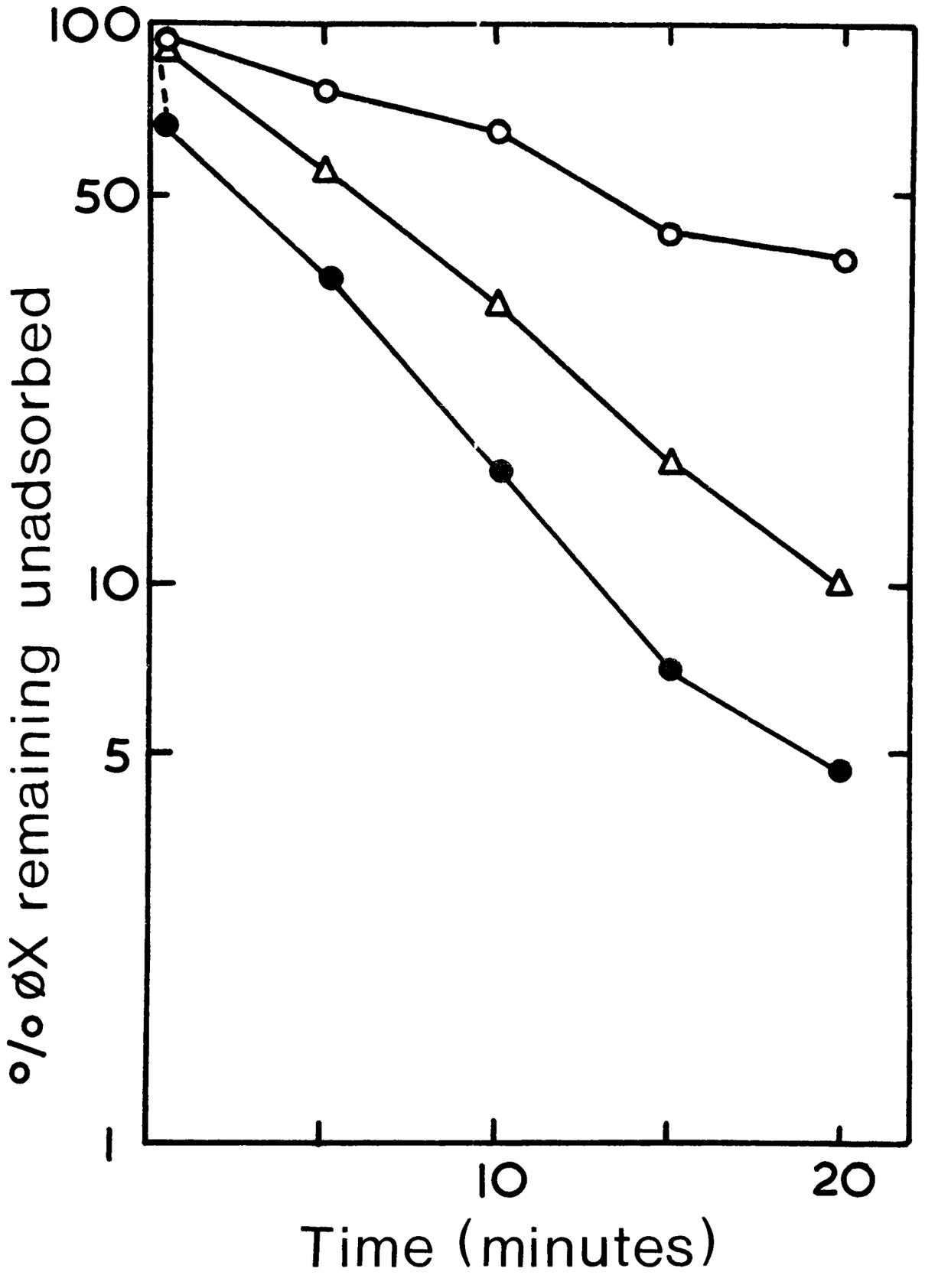

100
50
10
5
1
% øX remaining unadsorbed
10
20
Time (minutes)

Figure 11. Adsorption of øX to E. coli C cell walls at different temperatures.

øX (5×10^4 p.f.u./ml) were incubated in adsorption medium (a) for 15 min. with no cell walls ——o——o——; and with E. coli C cell walls at 0.1 units/ml. ——△——△——.

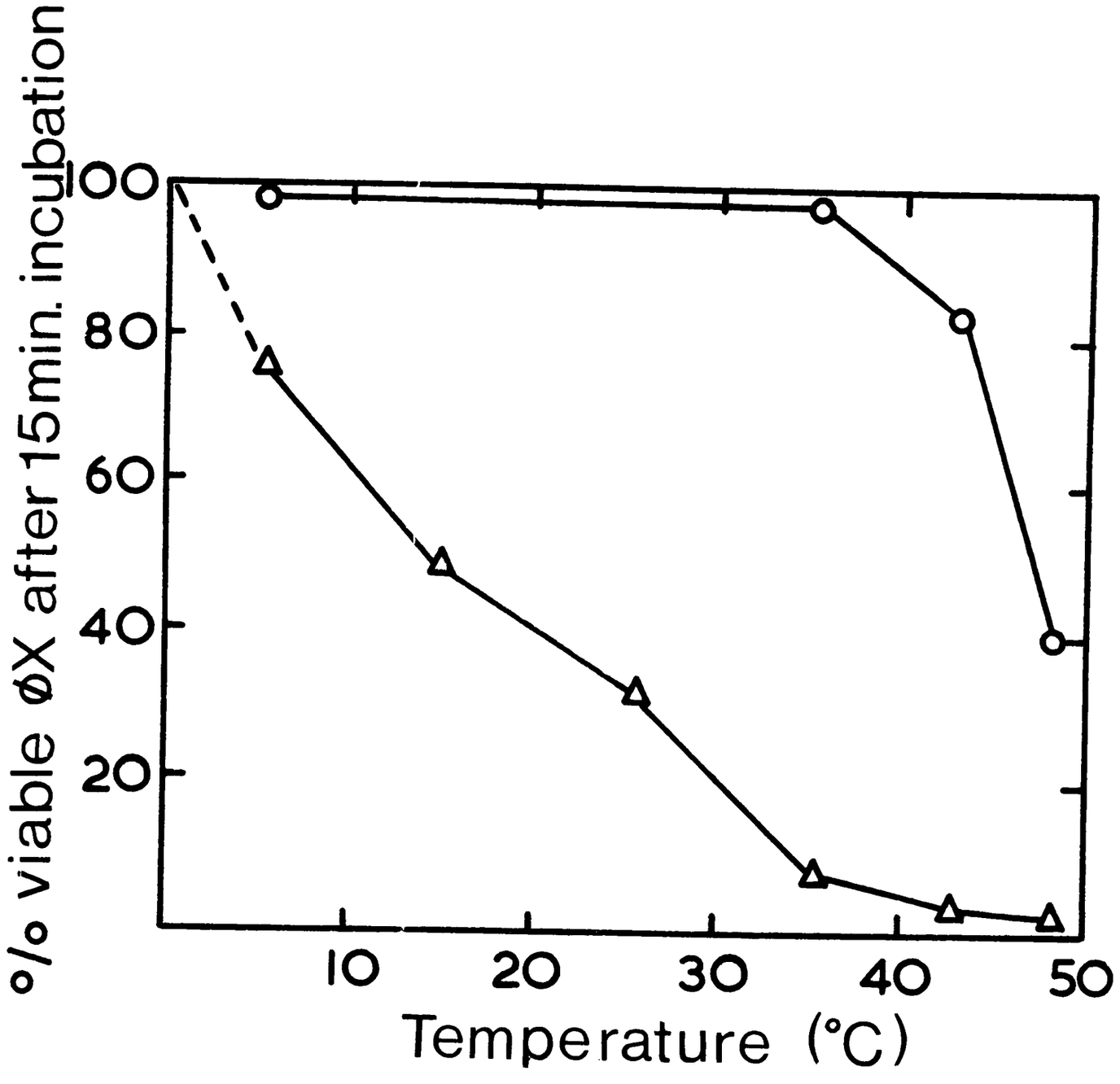

% viable ØX after 15min. incubation
100
80
60
40
20
10
20
30
40
50
Temperature (°C)

Figure 12. Adsorption of ØX to E. coli C cell walls at different pH values.

ØX (5×10^4 p.f.u./ml) were incubated in adsorption medium (a) (containing 0.1 M DMGA-NaOH, pH 4-7 or 0.1 M tris-HCl, pH 7-9) for 15 min. Samples with no cell walls, ——o——o——; samples with E. coli C cell walls at 0.1 unit/ml ——△——△——.

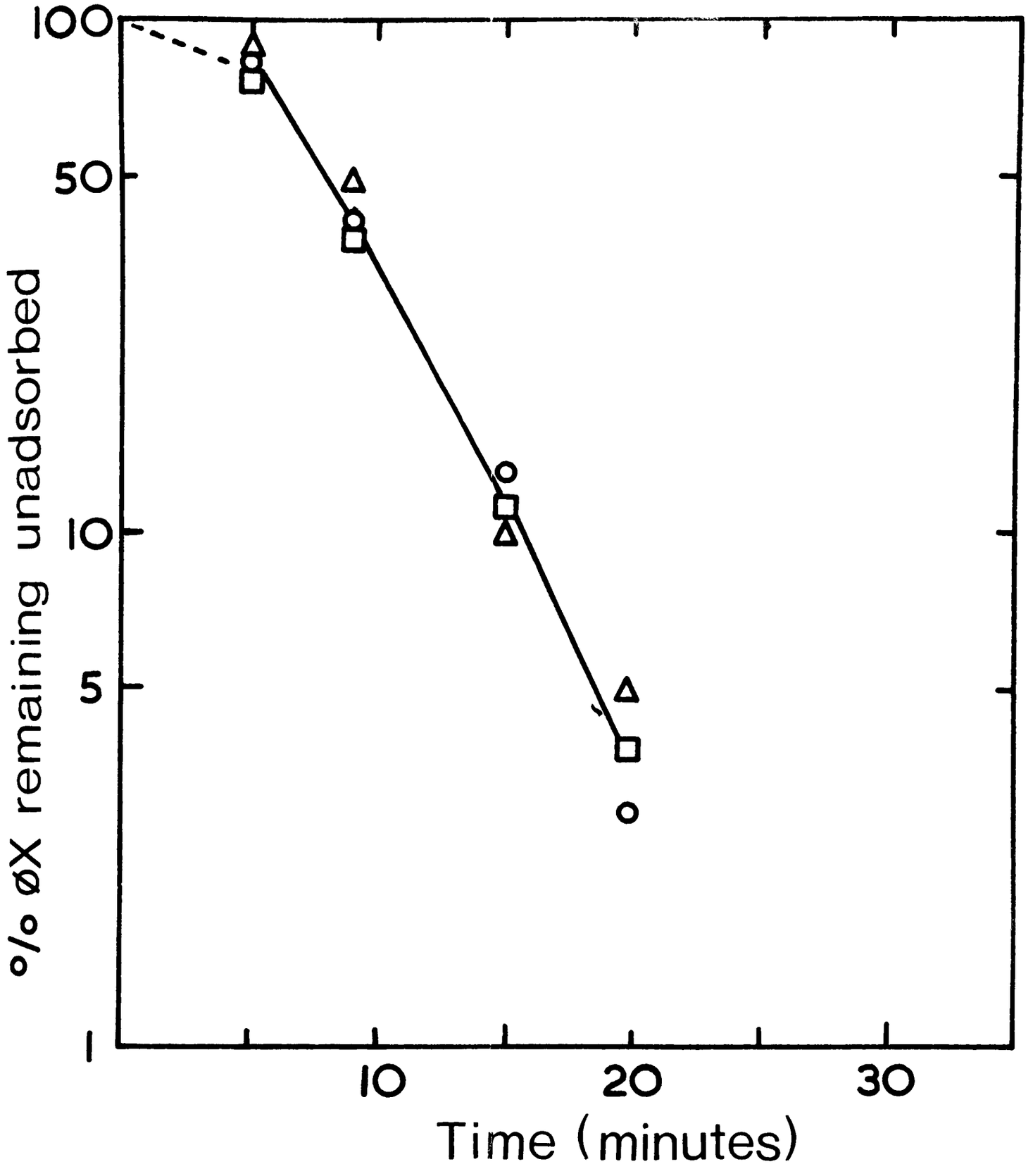
% viable øX after 15 min. incubation
100
80
60
40
20
5
6
7
8
9
pH

Figure 13. The effect of washing E. coli C cell walls after adsorption of ϕX.

ϕX (5×10^4 p.f.u./ml) were incubated at 37° in adsorption medium (a) with E. coli C cell walls at 0.1 unit/ml for 20 min. Samples were diluted (1) into dilution fluid, and plated immediately —△———△— ;

or, (2) into a solution of lysozyme (0.1 mg/ml in 0.25 M tris-HCl, pH 8.1) and EDTA (10^{-2} M) and left for 30 min. at room temperature before plating. —□—□— ;

or, (3) into $Na_2B_4O_7$ (5×10^{-2} M) and EDTA (3×10^{-3} M) and left overnight, before plating. —○———○—


100
50
10
5
1
% øX remaining unadsorbed
10
20
30
Time (minutes)

The release of DNA from ØX, after adsorption to cell walls

The previous results indicate that cell walls can provide a model of the bacterial surface, suitable for use in investigating penetration. If, in order to infect bacteria, DNA is released from ØX when they adsorb, it may also be released when cell walls are used. The principle of the following experiments was to determine whether any ØX DNA was released, thereby becoming sensitive to DNase, after adsorption. The enzyme converts DNA to acid soluble products, but any nucleic acid still inside intact ØX protein coats would be protected, and remain insoluble. The ratio of soluble material was determined by using ^{32}P-labelled ØX.

Experimental Procedure and Results

Samples of ^{32}P-labelled ØX were treated as described in Table 6. Acid soluble ^{32}P-labelled material is present in the ØX preparation, but after incubation at 37^{o} in the presence of DNase the quantity is increased from 8 to 21%. There is a further increase of 9%, if ØX are incubated with cell walls, before the DNase treatment. This suggests that DNA is released on adsorption.

Table 6. Distribution of Radioactivity before and after incubating ^{32}P-labelled ØX with cell walls of E. coli C.

^{32}P-labelled ØX (10^8 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37° for 30 min. in adsorption medium (a). DNase was added to a concentration of 1.0 µg/ml in the presence of 5×10^{-3} M $MgSO_4$, and the sample was incubated at 37° for a further 30 min. The reaction was stopped with $HClO_4$ (0.25 N) in the presence of B.S.A. (0.5 mg/ml) at 0°. After 15 - 30 min. the solutions were centrifuged and the pellet was re-suspended in N NaOH (acid insoluble counts). The supernatant contained acid soluble counts. Controls contained ØX incubated with DNase, in the absence of cell walls. ^{32}P-counts were estimated in the Lobetamat and expressed as percentages of the total ^{32}P-counts in the sample.

Sample	Acid soluble counts	Acid insoluble counts
1. ^{32}P-ØX preparation	8	87
2. ^{32}P-ØX, incubated in adsorption medium (a) and treated with DNase	21	73
3. ^{32}P-ØX, incubated with cell walls in adsorption medium (a), and treated with DNase	30	67

Treatment of the ϕX-cell wall complex with lysozyme

The DNA released from ϕX at adsorption may become entangled in the cell walls, and be partly protected from DNase activity. Lysozyme breaks the mucopeptide complexes of cell walls. It would therefore release any trapped DNA, and possibly make it more susceptible to DNase digestion, resulting in an increase in the proportion of acid soluble counts.

Experimental procedure and results

Samples of ^{32}P-labelled ϕX were treated as in Table 7. Lysozyme and DNase did not release extra acid-soluble material from the ϕX particles. In the presence of cell walls, however, lysozyme caused a rise of 13% above the level found for cell walls and DNase alone (Table 7). It was concluded that some ϕX DNA had been protected from DNase digestion, possibly because it was trapped in the cell walls, or because a complex between ϕX particles and the cell walls was disaggregated by the lysozyme, and the DNA became susceptible through the resultant breakdown of particles

Table 7. Distribution of radioactivity after treating ^{32}P-labelled ØX-cell wall complex with lysozyme.

^{32}P-labelled ØX (1×10^8 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37° for 30 min. in adsorption medium (a), pH 8.0. Lysozyme (0.1 mg/ml in 0.25 M tris-HCl, pH 8.1) and EDTA (10^{-2} M) were added. The samples were incubated at room temperature for 30 min. DNase was then added to a concentration of 1.0 µg/ml. in the presence of $MgSO_4$ (1.5×10^{-2} M). The samples were incubated for a further 30 min. The reaction was stopped with $HClO_4$ (0.25 N) in the presence of B.S.A. (0.5 mg/ml) at 0°. After 15 - 30 min the solutions were centrifuged and the pellet was resuspended in N NaOH (acid insoluble counts). The supernatant contained the acid soluble counts. Controls contained ØX incubated with DNase and lysozyme in the absence of cell walls. ^{32}P-counts were estimated in the Lobetamat and expressed as percentages of the total ^{32}P-counts in the sample.

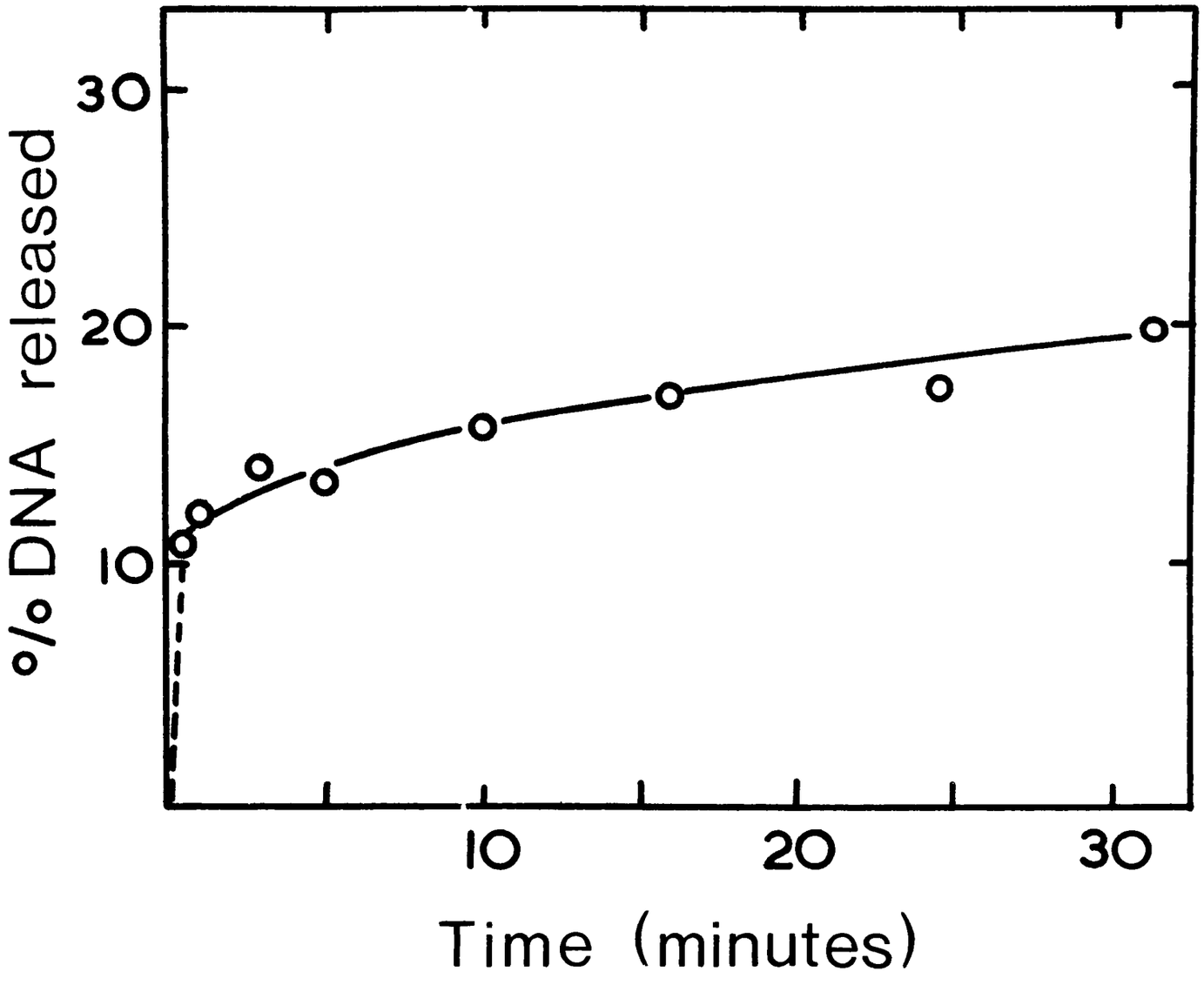
Sample	Acid soluble counts	Acid insoluble counts
1. ^{32}P-ØX preparation.	11	91
2. ^{32}P-ØX incubated in adsorption medium (a).	21	75
3. ^{32}P-ØX incubated in adsorption medium (a) and treated with lysozyme and DNase.	23	72
4. ^{32}P-ØX + cell walls incubated in adsorption medium (a) and treated with DNase.	37	61
5. ^{32}P-ØX + cell walls incubated in adsorption medium (a) and treated with lysozyme and DNase.	50	44

The time course of DNA release, from adsorbed ØX

The experiment was performed as described in Figure 14. The results show that DNase sensitive material was released during the first 5 min of adsorption.

The time course of DNase digestion was determined to ensure that this was not the rate-limiting factor in the previous experiment. ^{32}P-labelled ØX, and excess E. coli C cell walls were incubated together as in Table 6. During the incubation with DNase and $MgSO_4$, samples were taken at intervals, for dilution into B.S.A. (0.5 mg/ml) and $HClO_4$ (0.25 N). Maximum digestion by the DNase was measured after 0.5 min incubation, demonstrating that this reaction would not be rate-limiting in the previous experiment. The results agree with those of Guthrie and Sinsheimer (1960), that at concentrations greater than 1 enzyme molecule per molecule of DNA, the reaction is too rapid for the kinetics to be measured.

Figure 14. The time course of DNA release from adsorbed ϕX. ^{32}P-labelled ϕX (10^8 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37°, for 30 min. in adsorption medium (c). Samples were taken into DNase (1.0 μg/ml) and $MgSO_4$ (5×10^{-3} M) and incubated at 37° for 0.5 min. The reaction was stopped with $HClO_4$ (0.5 N) at 0°. After 15 min. samples were centrifuged at 5000 g. The pellet was resuspended in N NaOH (acid insoluble counts). The supernatant contained acid soluble counts. ^{32}P-counts were estimated in the Lobetamat. Results were expressed as % acid-soluble ^{32}P-counts released.


% DNA released
30
20
10
10
20
30
Time (minutes)

Infectivity towards spheroplasts of material obtained from ØX particles.

The DNA which becomes sensitive to DNase digestion at adsorption, may be produced by the mechanism which normally initiates infection of bacteria. If this mechanism is all that is needed to allow penetration of the cell walls, then, provided the DNA is intact, it should infect spheroplasts of E. coli K12W6, in the same way as isolated single-stranded ØX-DNA. The object of the following experiments was to test this by adding spheroplasts to ØX which had been incubated with cell walls. The system was the same as that tested with isolated DNA, and '70°-ØX'. (see Methods). The effect of treating the cell wall-ØX complex with lysozyme and DNase, before incubation with spheroplasts was also tested.

(a) Infectivity of ØX-cell wall complex

ØX at 5×10^8 p.f.u./ml were allowed to adsorb to cell walls in adsorption medium (b). After 30 min at 37° 1 ml spheroplasts in spheroplast stock medium (SS medium) was added to 1 ml of the ØX-cell wall complex. The mixtures were incubated for 15 min at 37°, and then 8 ml. protoplast nutrient medium (PAM medium) was added. Samples were assayed for free virus immediately after this dilution. These samples were further diluted through cold dilution fluid, or 0.1 M $Na_2B_4O_7$ to break the spheroplasts, and prevent any viral reproduction in infected ones. The experimental tubes were incubated for a further 90 min. before being diluted through $CHCl_3$-saturated dilution fluid, prior to estimation of the total number of virus. These samples included virus formed

in the spheroplasts, and any free virus particles which did not adsorb to the cell walls. The difference between the numbers of p.f.u. produced in these samples, and those taken before incubation showed the increase due to reproduction in the spheroplasts. Control tubes contained øX-cell wall complex, incubated in SS medium and PAM medium, but containing no spheroplasts.

The results of the experiment are shown in Table 8. When øX adsorb to cell walls, material is produced, which can infect spheroplasts of E. coli K12 W6. Intact virus do not do this.

(b) Infectivity of øX-cell wall complex after lysozyme treatment

After adsorption of øX to cell walls, 1 ml samples were added to 0.02 ml 4% EDTA. Lysozyme (0.2 ml of 0.1 mg/ml solution, in 0.25 M tris-HCl, pH 8.1) and EDTA (10^{-2} M final concentration) were added to the tubes which were incubated at room temperature for 15 min., before spheroplasts were added. The experiment was then completed as before. Controls consisted of øX-cell wall complexes incubated with spheroplasts, but without treatment with lysozyme.

The results are shown in Table 9. Lysozyme appeared to cause very little change in the infectivity towards spheroplasts, shown by the øX-cell wall complex.

(c) Infectivity of the øX-cell wall complex, after treatment with DNase

øX particles were allowed to adsorb to cell walls in the

Table 8. Infectivity of ØX towards spheroplasts of E. coli K12 W6 after adsorption to E. coli C cell walls.

ØX were incubated in adsorption medium (b) with 0.1 unit of cell walls, and added to 2.0×10^8 spheroplasts. After incubation spheroplasts were lysed in cold $CHCl_3$-saturated 0.1 M $Na_2B_4O_7$, before p.f.u.'s were assayed. Results are expressed as the total no. of viable ØX present in each sample.

Sample	p.f.u.
no. of ØX added to cell walls	4.0×10^8
no. of ØX unadsorbed after 30 min. incubation.	8.3×10^7

	Control (no spheroplasts)	Experiment (with spheroplasts)
no. ØX after 15 min. incubation on SS medium	6.0×10^7	4.0×10^7
no. ØX after 90 min. incubation on PAM medium	4.0×10^7	4.4×10^8

Table 9. Infectivity of øX towards spheroplasts of E. coli K12W6 after adsorption to E. coli C cell walls, followed by lysozyme treatment.

øX were incubated at 37° in adsorption medium (b) (pH 8.0) with 0.1 unit cell walls for 30 min. Lysozyme was added, to a concentration of 0.1 mg/ml in 0.25 M tris-HCl, pH 8.1 with 10^{-2} M EDTA. 2.0×10^8 spheroplasts were added. After 15 min incubation they lysed in cold $CHCl_3$-saturated 0.1 M $Na_2B_4O_7$, and the infectious units present were analysed. Results are expressed as the total no. of viable phage present in each sample.

Sample	p.f.u.
no. øX added to cell walls	1.0×10^8
no. øX unadsorbed after 30 min.	4.6×10^7

	Control (no lysozyme no spheroplasts)	Control (no lysozyme with spheroplasts)	Experiment (with lysozyme with spheroplasts)
no. øX after 15 min incubation in SS medium	6.4×10^6	2.8×10^6	3.0×10^6
no. øX after 90 min incubation in PAM medium	7.4×10^6	1.8×10^7	2.4×10^7

presence of DNase at a concentration of 1.0 μg/ml., and 10^{-2} M $MgSO_4$. The tubes were incubated at 37° for 30 min. and spheroplasts were then added in SS medium as before. In one experiment ^{32}P-labelled ØX am3 were used. The amount of acid soluble material produced, and the infectivity of spheroplasts were measured simultaneously. Control mixtures were not treated with DNase.

The results of the spheroplast infection are shown in Table 10. DNase treatment of ØX-cell wall complexes does not reduce their ability to infect spheroplasts, but, as in Table 6, there was a 10% increase in acid soluble counts demonstrating that DNase sensitive material was released. It was concluded that free ØX DNA was not responsible for the infectivity.

(d) Nature of the Infectious Material

After adsorption, the ØX-cell wall complex was centrifuged at 100,000 g for 2 hr. (Spinco model L, 40 rotor). This concentrated the complex, and free ØX, but any ØX-DNA completely released from the coat protein, remained in the supernatant. The pellet was resuspended in the original volume of adsorption medium (b). This suspension and the supernatant were then tested separately for infectivity towards spheroplasts.

The results (Table 11) show that all the infectivity was associated with the pellet, supporting the conclusion that, in these experiments, spheroplasts were not infected by released ØX-DNA.

Table 10. The effect of DNase on infectious material released by the ϕX-cell wall complex.

ϕX were incubated in adsorption medium (b), pH 7.2 with 0.1 unit cell walls in the presence of DNase at 1.0 μg/ml. and 10^{-2} M $MgSO_4$. 2.0×10^8 spheroplasts were added. After incubation they were lysed in cold $CHCl_3$-saturated 0.1 M $Na_2B_4O_7$, and total p.f.u.'s were measured. Results are expressed as the total no. of viable ϕX present in each sample.

Sample	p.f.u.
no. of ϕX added to cell walls	1.0×10^8
no. of ϕX unadsorbed after 30 min.	2.8×10^7

	Control (no DNase no spheroplasts)	Control (no DNase with spheroplasts)	Experiment (with DNase with spheroplasts)
no. ϕX after 15 min. incubation in SS medium	1.6×10^7	1.3×10^7	1.4×10^7
no. ϕX after 90 min. incubation in PAM medium	9.4×10^6	1.6×10^8	1.7×10^8

Table 11. Location of material producing infection of spheroplasts of E. coli K12W6 after ØX have adsorbed to cell walls of E. coli C .

ØX were incubated in adsorption medium (b) with 0.1 unit of E. coli C cell walls. One sample was treated as in Table 8. The other was centrifuged for 2 hr at 100,000 g, at 4° in the 40 angle rotor of the Spinco Model L centrifuge. The pellet was resuspended in the original volume of adsorption medium (b). This suspension, and the supernatant were then treated separately, as in Table 8.

Sample	p.f.u.
no. of ØX added to cell walls	1.9×10^8
no. of ØX unadsorbed after 30 min.	7.9×10^7

	Control (no centrifugation, no spheroplasts)	Control (no centrifugation with spheroplasts)	Experiment (resuspended pellet with spheroplasts)	Experiment (supernatant with spheroplasts)
no. ØX after 15 min. incubation in SS medium.	5.6×10^7	4.9×10^7	4.4×10^7	$< 10^5$
no. ØX after 90 min. incubation in PAM medium.	6.8×10^7	4.5×10^8	2.5×10^8	$< 10^5$

The structure of particles formed after adsorption of ØX to cell walls.

Sedimentation through sucrose density gradients fractionates particles according to their shape and size. øX has a sedimentation coefficient of 114S (Sinsheimer, 1959a) and particles of 70S and 45 - 60S have been reported (Carusi and Sinsheimer 1963; Guthrie and Sinsheimer, 1960; Maclean and Hall, 1961; Dann-Markert, Deutsch and Zillig, 1966). Since adsorption to cell walls produced components capable of infecting spheroplasts, some change must take place on contact with cell walls. If the change results either in any alteration in the DNA content, or the shape of the viruses, the resultant particles should move to different positions on sucrose gradients. To investigate the possible existence of different components, the mixtures obtained on incubating cell walls with ^{32}P- and ^{35}S-labelled øX were analysed by velocity sedimentation in sucrose density gradients. The sedimentation value of the components obtained was estimated by comparing the position with those of the known markers illustrated in Figure 3. The proportion of DNA in the different particles was determined by calculating the ratio of $^{35}S/^{32}P$-counts, an increase in the ratio indicating a loss of viral DNA.

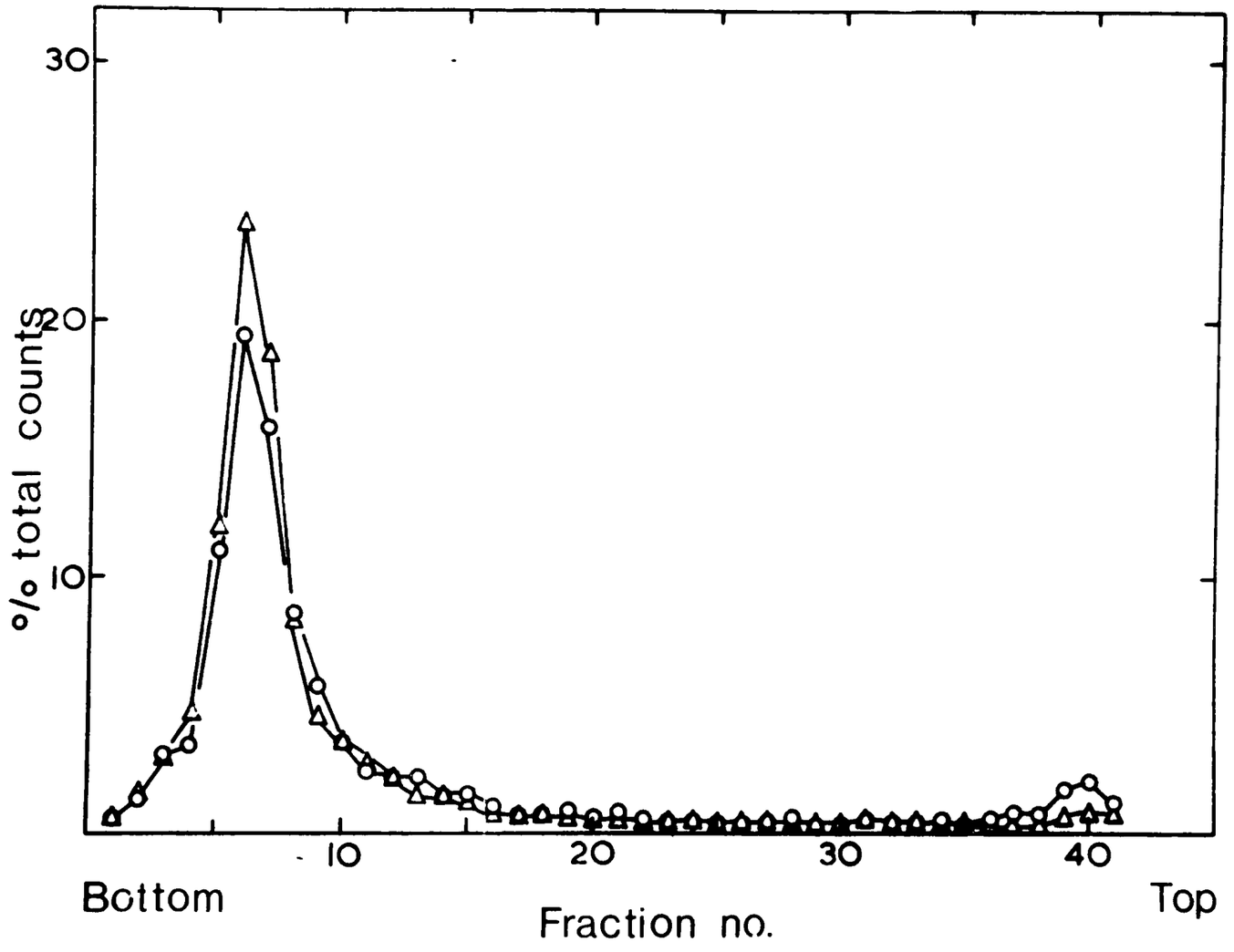
Experimental procedure

(a) øX particles

A 0.1 ml sample of ^{32}P, ^{35}S-labelled øX at 2.5×10^9 p.f.u./ml was put directly onto a sucrose gradient, and treated as described in Methods. A single peak was obtained in the position shown in Figure 15.

Figure 15. Sucrose density gradient analysis of a ^{32}P-, ^{35}S-labelled øX preparation.

The sucrose gradients (5-30% (w/v) in 5 x 10^{-2} M tris, pH 8.1 and 3 x 10^{-3} M EDTA) were run for 2 hrs. at 100,000 g, at 4°C in the SW39 rotor of the Spinco Model L centrifuge. Each fraction was assayed for ^{32}P and ^{35}S-counts. ^{32}P-counts —△—△—; ^{35}S-counts ——o——o——.


30
20
10
% total counts
10
20
30
40
Bottom
Top
Fraction no.

(b) Isolated ØX DNA

A sample of isolated ^{32}P-labelled ØX-DNA at 2 x 10^{10} molecules/ml was layered onto the surface of a 5 - 30% sucrose gradient, and centrifuged at 100,000 g for 2 hr. The result is shown in Figure 16. The main peak shows the position at which intact, circular ØX-DNA molecules move.

(c) '70°-ØX' and '70°-ØX' treated with DNase

Sucrose density fractionation was used to examine the effect of DNase on particles which had been partly disrupted by heating at 70° (Guthrie and Sinsheimer, 1960; Maclean and Hall, 1962). The results (Figure 17) show that heated particles sediment at 55 - 60S and that DNase treatment converts them to a form which sediments at 70S (Figure 18). The repetition of this work was done to demonstrate that DNA, associated with disrupted ØX particles, is sensitive to DNase under the conditions used throughout these experiments.

(d) The effect of incubation time on the ØX-cell wall interaction

Two samples of ^{32}P-, ^{35}S-labelled ØX at 10^9 p.f.u./ml. were incubated with cell walls in 2 ml adsorption medium (c). One sample was incubated at 37° for 5 min and the other for 40 min. They were then chilled and centrifuged at 0° for 30 min at 32,000 g. The supernatants were kept for sucrose gradient analysis, and the pellets, consisting of cell walls and adsorbed ØX, were resuspended in 0.25 ml 5 x 10^{-2} M $Na_2B_4O_7$ and 3 x 10^{-3} M EDTA. They were left at 4° overnight to elute particles from the cell

Figure 16. Sucrose density gradient analysis of ^{32}P-labelled øX-DNA.

DNA was extracted from ^{32}P-labelled øX with Borate-saturated re-distilled phenol at 70°. The sucrose gradients (5-30% (w/v) in 5 x 10^{-2} M tris, pH 8.1, and 3 x 10^{-3} M EDTA) was run for 2 hr. at 100,000 g, at 4° in the SW39 rotor of the Spinco Model L centrifuge. Fractions were assayed for ^{32}P-counts, ——△——△—

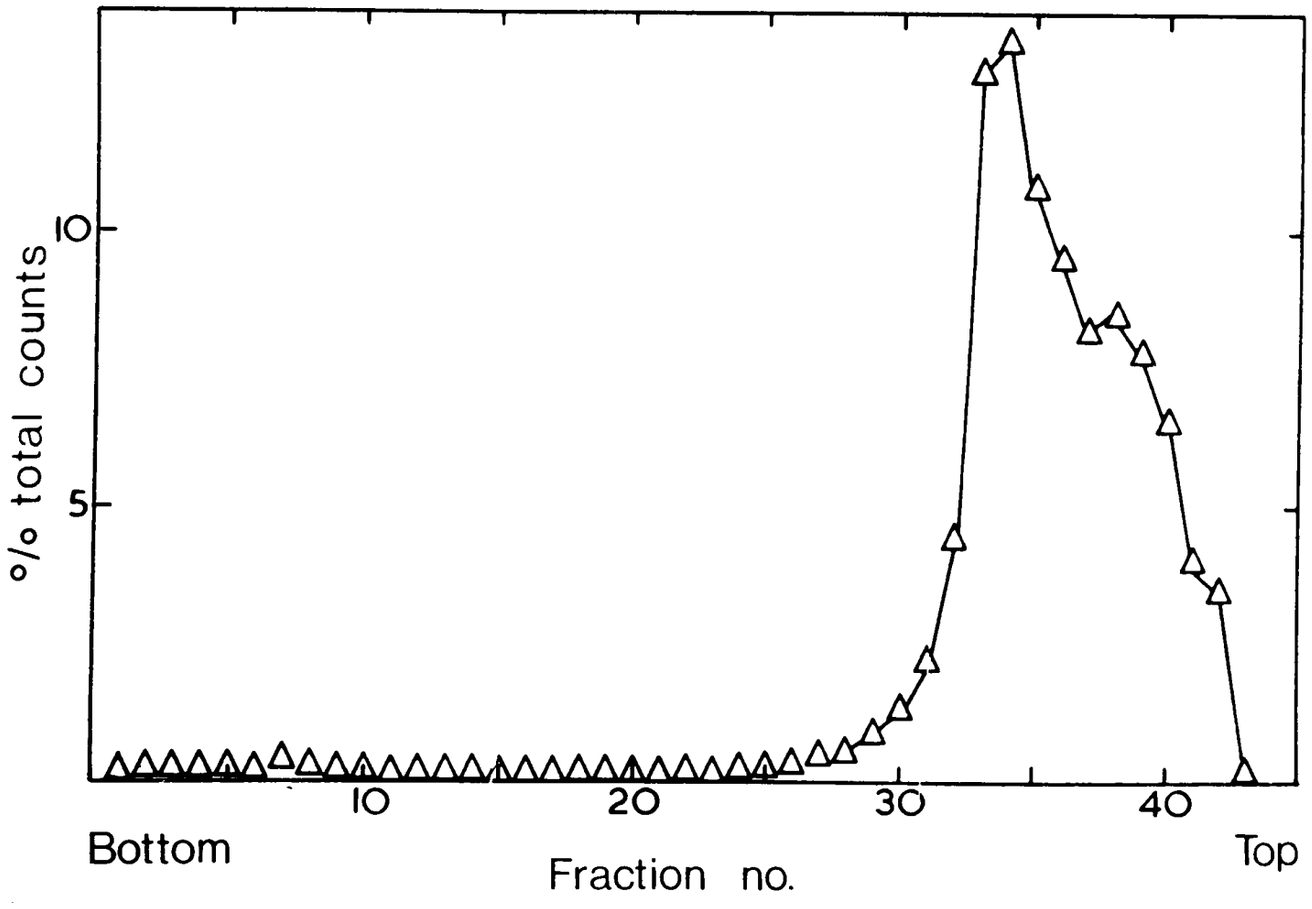
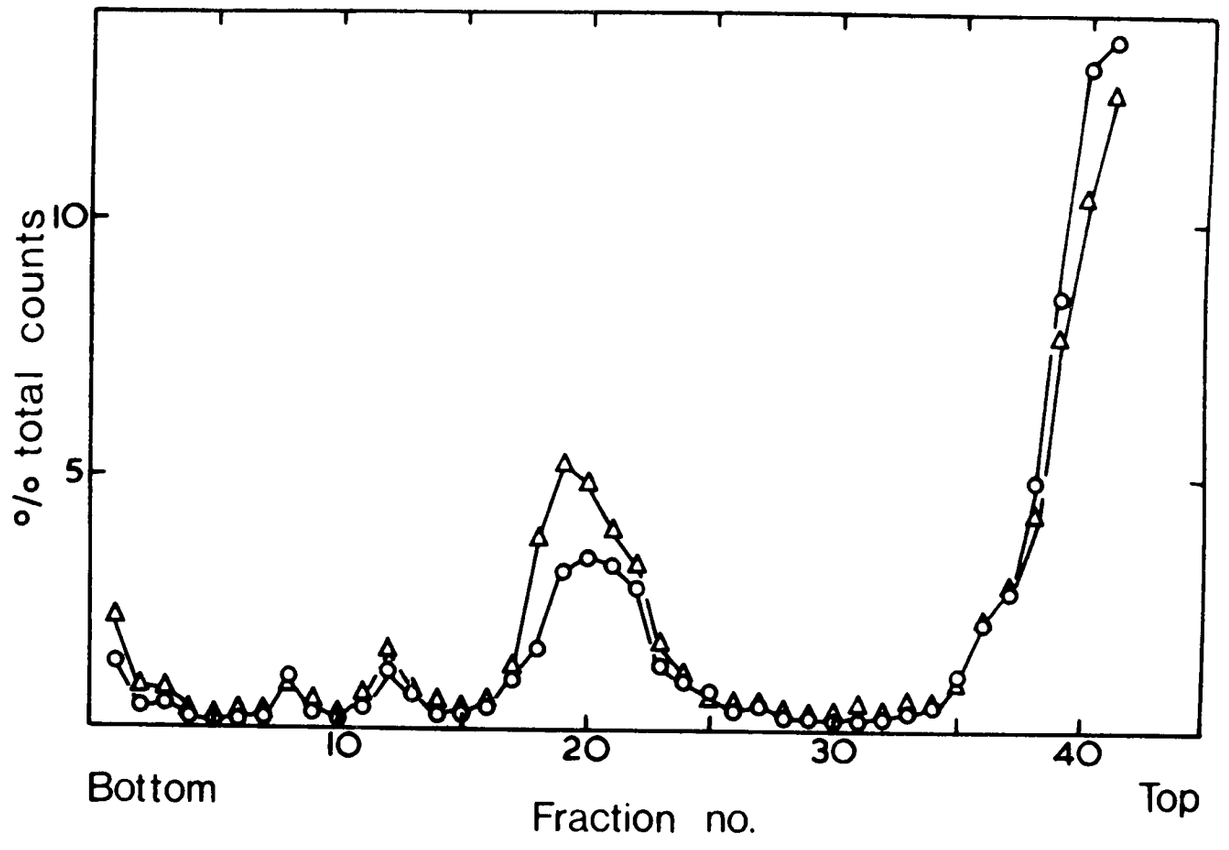
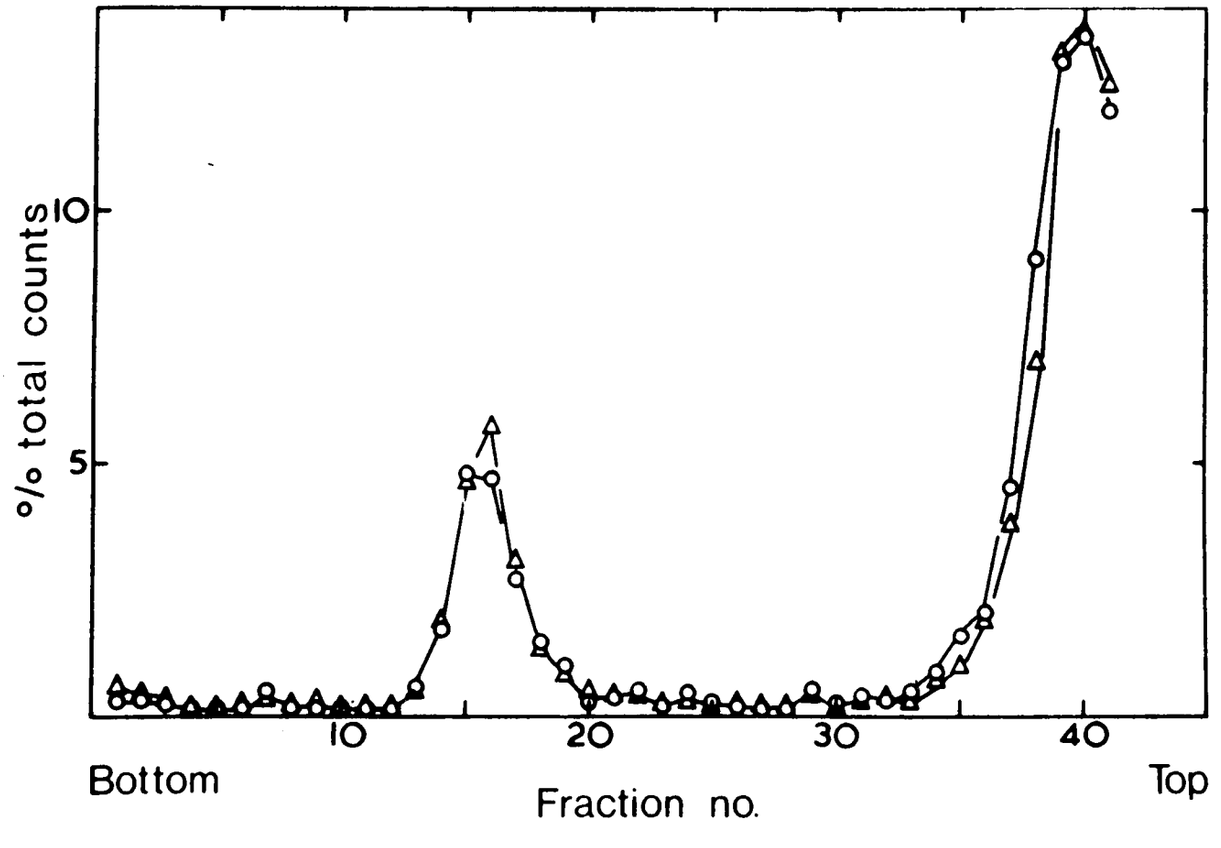

% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

Figure 17. Sucrose density gradient analysis of '70°-øX'
^{32}P-, ^{35}S-labelled øX in 5 x 10^{-2} M tris, pH 8.1, were heated at 70°, for 5 min.

Figure 18. Sucrose density gradient analysis of 70°-øX, treated with DNase.
^{32}P-, ^{35}S-labelled øX in 5 x 10^{-2} M tris, pH 8.1, were heated at 70°, for 5 min, followed by chilling and incubation at 37°, with DNase (1.0 µg/ml. in 10^{-2} M NaCl, 5 x 10^{-3} M $MgSO_4$) for 20 min.
The sucrose gradients (5-30% (w/v) in 5 x 10^{-2} M tris pH 8.1; and 3 x 10^{-3} M EDTA) were run for 2 hr at 100,000g, at 4° in the SW39 rotor of the Spinco Model L centrifuge. Each fraction was assayed for ^{32}P-counts ——△——△——; ^{35}S-counts ——o——o——.


% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top


% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

walls. Samples (0.1 ml) were layered onto the surface of gradients; 0.2 ml samples of the original supernatants were also run in gradients. Control tubes contained ØX and cell walls kept at 0°, and ØX with no cell walls.

The results (Figures 19 and 20) show that although intact ØX cannot be obtained from cell walls after 5 - 10 min. adsorption (Figure 13), altered particles are washed off. The products obtained after 5 min. incubation consisted of two components with sedimentation coefficients of 70 and 45 - 50S respectively. Longer incubation of ØX-cell wall complexes gave rise to material sedimenting at 70 - 75S (Figure 20).

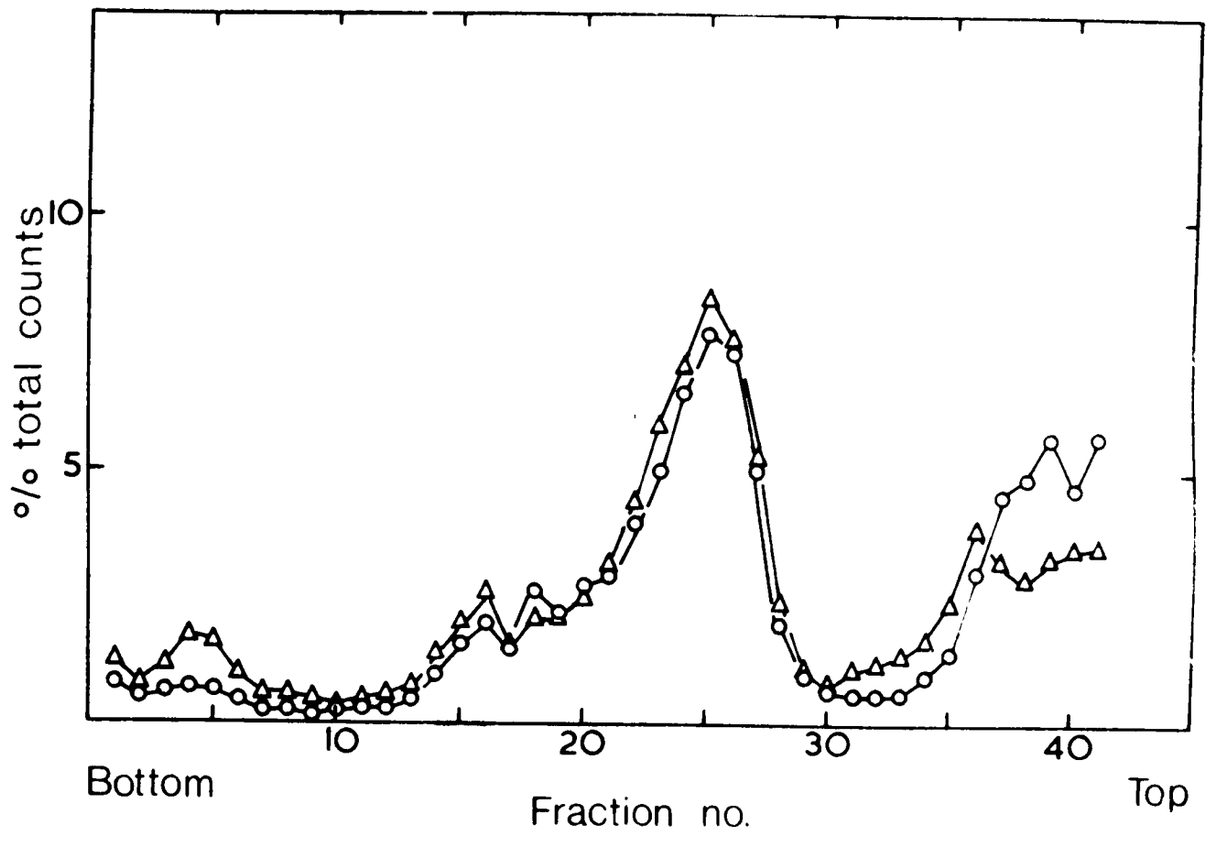
Velocity gradient analysis of the supernatants obtained after centrifuging the original ØX-cell wall suspension shows that very few virus are released from the cell wall without treatment with $Na_2B_4O_7$-EDTA, and that there is a high proportion of low molecular weight material present.

Similarly there are very few particles detectable in the gradients used to analyse the samples from ØX-cell wall complexes kept at 0° for 30 min (Figure 22), and those obtained after incubation of ØX with cell walls of E. coli B.

There was no peak in any of the gradients corresponding in position with that expected for free, circular molecules of DNA (see Figure 16), supporting earlier results that infectious molecules of ØX-DNA are not released.

Figures 19 and 20. Sucrose density gradient analysis of components formed from ØX particles incubated with cell walls of E. coli C.

^{32}P- ^{35}S-labelled ØX (10^9 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37°, in adsorption medium (c), for 5 min (Figure 19) and 40 min (Figure 20). They were chilled and centrifuged for 30 min at 32,000 g, at 4° in the 40 rotor of the Spinco Model L centrifuge. The pellets were resuspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA, and left at 4° overnight 0.1 ml samples were put onto sucrose gradients (5-30%(w/v) in 5×10^{-2} M tris, pH 8.1, and 3×10^{-3} M EDTA) and run for 2 hr., at 100,000 g, at 4° in the SW 50 rotor of the Spinco centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; ^{35}S-counts —o—o—.


% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

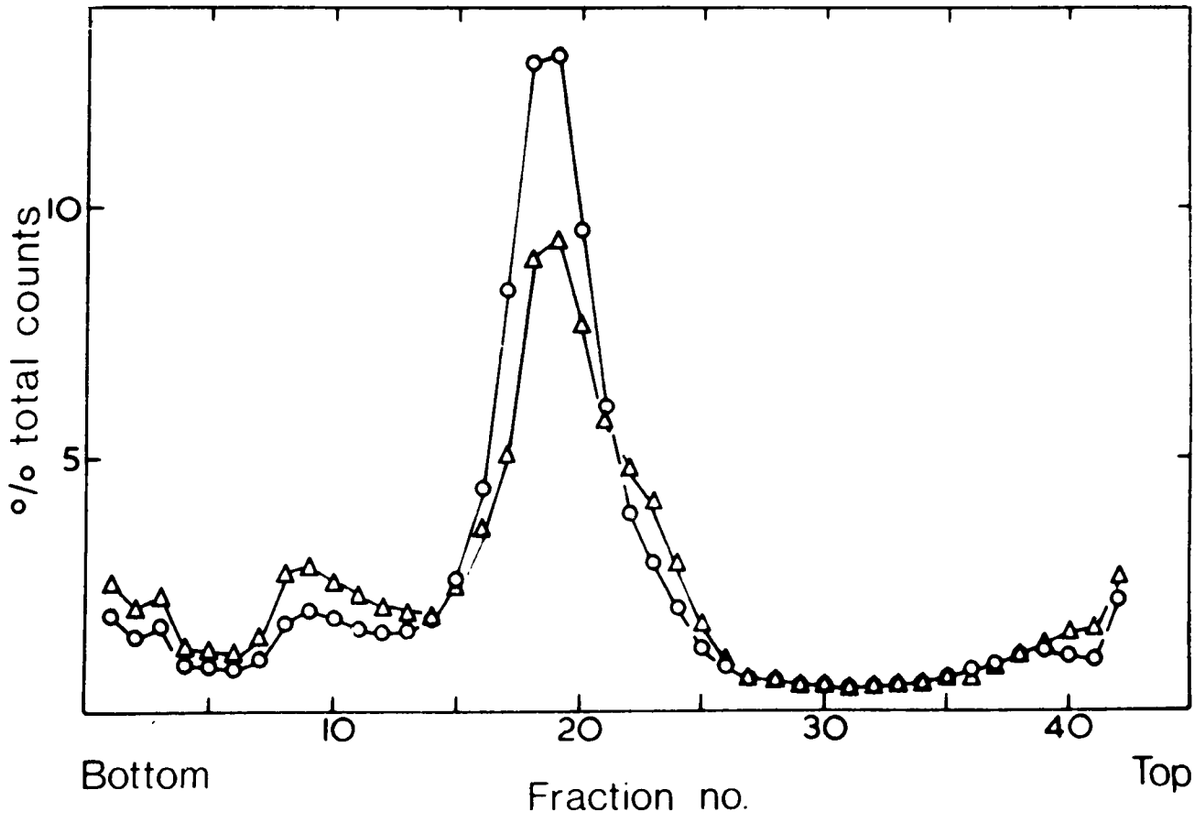

% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

Figure 21. Sucrose density gradient analysis of ØX particles in supernatant fractions.

^{32}P- ^{35}S-labelled ØX (10^8 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37°, in adsorption medium (c) for 40 min. They were chilled and centrifuged for 30 min. at 32,000 g, at 4° in the 40 rotor of the Spinco Model L centrifuge. 0.2 ml samples from the supernatants were put onto sucrose gradients (5-30% (w/v) in 5×10^{-2} M tris, pH 8.1, and 3×10^{-3} M EDTA) and run for 2 hr. at 100,000 g, at 4° in the SW 50 rotor of the Spinco centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; ^{35}S-counts —o—o—

Few counts were found on the gradient, for example 135 ^{32}P-counts, at fraction 16.

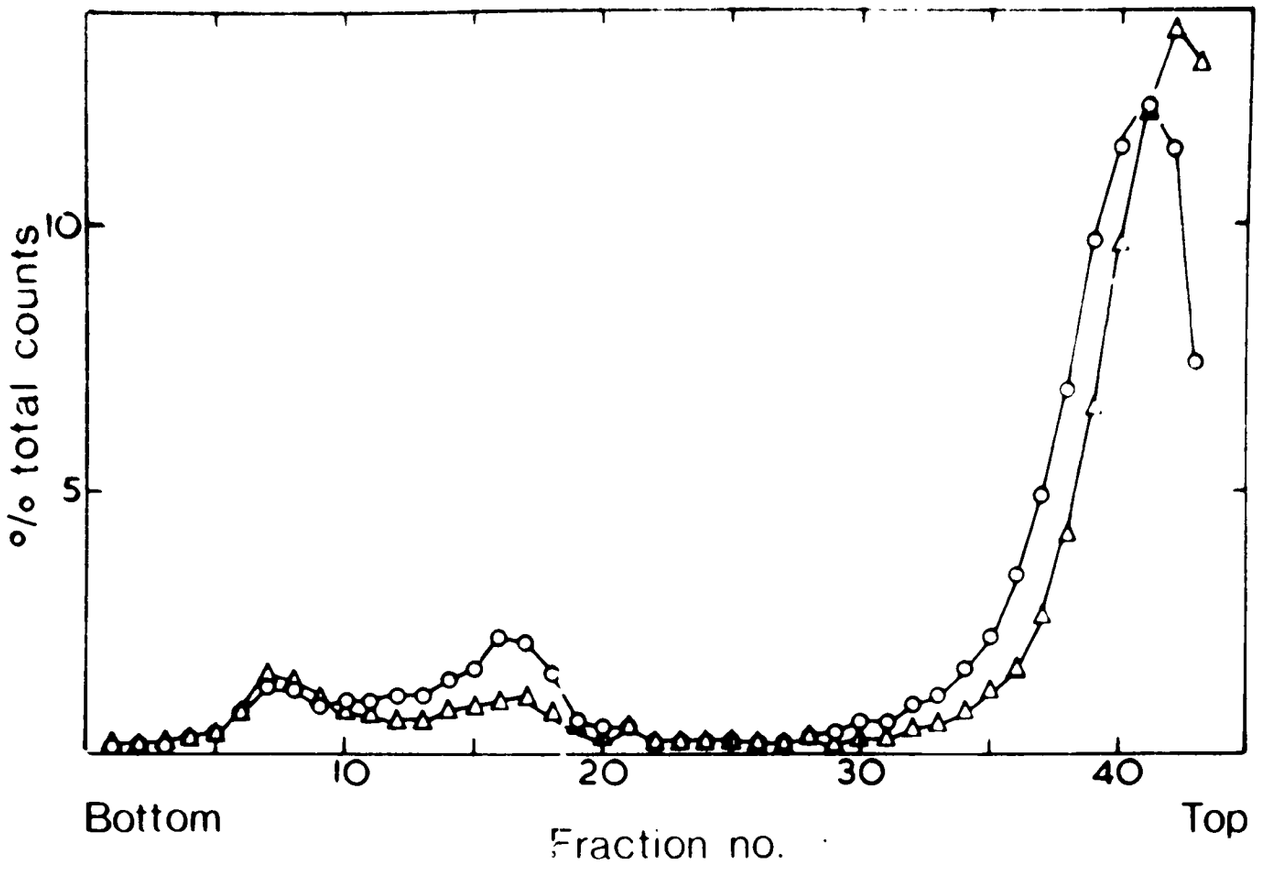
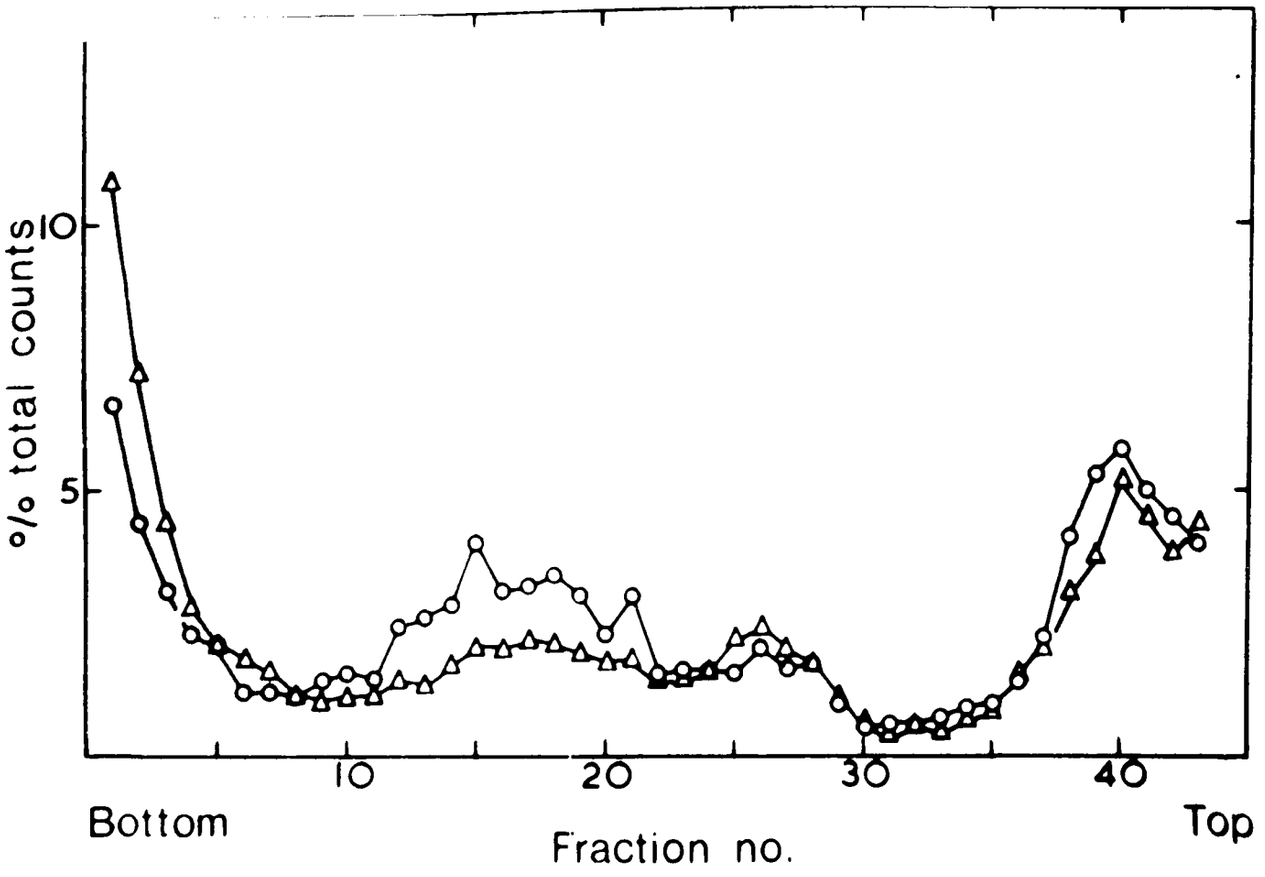

% total counts
10
5
10
20
30
40
Bottom
Top
Fraction no.

Figure 22. Sucrose density gradient analysis of øX particles kept at 0°.

^{32}P- ^{35}S-labelled øX (10^9 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were kept at 0°, in adsorption medium (c). They were centrifuged for 30 min. at 32,000 g., at 4° in the 40 rotor of the Spinco Model L centrifuge. The pellet was re-suspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA and left overnight at 4°. 0.2 ml samples were put onto sucrose gradients (5-30% (w/v) in 5×10^{-2} M tris, pH 8.1, and 3×10^{-3} M EDTA) and run for 2 hr at 100,000 g, at 4° in the SW 50 rotor of the Spinco centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; ^{35}S-counts —o—o—

Few counts were found on the gradient for example 324 ^{32}P-counts on fraction 15.


% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

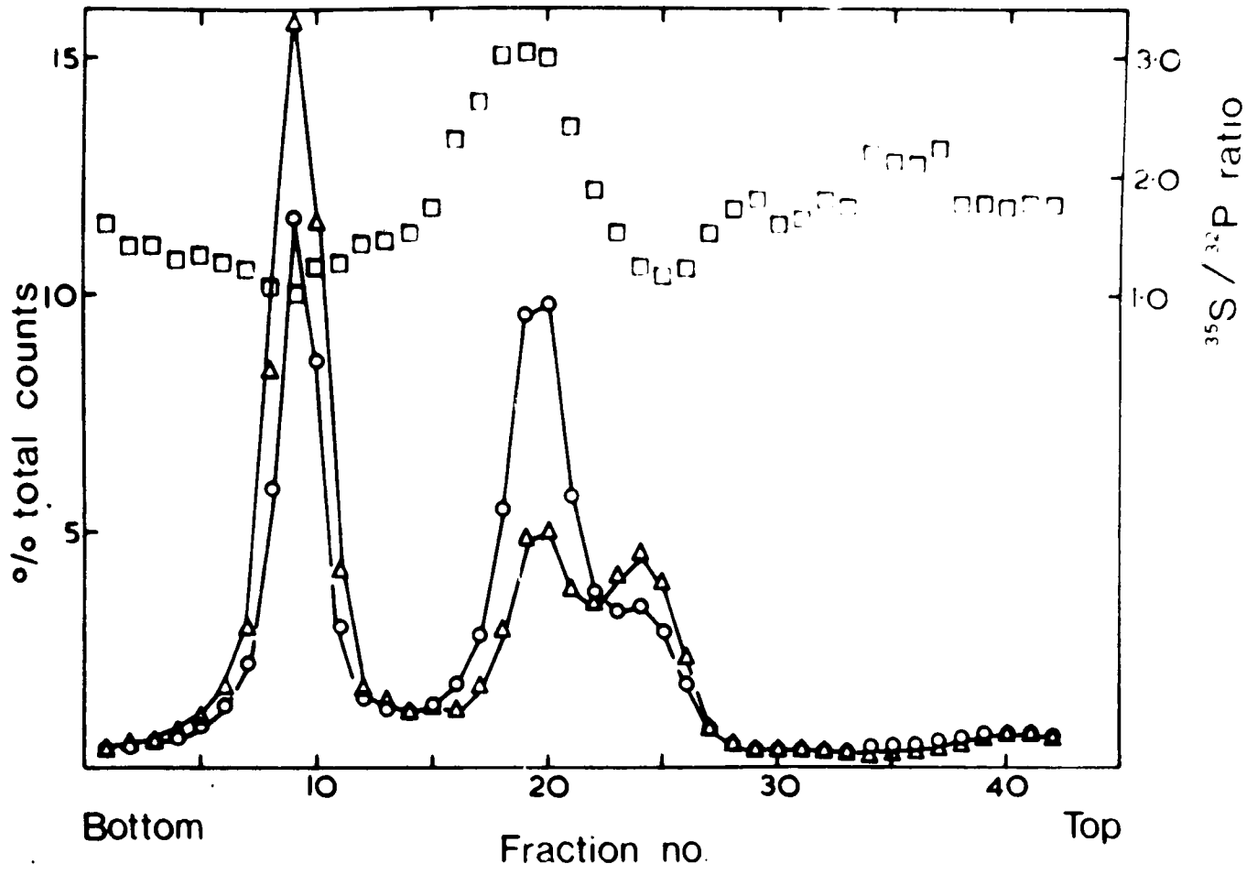
(e) Treatment of the ϕX-cell wall complex with DNase

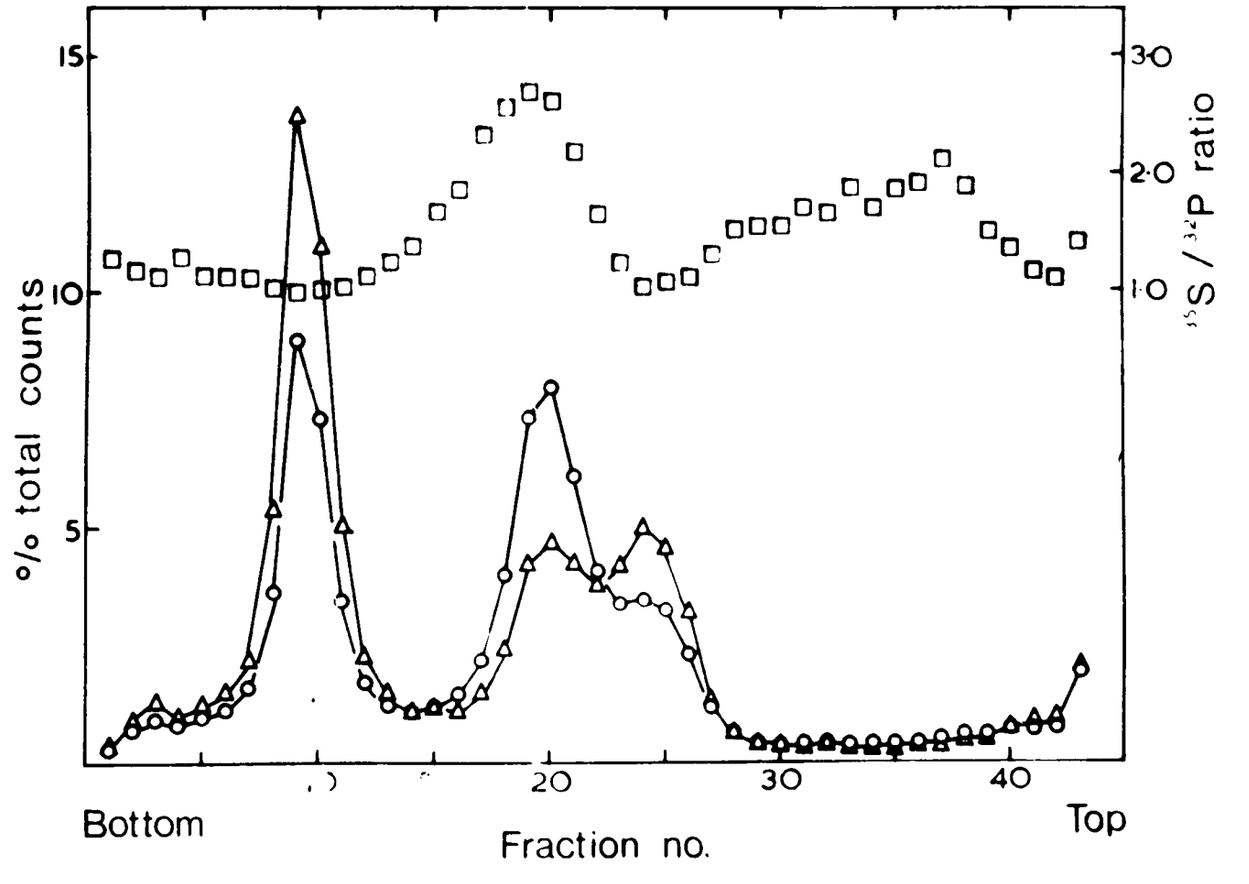
^{32}P-, ^{35}S-labelled ϕX were incubated with cell walls in 2 ml. adsorption medium (c) in the presence of 0.1 mg/ml DNase, and 0.1% $MgSO_4$ solution. The samples were incubated for 2 - 3 min. and then chilled, centrifuged and resuspended in $Na_2B_4O_7$-EDTA, and treated as before. Control samples with no DNase treatment were run simultaneously.

The results of incubating ϕX and cell walls for 2 - 3 min, with and without DNase are shown in Figures 23 and 24. Intact virus are still present after this short incubation. Neither the distribution of particles, nor the $^{35}S/^{32}P$ ratio is altered by treating the sample with DNase

Figures 23 and 24. Sucrose density gradient analysis of components formed from ØX particles incubated with cell walls in the presence of DNase.

^{32}P-, ^{35}S-labelled ØX (10^9 p.f.u./ml) and E. coli C cell walls (0.1 unit/ml) were incubated at 37° in adsorption medium (c) (Figure 23). DNase (1.0 µg/ml) and $MgSO_4$ (5×10^{-3} M) was added to a second sample (Figure 24). Both were incubated for 2-3 min before being chilled and centrifuged for 30 min at 32,000 g. at 4° in the 40 rotor of the Spinco Model L centrifuge. The pellets were resuspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA, and left at 4° overnight. 0.1 ml samples were put onto sucrose gradients (5-30% w/v) in 5×10^{-2} M tris, pH 8.1, and 3×10^{-3} EDTA) and run for 2 hr at 100,000 g, at 4° in the SW50 rotor of the Spinco centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; ^{35}S-counts —o—o— ; and the ratio of $^{35}S/^{32}P$ □·□ □ □


15
10
5
% total counts
3·0
2·0
1·0
^{35}S / ^{32}P ratio
10
20
30
40
Bottom
Top
Fraction no.


15
10
5
% total counts
3·0
2·0
1·0
^{35}S / ^{32}P ratio
20
30
40
Bottom
Top
Fraction no.

The morphology of particles found after adsorption of øX to cell walls.

Adsorption to cell walls produces ØX particles with altered sedimentation rates. To determine whether the particles had a changed morphology, or whether any extra material was attached to their surface, samples were examined by electron microscopy.

Experimental procedure

^{32}P-, ^{35}S-labelled ØX at 10^9 p.f.u./ml. and unlabelled ØX am3 at 10^{12} p.f.u./ml. were incubated with cell walls, and prepared for velocity sedimentation in sucrose gradients. After centrifugation alternate drops were collected onto filter paper for scintillation counting, or directly into vials for electron microscopy. Samples for electron microscopy were prepared as described in Methods.

The distribution of radioactivity is shown in Figure 25. Photomicrographs, (Plates 2 - 4) were taken from the points marked in Figure 25, to show intact ØX, particles from the 70S and 45 - 50S peaks. '70°-ØX' and '70°-ØX' treated with DNase were also examined in the electron microscope (Plates 5 and 6).

The results show that the preparations do not contain extraneous material but they are inconclusive because no consistent differences exist between the majority of particles in any of the samples examined. Three types of particles were seen.

(a) Plate 2A., shows a virus where no stain has penetrated. It is assumed to be an intact 114S particle.

(b) Plate 3B, shows that stain has penetrated the coat, and is filling the central space. The virus is assumed to have lost much of its DNA.

(c) Plate 4C, is a particle with an intermediate form. A ring of stain can be seen in the virus, but it does not fill the space. This particle is assumed to have been altered slightly.

Plates 5 and 6 both show all three types of particle (A,B,C) and another common feature (D), where material appears to have stuck to the surface of the ϕX. This phenomenon is not understood.

It was concluded that any change in morphology induced by adsorption is either too subtle to be observed at this magnification, or is masked by a change in configuration during fixing.

Figure 25. Sucrose density gradient analysis of ØX particles, for electron microscopy.

^{32}P- ^{35}S-labelled ØX (5×10^9 p.f.u./ml) and unlabelled ØX am3 (10^{12} p.f.u./ml) were incubated with E. coli C cell walls at 37°, for 2-3 min. before being chilled and centrifuged at 32,000 g, at 4° in the 40 rotor of the Spinco Model L65 centrifuge. The pellets were resuspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA, and left at 4° overnight. 0.2 ml samples were put onto sucrose gradients (5-30% (w/v) in 5×10^{-2} M tris, pH 8.1 and 3×10^{-3} M EDTA) and run for 2 hr. at 100,000 g. at 4° in the SW 39 rotor of the Spinco L65. Each fraction was assayed for ^{32}P-counts ——△——△— and ^{35}S-counts ——o——o— . Samples were taken from points marked (I, II, III) for electron microscopy.

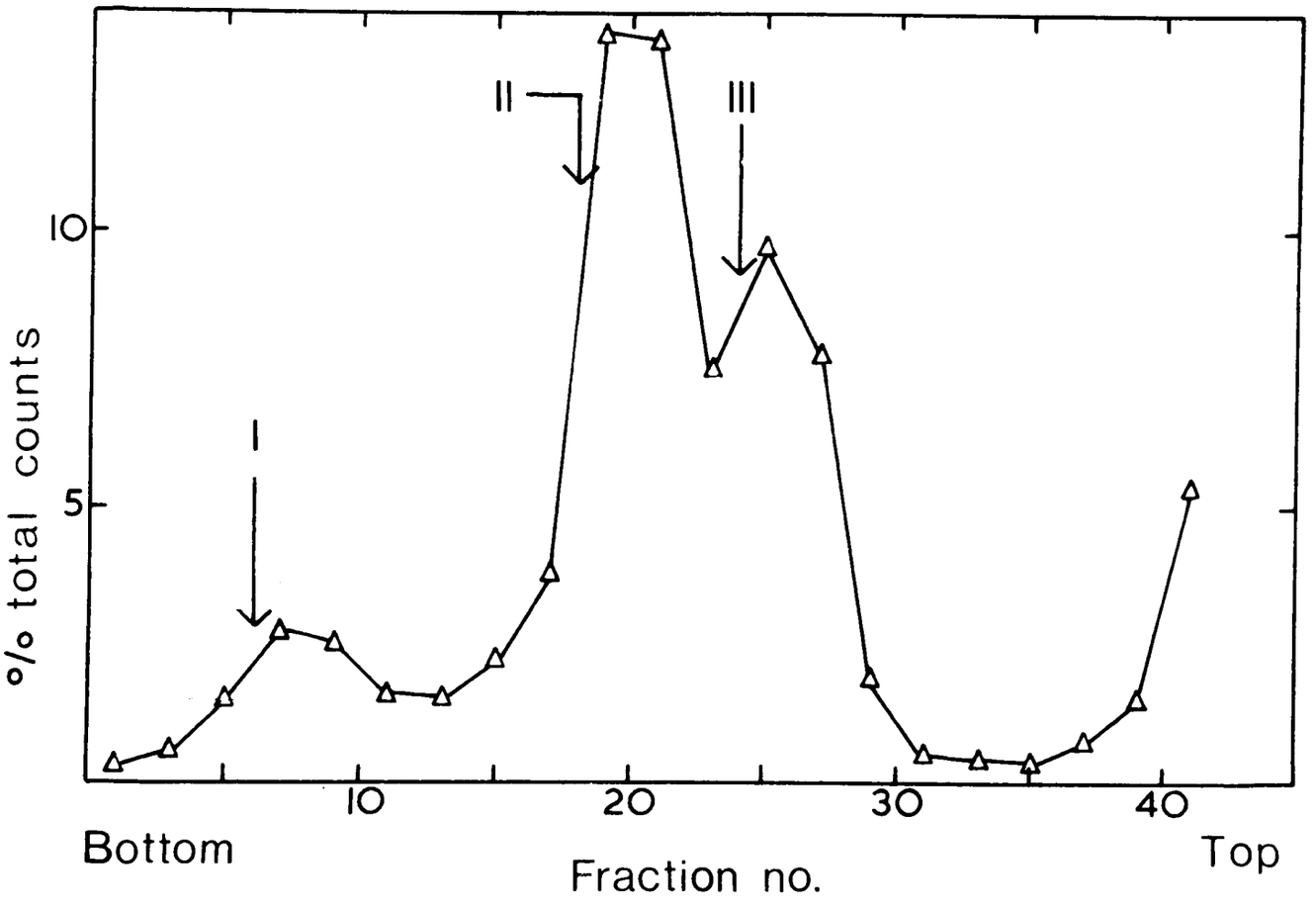

I
II
III
10
5
% total counts
10
20
30
40
Bottom
Fraction no.
Top

Plate 2. Electron micrograph of 114S ØX particles.

Samples were prepared as described for Figure 25, and taken at point I. They were fixed in 0.25 M formaldehyde and 1.5×10^{-3} M $MgCl_2$ for 5 min. Uranyl acetate (1% solution) was used as negative stain. Samples were examined in a Phillips 200 Electron Microscope Magnification x 222,000.

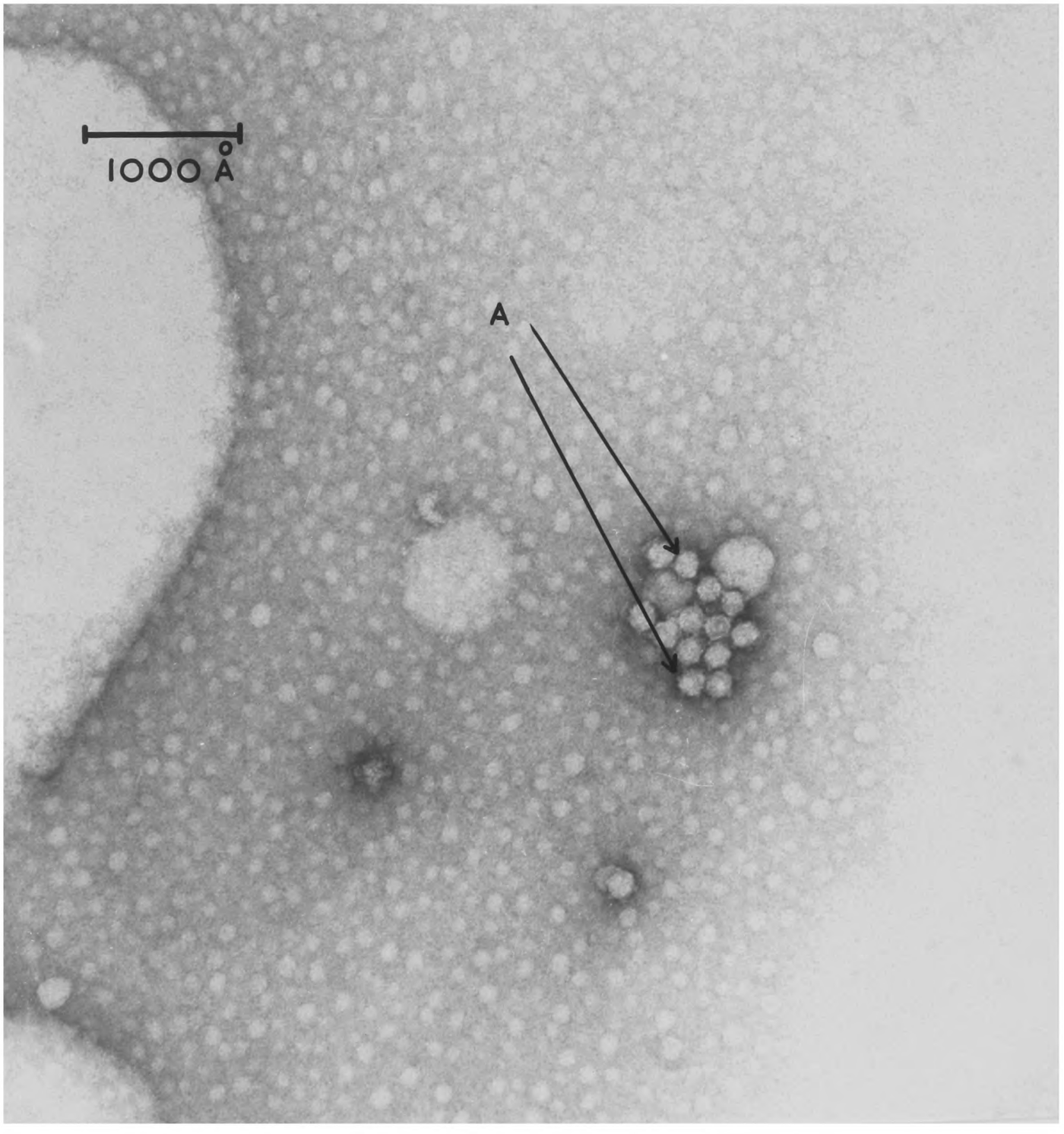

1000 Å
A

Plate 3. Electron micrograph of 70S øX particles.

Samples were prepared as described for Figure 25, and taken at point II. They were fixed in 0.25 M formaldehyde and 1.5×10^{-3} $MgCl_2$ for 5 min. Uranyl acetate (1% solution) was used as negative strain. Samples were examined in a Phillips 200 electron microscope. Magnification x 222,000

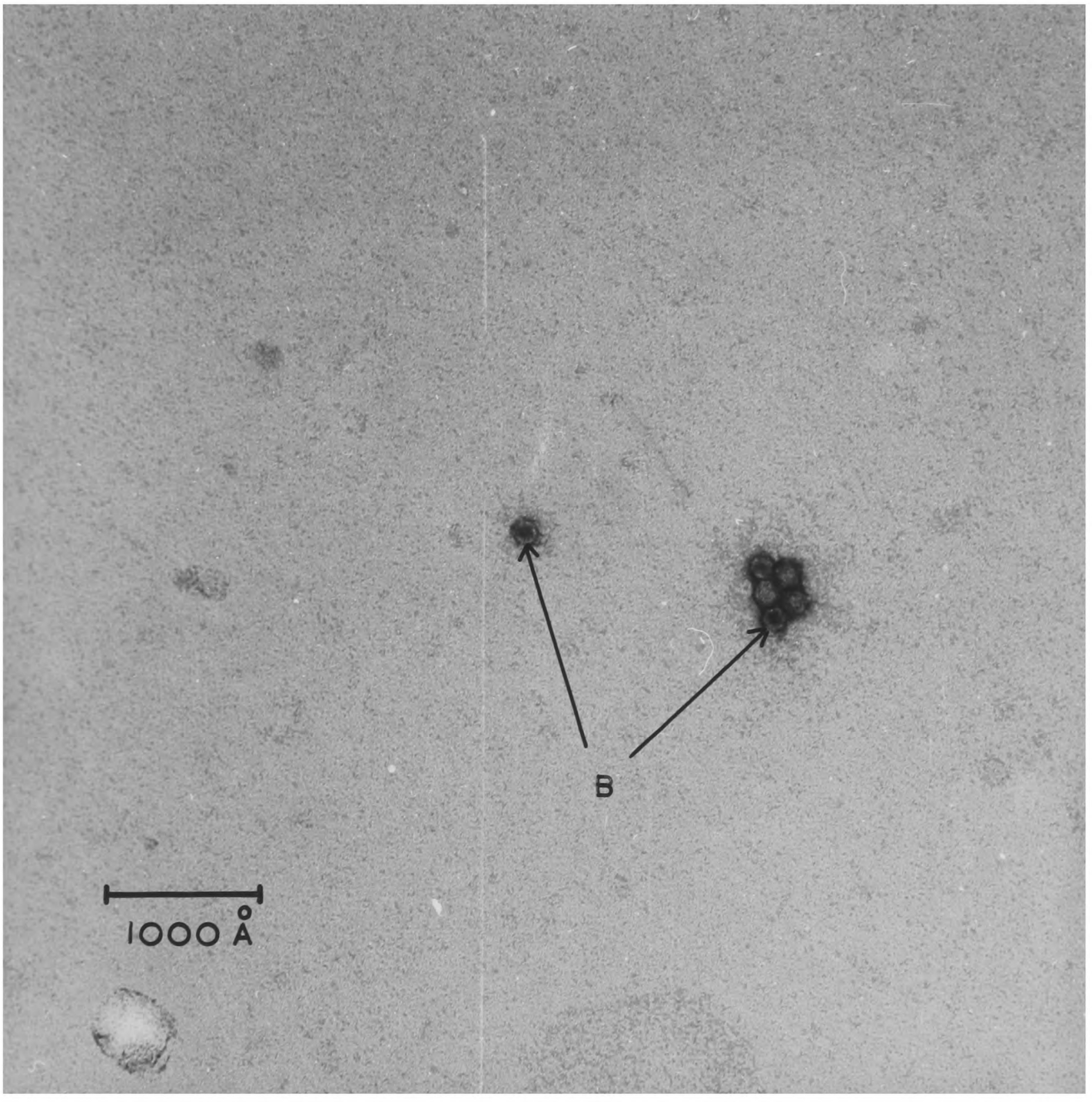

B
1000 Å

Plate 4. Electron micrograph of 45-50S øX particles.

Samples were prepared as described for Figure 25, and taken at point III. They were fixed in 0.25 M formaldehyde and 1.5×10^{-3} M $MgCl_2$ for 5 min. Uranyl acetate (1% solution) was used as negative stain. Samples were examined in a Phillips 200 electron microscope. Magnification x 222,000.

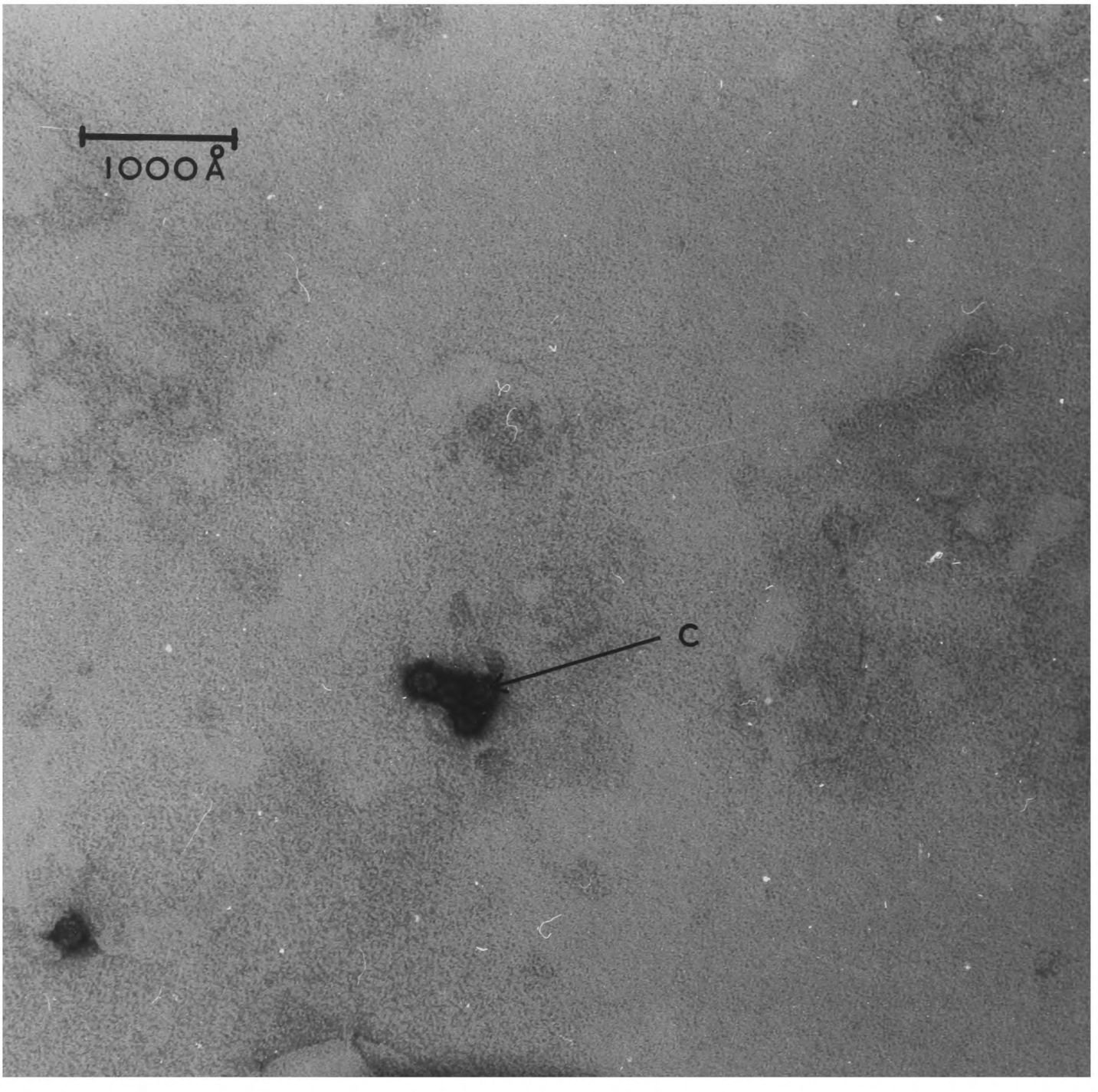

1000 Å
C

Plate 5. Electron micrograph of '70°-øX'.

øX at 10^{12} p.f.u./ml. in 5 x 10^{-2} M tris, pH 8.1, were heated to 70°, for 5 min. They were fixed in 0.25 M formaldehyde and 1.5 x 10^{-3} M $MgCl_2$ for 5 min. Uranyl acetate (1% solution) was used as negative stain. Samples were examined in a Phillips 200 Electron Microscope. Magnification x 222,000.

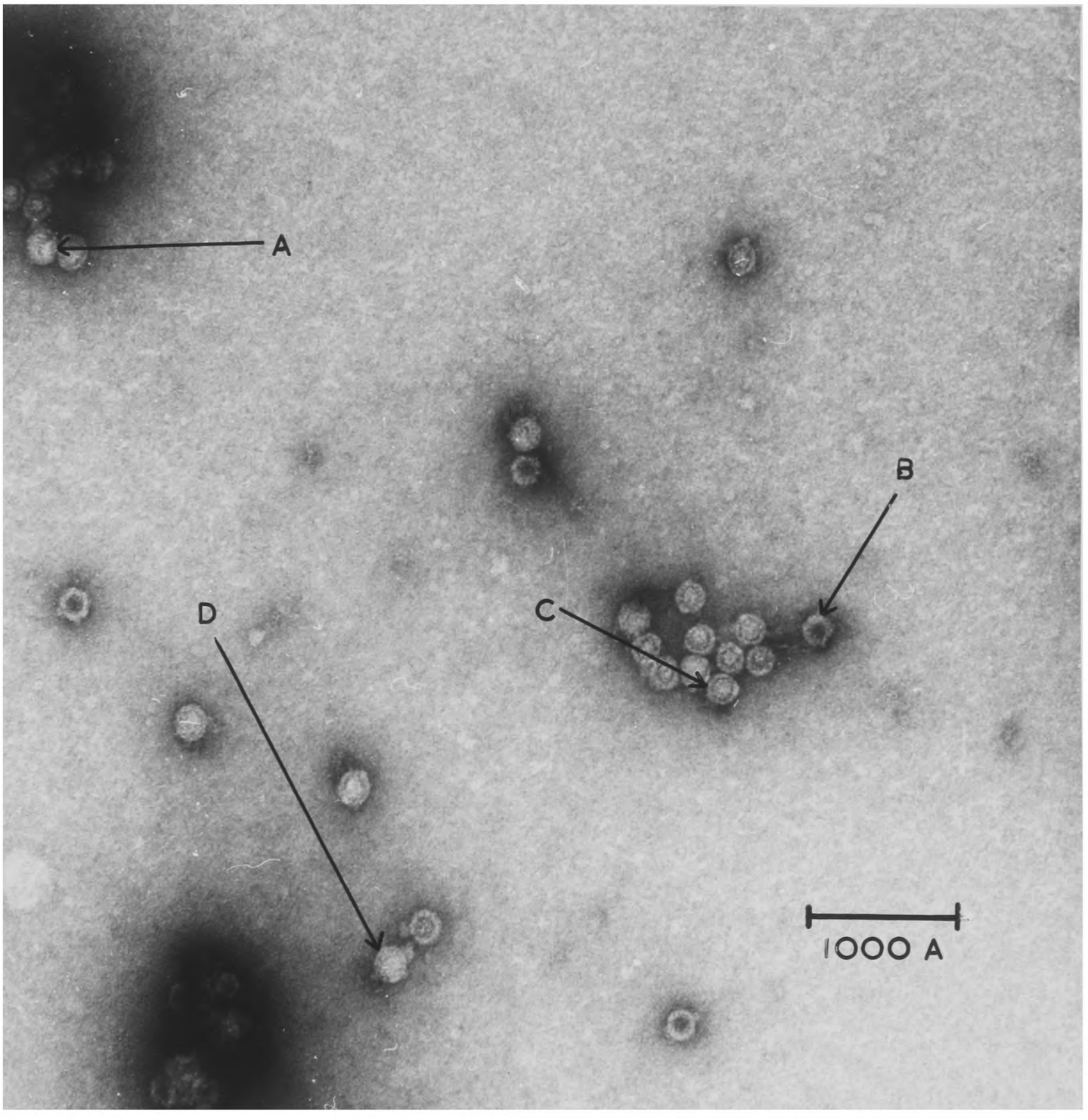
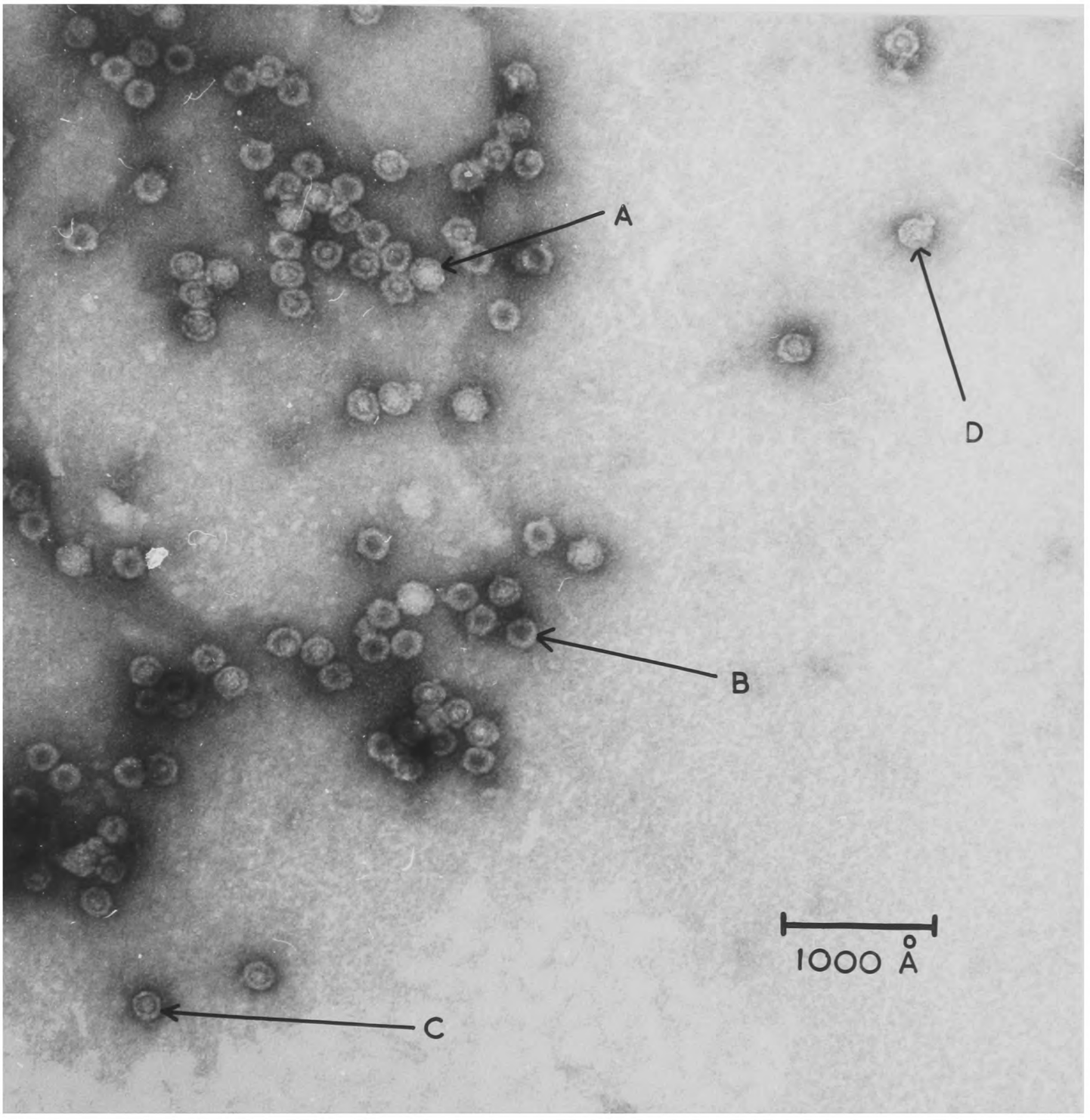

A
B
C
D
1000 A

Plate 6. Electron micrograph of '70°-øX' incubated with DNase. øX at 10^{12} p.f.u./ml. in 5 x 10^{-2} M tris, pH 8.1, were heated at 70°, for 5 min. They were chilled and then incubated at 37°, with DNase (1.0 µg/ml. in 10^{-2} M NaCl, 5 x 10^{-3} M $MgCl_2$, for 5 min. Uranyl acetate (1% solution) was used as negative stain. Samples were examined in a Phillips 200 Electron Microscope. Magnification x 222,000.


A
D
B
C
1000 Å

The structure of particles formed after adsorption to *E. coli* C cells.

Particles produced when ØX adsorb to cell walls may be an artefact, not produced with viable cells of E. coli C. To check this, components released after short term incubation of phage with whole bacteria were examined in sucrose density gradients.

Experimental procedure

E. coli C was grown and prepared as for adsorption experiments; 0.4 ml of the stock was added to ØX, suspended in adsorption medium (c). Control samples were kept cold, or incubated with E. coli B cells. Experimental samples were incubated at 37° for 3 - 5 min., with and without DNase treatment; or for 40 min. without DNase. They were then chilled, and centrifuged at 18,000 g for 15 min. The supernatant was kept for sedimentation analysis. The pellet was resuspended in 0.25 ml. 5×10^{-2} M $Na_2B_4O_7$ and 3×10^{-3} M EDTA, and left cold overnight. The cells were lysed in $Na_2B_4O_7$. The bacterial DNA released made the suspension very viscous. The viscosity was reduced by agitation on a vortex mixer (Scientific Industries International, Inc. (U.K.) Ltd.). Samples of 0.1 ml were then put onto gradients. Samples of 0.2 ml from the supernatants saved earlier were layered onto separate gradients.

Results

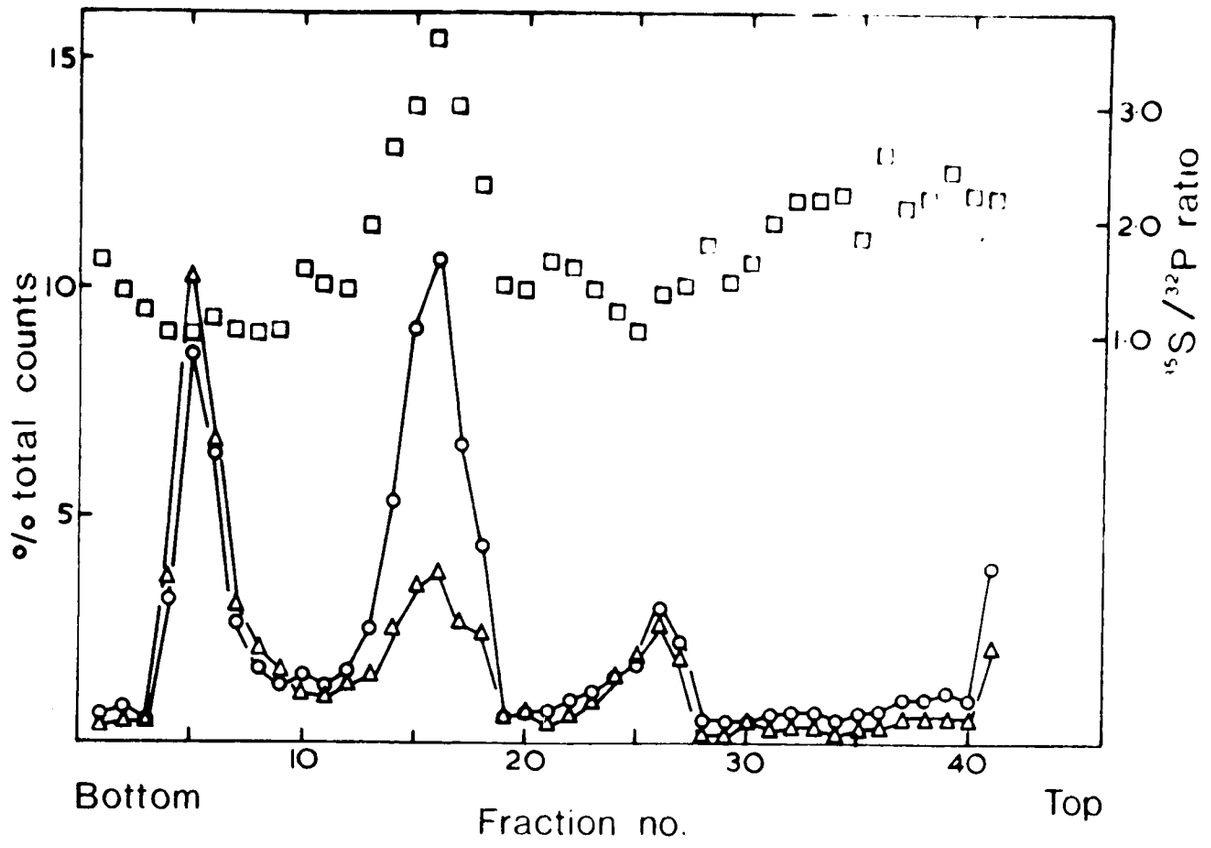
Control gradients, and those containing material from

supernatants, showed similar results to those obtained with cell walls.

Results of the experiments are shown in Figures 26 - 28. The same three peaks were found as before after short incubations (Figure 26) and their distribution was not altered by DNase treatment (Figure 27). Incubation for 40 min. produced a single peak at c. 70 - 75S. The peaks obtained with whole cells of E. coli C were not as well defined as those found on cell walls, but they are similar, and show a similar value for the $^{35}S/^{32}P$ ratio.

Figures 26 and 27. Sucrose density gradient analysis of ØX particles incubated with E. coli C cells for 3-5 min.

^{32}P- ^{35}S-labelled ØX (10^9 p.f.u./ml) and E. coli C (1×10^8 cells/ml) were incubated at 37°, in adsorption medium (c) for 3-5 min (Figure 26). DNase (1.0 µg/ml) and $MgSO_4$ (5×10^{-3} M) was added to the second sample (Figure 27). They were chilled and centrifuged at 8,000 g for 10 min. The pellets were re-suspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA, and left at 4° overnight. The viscosity of the solution was reduced by agitation on a vortex mixer, and 0.2 ml samples were put onto sucrose gradients (5-30% (w/v) in 5×10^{-2} tris, pH 8.1, and 3×10^{-3} M EDTA) and run for 2 hr at 100,000 g, at 4° in the SW 50 rotor of the Spinco Model L centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; ^{35}S-counts ——o—— —o——. The ratio of $^{35}S/^{32}P$ □ □ □ □


15
10
5
% total counts
3·0
2·0
1·0
$^{35}S/^{32}P$ ratio
10
20
30
40
Bottom
Top
Fraction no.

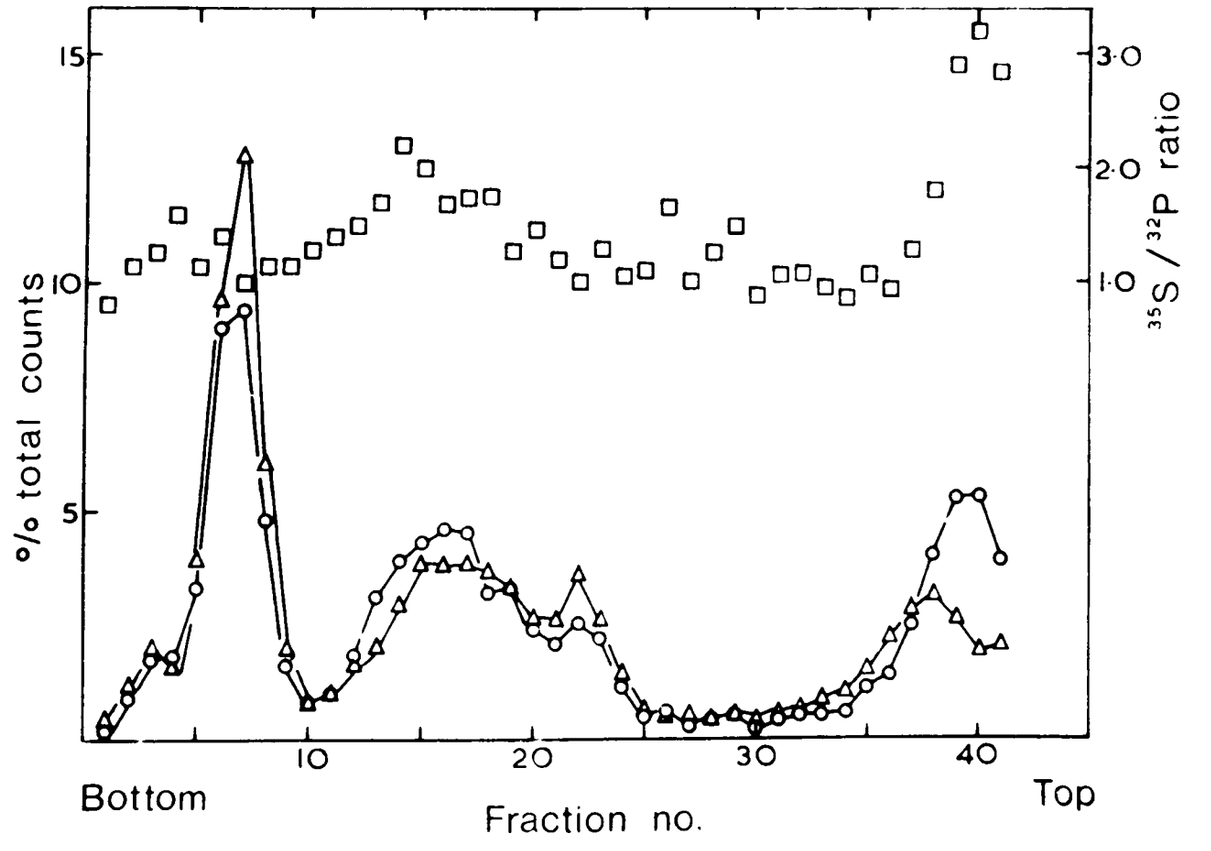
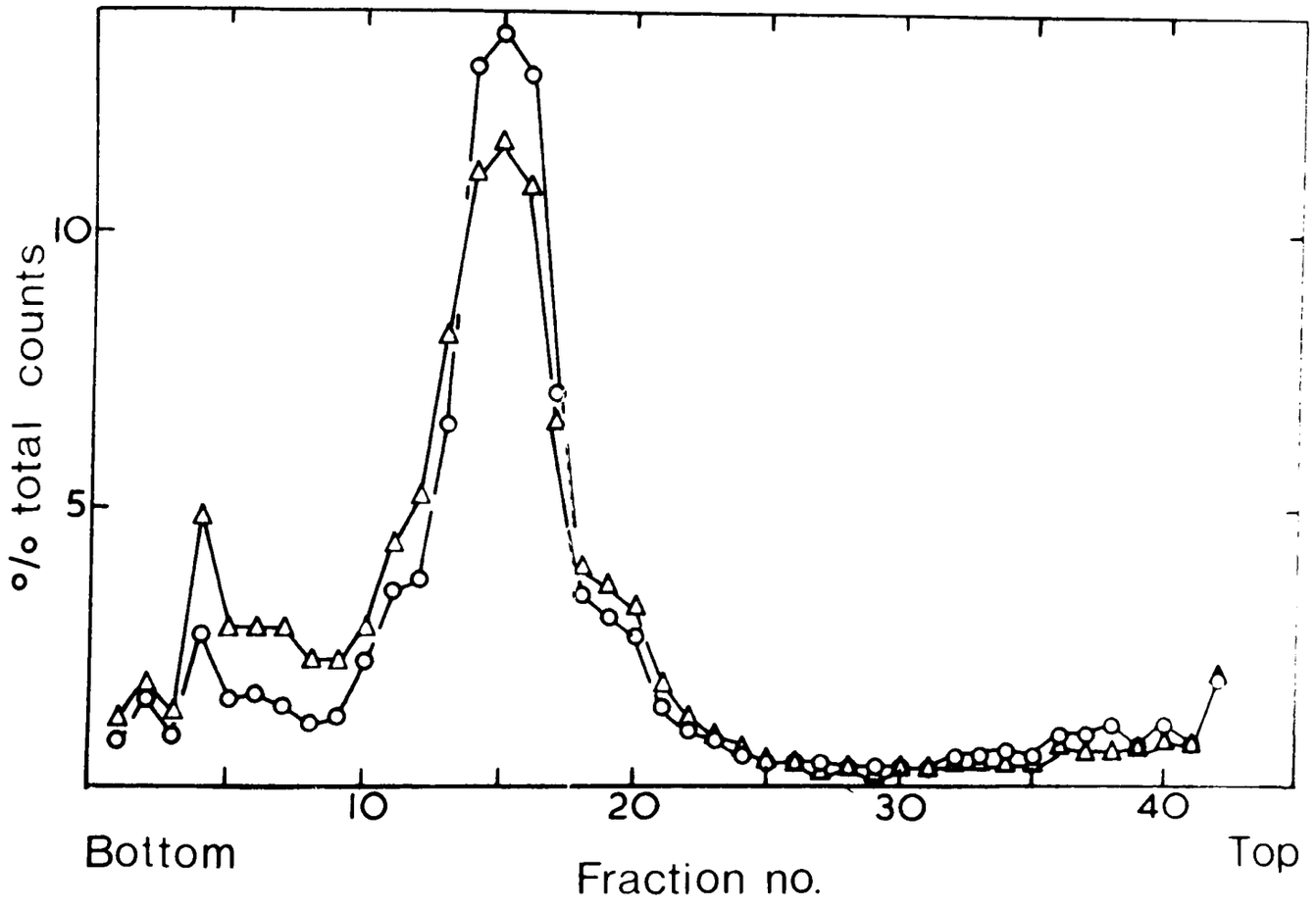

15
10
5
% total counts
3·0
2·0
1·0
$^{35}S/^{32}P$ ratio
10
20
30
40
Bottom
Top
Fraction no.

Figure 28. Sucrose density gradient analysis of ØX particles incubated with E. coli C cells for 40 min.

^{32}P- ^{35}S-labelled ØX (10^9 p.f.u./ml) and E. coli C (1×10^8 cells/ml) were incubated at 37°, in adsorption medium (c) for 40 min. They were chilled, and centrifuged at 8,000 g for 10 min. The pellets were resuspended in 5×10^{-2} M $Na_2B_4O_7$ and 5×10^{-3} M EDTA, and left at 4° overnight. The viscosity of the solution was reduced by agitation on a vortex mixer, and 0.2 ml samples were put onto sucrose gradients (5-30% (w/v) in 5×10^{-2} M tris, pH 8.1, and 3×10^{-3} M EDTA) and run for 2 hr. at 100,000 g., at 4° in the SW 50 rotor of the Spinco Model L centrifuge. Each fraction was assayed for ^{32}P-counts —△—△— ; and ^{35}S counts ——o——o— .


% total counts
10
5
10
20
30
40
Bottom
Fraction no.
Top

Infectivity of øX particles recovered after velocity sedimentation in sucrose density gradients.

The final experiment was performed to determine whether either the 70S or the 45 - 50S particles, were responsible for the spheroplast infectivity that follows adsorption by ϕX.

Experimental procedure

^{32}P- ^{35}S-labelled ϕX (10^9 p.f.u./ml) and unlabelled ϕX am3 (2×10^{11} p.f.u./ml) were incubated with cell walls in 2 ml. adsorption medium (c). The large number of unlabelled ϕX were added to ensure that the number in each sample of the gradient was great enough for successful spheroplast infection, provided the particles were infectious.

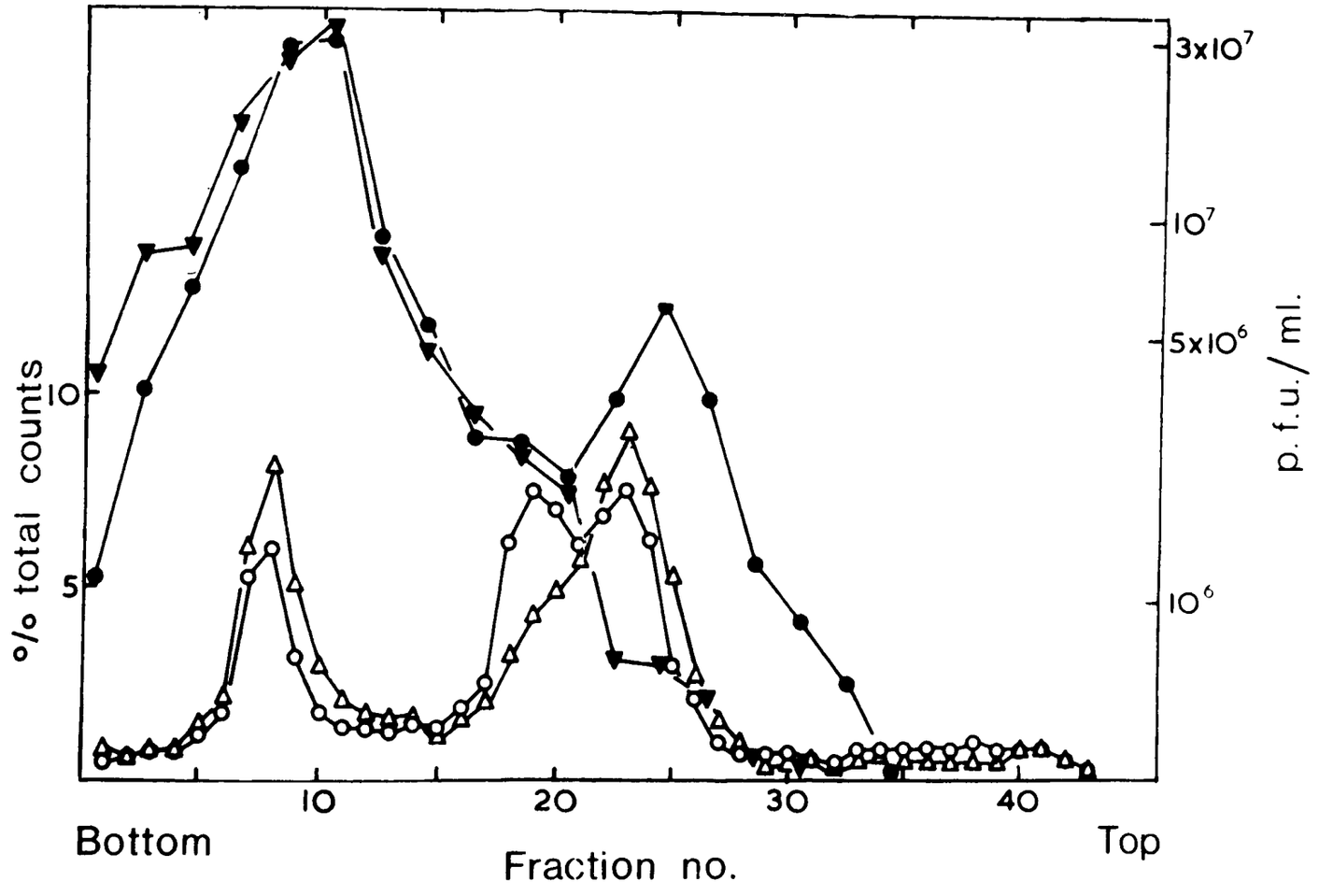
The samples were incubated at 37° for 2 - 3 min and then treated as before. When collecting the gradients, every alternate drop was diluted into 0.6 ml. dilution fluid. The others were collected for scintillation counting. The samples in dilution fluid were then tested for their ability to infect bacteria and spheroplasts. The control contained ϕX which were not incubated with cell walls.

Results

The results of the experiment are plotted in Figure 29. The 45 - 50S particles were infectious in the spheroplast assay. In the control, no 45 - 50S particles were produced, and there was no infection of spheroplasts.

Figure 29. Infectivity of particles after sucrose density gradient analysis.

^{32}P- ^{35}S-labelled ϕX (5 x 10^9 p.f.u./ml) and unlabelled ϕX am 3 (10^{12} p.f.u./ml) and E. coli C cell walls were incubated in adsorption medium (c) for 2-3 min. at 37°. They were centrifuged cold at 32,000 g 30 min in the 40 rotor of the Spinco Model L centrifuge. The pellets were resuspended in 5 x 10^{-2} M $Na_2B_4O_7$ and 5 x 10^{-3} M EDTA, and left at 4° overnight. 0.1 ml samples were put onto sucrose gradients (5-30% (w/v) in 5 x 10^{-2} M tris, pH 8.1, and 3 x 10^{-3} M EDTA) and run for 2 hr at 100,000 g, at 4° in the SW 50 rotor of the Spinco centrifuge. Alternate drops were assayed for ^{32}P-counts —△—△— ; and ^{35}S-counts —o—o— . The intermediate drops were assayed for total p.f.u. —▽—▽— ' and p.f.u. after incubation with spheroplasts.—ⓒ—ⓢ—


3x10⁷
10⁷
5x10⁶
10⁶
p.f.u./ml.
% total counts
10
5
10
20
30
40
Bottom
Top
Fraction no.

DISCUSSION

The results of this work confirm that ϕX will adsorb to cell walls under the same conditions of temperature, pH and ionic strength as are required to give successful infection of intact bacteria. They show that adsorbtion to isolated cell walls is alone sufficient to cause release of viral DNA in a form accessible to DNase. At the same time as the DNA is released, particles are formed, which have different sedimentation properties from those of intact ϕX. The 70S particles show no infectivity, but the 45 - 50S component (for convenience, referred to hereafter as 50S particles) do show infectivity towards spheroplasts, although not to whole cells.

Since DNA release and the formation of the modified particles occur with isolated cell walls, they must arise in the absence of any metabolic process contributed by the host cell. The problem, therefore, is to determine whether these components participate in normal infection of bacteria by ϕX, or whether they are artefacts associated with attachment to cell walls. Before discussing the possible participation of the particles in normal ϕX penetration, however, it is necessary to summarize the facts relating to the structure, and to compare their properties with those of other particles derived from ϕX, and described by other investigators.

It seems unlikely that the low sedimentation coefficient of the 50S particles is caused by adhering cell wall material or other debris, since electron micrographs fail to reveal the

presence of contaminating material of this kind. However, few particles were examined in this way, and it is impossible to be certain that small amounts of material which might modify sedimentation behaviour, would be detectable. An alternative explanation for the sedimentation properties of the 50S component is that it arises from øX particles which have undergone a conformational change resulting from an alteration in the arrangement of the protein subunits, but which is too subtle to be detected by electron microscopy. Such changes might be expected to result in the partial release of DNA from the protein coat, or an increase in its accessibility to DNase, as is found, for example with øX particles heated to 70°. (Guthrie and Sinsheimer, 1960). In contrast with the results using '70°-øX', however, the S-value does not change following treatment of the particles with DNase, and the ratio of the $^{35}S/^{32}P$-counts remains the same as that of intact virus. This suggests that the sedimentation properties of the 50S particles probably result only from changes in the coat protein of the whole phage.

Further evidence that changes take place in the coat protein is provided by the altered infectivity properties of the 50S particles. Unlike intact øX, 50S particles have lost the ability to infect whole bacteria, but they can infect spheroplasts. It was not possible to obtain an estimate of efficiency of 50S particle infectivity towards spheroplasts, so that the proportion of infectious particles in this position on the sucrose gradients

could not be measured.

Particles showing some resemblances to the 50S component have been described by other workers, the 47S particle, for example, which Guthrie and Sinsheimer (1960), obtained by heating øX at 70° for 5 min. Some of the properties of these '70°-øX' have already been mentioned. They are consistent with the particles possessing a trailing strand of DNA which is removable by DNase treatment, giving a further particle which moves at 70S. It is this property which distinguishes the '70°-øX' from the 50S component described here.

A different particle, obtained from øX lysates, was described by Dann-Markert, Deutsch & Zillig (1966). The particles moved at 57S on sucrose density gradients, and were infective towards spheroplasts. Electron microscopy revealed partly extruded DNA which was removable with DNase treatment, producing a non-infectious 70S particle. The origin of the 57S particles was not established, but again they differ from the 50S particles described in this thesis, in their sensitivity to DNase.

The 50S component arising from øX particles after attachment to cell walls appears, therefore, to consist of a hitherto undescribed particle with a full complement of viral DNA, but with a modified protein coat. Its possible participation in the natural infective process will be discussed later.

Another viral component which appears after short periods

(2 - 5 min) of incubation of ØX with cell walls is DNA, as shown by the release of DNase-sensitive ^{32}P-labelled material. None of the experiments described here were capable of indicating whether whole infectious molecules, as opposed to fragments of ØX DNA, were released. The latter possibility seems to be more likely, because DNase treatment of ØX-cell wall complexes did not destroy any of the infectious material detectable in the spheroplast assay. It was concluded that all the infectivity towards spheroplasts, which is released when ØX are incubated with cell walls, resides in the 50S particles.

The third viral component which appears on incubating ØX with cell walls seems to be identical with the 70S "top component" originally identified by Sinsheimer (1959a) in ØX preparations, and subsequently by several others (Maclean & Hall, 1962; Daems, Eigner, Van der Sluys, Cohen, 1962; Eigner, Stouthamer, Van der Sluys, Cohen, 1963; Stouthamer, Daems, Eigner, 1963; Bleichrodt & Knijnenburg, 1969).

In attempting to decide whether the formation of the different ØX-derived components plays any part in the natural infectious process, it is important to know whether the different particles also arise on attachment of ØX to whole bacteria. The results described here show that both 50S and 70S particles can be eluted from whole cells following adsorption of ØX. Also, since 70S, although not 50S particles, have been found regularly in cell lysates in a number of different investigations, their

production in these experiments provides further evidence that the cell wall system is an acceptable model for the initial stages of ØX infection. They seem to be typical products of ØX adsorption, although their reduced DNA complement precludes their being intermediates in the transfer of DNA to host cells.

The role of the 50S components is extremely difficult to evaluate. Two possibilities seem to exist: either the particles are true intermediates in the disruption of the virus to yield infectious DNA, which then passes across the host cell membrane; or they are side products which play no part in the natural infectious process. If the 50S particles are side products then they might be expected to remain present in constant amounts, or possibly to accumulate during prolonged incubation of virus with cell walls and bacteria. Instead, examination of eluates made after 30 min. incubation of ØX with bacteria or with cell walls reveals the presence of 70S particles only. This suggests that 50S particles may be precursors of those of 70S, but since the 50S particles are insensitive to DNase it is hard to envisage how they could be converted to 70S particles without the intervention of some intermediate type of particle, for which no evidence exists. Furthermore, it seems unlikely that sufficient DNA is released in the period 5 - 30 min. after mixing, to account for the conversion of 50S to 70S particles. In attempting to study this further, a more detailed study of the time course of the appearance and disappearance of the different viral products formed during

incubation might be useful, although technically difficult if quantitative results are to be obtained.

If the 50S particles are true intermediates in the infectious process, they might be expected to accumulate in eluates from cell walls, but to disappear during incubation with live bacteria. Knippers, Salivar, Newbold & Sinsheimer (1969) have demonstrated that successful penetration by ϕX does not occur with starved bacteria. They state that DNA can enter cells in the presence of 0.1 M potassium cyanide, or 0.1 M sodium azide, indicating that active bacterial metabolism is not essential for penetration, and they suggest that the surface may be reversibly altered during starvation in such a way as to preclude the entry of foreign nucleic acids. The bacteria used in the experiments described in this thesis had been starved, and it therefore now seems unlikely that they could take up DNA from ϕX. This would suggest that 50S particles, if they are intermediates in infection, would accumulate in these bacterial eluates. Since, however, they are not found in cell wall and bacterial eluates after 5 - 10 min., they must either become so firmly attached to the surface that washing with $Na_2B_4O_7$-EDTA fails to remove them; or they must be very unstable, if, once attached to the cell surface, they are kept at 37° under conditions where penetration is not favoured. The use of spheroplasts has shown that under conditions where 50S particles are stabilised, in $Na_2B_4O_7$ at 0°, they are infectious towards metabolising cells. It is thus possible to

predict that if these particles are true intermediates, interaction between ØX and actively metabolising cells would yield few or no 50S particles, even in 3 - 5 min. incubation, because subsequent stages in penetration would no longer be blocked. Alternatively, if their production is incidental to penetration, numbers comparable with those already found should be produced even with metabolising cells.

It has not been possible to elucidate how penetration is achieved, but production of the 50S particles is of considerable interest. Although these particles may be a by-product, and not a genuine intermediate in penetration, their production has shown that adsorption to cell walls does trigger a change in morphology of the virus, as it does in the T-even viruses. The use of cell walls and spheroplasts, although possibly an oversimplification, has been valuable in demonstrating the conformational change in whole ØX particles. Development of this work must now involve fully metabolising bacteria.

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