Winter 1973

MECHANISM OF VIRAL INACTIVATION IN ESTUARINE WATER

EDWARD MARTIN CARNEY

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73-16,744

CARNEY, Edward Martin, 1942-
MECHANISM OF VIRAL INACTIVATION IN ESTUARINE
WATER.

University of New Hampshire, Ph.D., 1973
Microbiology

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MECHANISM OF VIRAL INACTIVATION IN

ESTUARINE WATER

by

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B.S., Southern Connecticut State College, 1965
M.S., University of Montana, 1969

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Microbiology

January, 1973

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Date

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ACKNOWLEDGEMENTS

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ABSTRACT

MECHANISM OF VIRAL INACTIVATION IN ESTUARINE WATER

by

EDWARD M. CARNEY

Information on the potential public health significance
associated with the presence of adenoviruses in estuarine
environments prompted studies of survival of adenovirus 5 in
a New Hampshire estuary. A virus inactivating capacity (VIC)
of estuarine water limiting survival to 15 days was described.
Profiles of inactivating influences led to studies of the VIC
of estuarine bacteria. A direct relationship between virus
inactivation and bacterial populations was shown.

Bacillus subtilis phage 41C was used in place of Adeno-
virus 5 in an attempt to determine the mechanism of inactivation.
The bacterial virus nucleic acid was labeled with ^{32}P. Bacterial
species of estuarine origin were shown capable of disrupting
the protein coat of the bacterial virus, leading to release of
DNA and loss of infectious titer. An inactivation of 79 to 93
per cent of infectivity was accompanied by release of 47 to 67
per cent of nucleic acid bound ^{32}P.

Inhibition of both bacterial virus and adenovirus in-
activation was obtained by addition of chloramphenicol to
estuarine water.

Electron micrographs of bacterial virus in test and
control studies revealed structural alteration of virions
occurring only in the presence of estuarine bacteria.

x

The demonstrated release of DNA, with supporting electron microscope evidence of structural alteration of bacterial virus, combined with inhibition of both effects by chloramphenicol, led to the following conclusion. The mechanism of bacterial virus inactivation was believed to consist of enzymatic degradation of coat protein by species of estuarine bacteria seeking to use the virion as a source of nutrient and energy.

CHAPTER I

INTRODUCTION

In the past 20 years there has been an increasing con-
cern over the presence of human viruses in water and their
potential transmission to man via the water route. Initially
viruses which comprise the enteric virus group and include
members of the poliovirus, echovirus, coxsackievirus, reovirus
and adenovirus groups were found to be shed in fecal material.
Refinement of tissue culture procedures and sampling techniques
led to isolation of enteric viruses from raw sewage, waste
treatment plant effluents, river, estuarine and seawater. En-
teric viruses have been isolated also from shellfish in areas
polluted by domestic waste discharges. The most recent demon-
stration of a presumed transmission of virus by water occurred
with well water in Michigan. Poliovirus II was isolated from
the water following an outbreak of illness which occurred within
30 hours following its consumption. The well water had been
bacteriologically certified as safe for human consumption.

As a result of repeated isolations of human viruses
from aquatic environments and the potential public health sig-
nificance associated with these viruses, a number of investigators
have become interested in studying the effects of receiving
water on viral pollutants. In these investigations polioviruses
have been used extensively. Echoviruses, coxsackieviruses and
Theilers virus have been used also. More recently bacterio-
phages have served as models in studying viral inactivation.

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This investigation was prompted in part by the con-
tinued isolation of human viruses from the New Hampshire
estuarine environment and by an interest in the mechanisms
by which animal viruses were being inactivated in estuarine
water. The objectives of this study were: 1) to evaluate
various methods for studying virus inactivation, 2) to deter-
mine the inactivation profiles of adenovirus type 5 and Bacillus
subtilis phage 41C in estuarine water, 3) to evaluate various
factors considered to influence viral inactivation and 4) to
determine if any detectable structural or biochemical altera-
tions occurred to the viral particle during the inactivation
process.

A representative from the adenovirus group was selected
for this investigation because, in addition to representing a
very interesting group of enteric viruses, the stabilities in
surface waters of adenoviruses have received very limited con-
sideration. Thirty-one adenovirus serotypes are recognized
and are the only DNA viruses within the enteric group. Although
infections of the upper respiratory tract and eye are most
common, adenoviruses, including serotype 5 have been isolated
from patients with infectious hepatitis. At the present time
there is no evidence that adenoviruses, directly or indirectly
cause infectious hepatitis. Some members of the adenovirus
group induce tumors in neonatal hamsters and transform cells
in vitro. Adenovirus type 5 was chosen for use in this study
because it is considered to be non-oncogenic and replicates
to high titer in the HEp-2 cell line.

Bacillus subtilis phage 41C which is a DNA phage was selected primarily to adapt and evaluate radioisotope and electronmicroscopic techniques to study viral inactivation in estuarine water systems. The data anticipated from the use of these techniques was considered essential to a more precise and in-depth understanding of the phenomenon of viral inactivation.

CHAPTER II

REVIEW OF THE LITERATURE

It has been recognized for over a century that water can act as a vehicle for the transmission of infectious agents capable of causing disease in man. In the London epidemic of 1854, John Snow demonstrated that water was responsible for the transmission of cholera. It was not however until the latter half of the 19th century that the causative agents of typhoid and paratyphoid fever, shigellosis and cholera were isolated, defined and characterized.

Throughout the first half of the 20th century and to a significant degree up to and including the present, the focus of attention with respect to microbial water pollution has centered primarily with bacteria. Epidemiological findings linking water transmitted infections to pollution of water with human feces led to the concept that coliform bacteria could be used as an indicator of the sanitary quality of a water supply.

By the early 1940's evidence was beginning to accumulate which suggested that infectious virus could be transmitted also to man via the water route (Neefe and Stokes, 1945). In studying the course of infection with poliovirus, it was discovered that infected individuals shed large quantities of infectious virus in fecal material (Paul and Trask, 1942). Sabin (1955) reported concentrations of 100,000 tissue culture infectious dose$_{50}$ (TCID$_{50}$) per gram of feces. This observation was complemented by the report of Paul, Trask, and Culotta (1939)

on the isolation of poliovirus from sewage. Since 1939 many
investigators have confirmed the presence of all three types
of poliovirus in sewage (Kelly et al., 1957; Gravelle and Chin,
1961).

Viruses from the echovirus, reovirus, coxsackievirus
and adenovirus groups were later discovered and reported to
display similar shedding profiles in infected individuals
(Chang, 1968; Seigland et al., 1966; Clark and Chang, 1959).
Members from these virus groups have also been isolated from
sewage (Melnick et al., 1954; Clark and Chang, 1959; Lund et
al., 1966; Lund et al., 1969; Malherbe and Strickland-Chalmley,
1967; Nupen, E., 1970).

With high levels of infectious virus being shed in
fecal material, it was not difficult to envision that water
contaminated with virus laden sewage could serve as a potential
reservoir of human infection. Of special interest with respect
to these virus groups was the demonstration of their capability
upon ingestion to initiate an infection via the gastrointestinal
tract (Chang, 1968).

As tissue culture procedures were refined and sampling
techniques improved, the presence of human viruses was demon-
strated in river, estuarine and seawater and in shellfish pol-
luted by domestic sewage discharges (Metcalf and Stiles, 1968;
Shuval et al., 1970). Metcalf and Stiles (1968) were able
repeatedly to demonstrate poliovirus in sewage effluent, es-
tuarine water and in shellfish lying beneath this water on the
same sampling day.

The danger of waterborne viral disease is heightened by the ever increasing human population. Increased sewage volume resulting from increased population density in many communities has severely impaired the capacity of sewage treatment facilities to render discharged sewage effluent free of infectious virus (Chang, 1968). In addition to inadequate sewage treatment, environmental areas, (in particular the marine environment) receiving large volumes of sewage effluent, have shown a greatly reduced capacity for decreasing the concentration of human pathogens in the water column (Mitchell, 1968).

At the present time, epidemiological evidence has shown only limited correlation between virus in water and the occurrence of clinical disease in individuals exposed to this water (Mosley, 1967; Chang, 1968; Berg, 1970). The most convincing evidence of a waterborne virus disease is that of infectious hepatitis. Approximately 45 epidemics of infectious hepatitis have been linked to polluted water (Mosley, 1967). In the New Delhi, India, epidemic of 1955-1956 between 30,000 to 50,000 cases of hepatitis resulted from sewage contamination of the city's water supply. There is also some epidemiological evidence that 6 epidemics of poliomyelitis may have been the result of viral transmission via water (Mosley, 1967). With respect to other virus groups under consideration, only adenovirus at the present time gives suggestive epidemiological evidence of possible waterborne transmission (Clark and Chang, 1959).

Although the epidemiological profile does not comple-
ment the observed picture of viral pollution, this may reflect
a limitation in the effectiveness of the current epidemiological
approaches to detect viral infection and disease. Mosley (1967)
in considering the difficulties of obtaining rigorous epidemio-
logical proof of waterborne transmission of viral diseases,
concluded that the difficulties encountered were inversely
proportional to the size of the outbreak and directly propor-
tional to its duration.

In separate reports Berg (1970) and Chang (1968) also
commented on the possible reasons for this lack of epidemio-
logical evidence in regard to waterborne viral disease. Chang
suggested that in the majority of cases where individuals are
infected by waterborne enteric viruses, the infections remain
subclinical and go undetected. In the New Delhi epidemic
Chang considered the number of subclinical cases of infectious
hepatitis to be 10 times the number of clinical cases. He
further stated with respect to poliovirus epidemics that only
1-2% of the poliovirus infections could be diagnosed on clinical
grounds while 90 to 98% of the remaining cases may go unrecog-
nized and unreported.

A second possibility suggested by Berg was that the
majority of viral infections may appear in secondary contacts
while the original water source from which the primary infec-
tion occurred may go unrecognized. At the present time Berg's
suggestion appears to be consistent with a possible low level
transmission of virus by water in areas where water quality

standards are high. Chang's view may be more applicable to regions where water standards and quality are poor.

As a result of the repeated isolation of human viruses from aquatic environments and the public health implications involved, a number of investigators began to look at virus survival or its rate of inactivation in aqueous systems. Initially, studies were carried out in fresh water systems (Clark et al., 1956; Gilcreas and Kelly, 1955). When enteric viruses were demonstrated in estuarine (Metcalf and Stiles, 1968) and coastal seawater environments (Shuval and coworkers, 1970), other investigators began to look at virus in these systems. The problems considered by various investigators included: virus survival in different aqueous systems and the possibility of differences in survival behavior between individual viruses.

Previous studies involved river water of varying degrees of pollution, autoclaved river water and distilled water. The viruses used to study inactivation in these systems included coxsackieviruses A2 and A5, Theilers virus and poliovirus. Results from these studies indicated that the viruses differed with respect to their survival time (Clark and Chang, 1959). It was also observed that the extent of fecal pollution affected virus survival. In water samples representing varying degrees of pollution, the investigators observed that as the degree of fecal pollution increased the rate of virus inactivation decreased. The incubation temperature was shown to influence inversely virus survival. However, heating or autoclaving the water prior to introducing virus increased greatly the survival time of the enteric virus. Commenting on this

effect of heat Clark and coworkers (1956) suggested that virus inactivation was in some way correlated with the normal microbial flora of the fresh water environment and that any treatment which destroys, removes or alters this flora influences virus survival.

Because of viral pollution of New Hampshire estuarine waters and shellfish, Metcalf and Stiles (1967) undertook a study to determine the survival potential of representative enteric viruses in estuarine water. Included in their study were coxsackievirus B3, poliovirus type I and echovirus 6. Results from studies conducted under laboratory conditions showed no difference in virus survival between sterile and nonsterile estuarine water. Individual viruses however, were shown to vary in their resistance to inactivation. Temperature was shown to affect inversely virus survival and was considered a determinant factor in the inactivation process.

When virus survival was studied in dialysis bags immersed in the estuary, virus survival was shown again to be decisively influenced by the temperature of the estuarine water. In addition to temperature, the authors suggested a possible correlation between virus inactivation and the biotic flora.

The effect of pollution on virus survival was studied by suspending virus in a diluent of sewage effluent and comparing its survival to virus suspended in estuarine water. Both systems were immersed in the estuary and their rate of inactivation followed. The effect of pollution on virus survival in the estuary was similar to that reported by Clark

and coworkers (1956) for polluted fresh water. Survival times
for all 3 viruses were increased significantly when they were
suspended in sewage effluent. Although the studies by Metcalf
and Stiles (1967) and Clark and coworkers (1956) were carried
out in different ways using different systems, they both agreed
on the influence of viral identity, temperature and pollution
on virus survival. They also implicated the natural microbial
flora as contributing to the observed virus inactivation in
the test systems.

In comparison to studies on virus inactivation in fresh
and estuarine water, the most extensive work on virus inacti-
vation has been carried out using marine water systems. Plissier
and Terre (1961) were the first to report on virus inactivation
in seawater. Using seawater sterilized by Seitz filtration
they were not able to detect a significant difference between
virus inactivation in seawater and tap water from the city of
Nice.

In 1964 Hedstrom and Lycke while experimentally study-
ing virus carriage by the oyster noted that poliovirus III was
rapidly inactivated in seawater. Lycke and coworkers in 1965
reported that three logs of poliovirus infectivity disappeared
from seawater in 4 days at 23 C. In attempting to elucidate
the factors responsible for this inactivation, they observed
that the virus inactivating capacity (VIC) of seawater was
heat sensitive. Heating the seawater to 90 C for one hr re-
sulted in no detectable inactivation when virus was held at
23 C. The VIC of the water was relatively stable between pH
5.5 and 8.5. Proteinaceous material including calf serum,

skimmed milk, lactalbumin hydrolysate as well as amino acids were shown to inhibit the VIC of seawater if added to the water before or at the same time as the virus. Increasing concentrations of glycine (0.2 - 6 mg/ml), but not glycocolic acid inhibited viral inactivation in seawater. The amino group of glycine was implicated in the inhibition. The viral inactivating capacity was not restricted to seawater from a specific location nor a specific sampled depth (Magnusson et al., 1966; Shuval et al., 1970). Seawater samples with the same viral inactivating capacity differed with respect to their ability to retain their VIC following serial dilutions. In comparing VIC of a sample of seawater for different viruses (Polioviruses 1, 2, and 3, coxsackievirus B5, echovirus 32, adenovirus 7, vaccinia, influenza A2 and Escherichia coli phage T1), the viruses were shown to vary in resistance to inactivation; however, all of the viruses were found to be un- stable in the seawater used. When E. coli phage T1, which appeared to be the most stable, was tested in other seawater samples exhibiting VIC, a marked reduction in virus infectivity occurred. The investigators (Magnusson et al., 1966) suggested that the phage displayed only a relative difference in sensi- tivity to inactivation when compared to animal viruses.

Poliovirus 1 was reported to be considerably more un- stable in seawater than in salt solutions of the same salinity and pH. Seawater samples with the same salinity, however, dis- played marked differences in VIC (Magnusson et al., 1966). It therefore was considered unlikely that salts present in seawater were the principal agents responsible for the low

stability of the suspended virus. Evidence was presented which
suggested that salinity may play an indirect but important role
in viral inactivation by providing an environment for the
optimal action of VIC factors (Magnusson et al., 1966). These
unspecified factors appeared ineffective below NaCl concentra-
tions of 0.1 M.

Physical treatments such as sonication and repeated
freezing and thawing did not influence the VIC of the water.
In concluding their 1966 study Magnusson and coworkers suggested
that it was unlikely that the reduction in infectious virus
was a result of adsorption by water microorganisms. The re-
sults suggested reaction(s) of a biochemical nature as
responsible for the observed viral inactivation.

Other investigators (Matossian and Garabedian, 1967)
reported that the VIC property of seawater was filtrable.
They observed that Seitz-filtered seawater incubated with virus
at 25 C displayed the same VIC as untreated seawater. The
investigators suggested that microorganisms and particulate
matter had no appreciable effect on the inactivation rate.
Studies conducted in numerous salt solutions and artificially
prepared seawater however did not approach the rate of inac-
tivation recorded for untreated seawater. Inactivation of 10^4
$TCID_{50}$/ml of poliovirus was similar in 2.8% NaCl and artificially
constituted autoclaved seawater. Inactivation of poliovirus
in artificially prepared Lyman and Fleming's (1940) artificial
seawater was slightly faster than in artificial seawater of
Constan. The difference between the two seawaters was con-
sidered to reflect the presence of additional ionic species

in the Lyman and Fleming's seawater. Emphasis was placed on
the multiplicity of chemicals rather than their concentration
as possibly influencing the inactivation of the poliovirus
(Matossian and Garabedian, 1967).

In studying the effect of boiling and aging on virus
inactivation it was observed that poliovirus was inactivated
at a slower rate in boiled or aged seawater than in fresh
untreated seawater. Boiling artificial seawater did not affect
its virucidal efficiency (Matossian and Garabedian, 1967). In
studies using marine bacterial lysates no significant increase
in the rate of inactivation of poliovirus was detected in the
presence of the lysates when compared to controls. In sum-
marizing their results Matossian and Garabedian (1967) stated
that apart from its chemical composition there may be a factor
in natural seawater which is heat labile and has virucidal
properties.

Still pursuing the question of viral inactivation
Magnusson and coworkers (1967) reported on the possible rela-
tionship of marine bacteria to the VIC of seawater. They
isolated 90 microorganisms from a seawater sample displaying
a high viral inactivating capacity. Of the ninety isolates
an unspecified number were lost in passage or were otherwise
discarded because of their possible terresterial or fresh water
origin. Of the remaining 32 bacterial and fungal strains, one
bacterial strain described as Vibrio marinus and later identi-
fied as Vibrio parahaemolyticus (Shuval et al., 1972) was
demonstrated to have a high viral inactivating capacity when
placed along with poliovirus type 3 in heat treated seawater.

Other bacteria showing lower VIC were not considered. The
organisms were screened for antiviral activity at a concen-
tration of 1 x 10^5/ml. It was noted that the Cl *Vibrio*
parahaemolyticus isolate lost its VIC when passaged at 25 C
but regained it when subcultured at 4 C.

Studies were conducted comparing natural seawater and
the Cl bacterium in heat-treated seawater under various condi-
tions (Magnusson et al., 1967). Both systems when subjected
to 50 C showed a marked reduction in VIC. The systems also
responded similarly to the addition of glycine or treatment
with ether. Sonication had no apparent effect on either system
thus reaffirming earlier results and extending them to a more
isolated system. Based on these observations the investigators
(Magnusson et al., 1967) suggested that it was tempting to
assume that the reactions being observed in the bacterial sus-
pension were similar to those responsible for the virus
inactivation in seawater. However, they emphasized that with
the available data no such statement could be made and the
question remained unanswered.

The size of the virus inactivating component appears
to be considerable since it has been reported to be retained
by a 0.45 micron membrane filter (Magnusson et al., 1966;
Shuval et al., 1970). The results concerning the non-filtra-
bility of the factor are in some disagreement with the obser-
vations of Matossian and Garabedian (1967) and Mitchell and
Jannasch (1969). The component has also been reported to be
non-dializable (Shuval et al., 1970).

Studies reported by Shuval and coworkers (1970) confirmed much of the results reported by Magnusson and his colleagues. Seawater samples displaying VIC were reported to lose this capacity when membrane filtered, treated with ether or heated to 90 C. The investigators linked maximum bacterial growth with the more rapid phase of virus inactivation.

Eight organisms were isolated by the research group (Shuval et al., 1970) which exhibited VIC. The organisms displayed widely differing morphological and biochemical characteristics which suggested that the VIC was associated with more than one marine bacterial species. The VIC expressed by 8 marine bacteria appears to be an unstable characteristic since, prior to reporting the study, the eight isolates had lost their viral inactivating capacity. The C1 isolate of Magnusson also appears to have lost its VIC (Shuval, personal communication).

The viral inactivating capacity of seawater may be the result of an enzyme produced by certain bacteria. Recent attempts to obtain VIC in the absence of marine bacteria have failed (Shuval et al., 1970).

In 1969 Mitchell and Jannasch using Escherichia coli bacteriophage ØX174 reported that seawater filtered through a 0.45 micron filter retained VIC activity which was, in part, attributed to marine bacteria present in the filtrate. Anderson and Heffernan (1965) reported the passage of some marine bacteria through a 0.3 micron filter. When inoculated into seawater or autoclaved seawater which had been filtered through a 0.22 micron filter, ØX174 was observed to be rapidly

inactivated. It was suggested that this chemical inactivation may be due to salinity or the presence of heavy metals in the filtered water. In assessing the observed results the investigators (Mitchell and Jannasch, 1969) concluded that three factors were affecting the inactivation of $\emptyset$X174: marine microorganisms, chemicals present in the seawater and a protective action resulting from suspended organic material to which phage may adsorb. It was considered that the biological and chemical effects would be sufficient to overcome the protective influences of the suspended organic matter and lead to a rapid inactivation of the virus in natural seawater.

In studies using E. coli bacteriophage T2 Gerba and Schaiberger (1972) demonstrated that when seawater was autoclaved and centrifuged at 16,000 RPM for 2 hr, 90% of the initial virus titer was lost within 6 days. In the uncentrifuged sample only 10% inactivation occurred. The T2 bacteriophage was reported to adsorb rapidly to clay (kaolinite) particles in artificial seawater and to be eluted from the clay with changes in salinity. It therefore was suggested (Gerba and Schaiberger, 1972) that suspended particulate matter may play an important role in viral survival in seawater.

CHAPTER III

METHODS AND MATERIALS

Cell line

An established human epithelial carcinoma cell line, HEp-2, obtained from BioQuest, Inc., Baltimore, Maryland, was used for the propagation and assay of adenovirus type 5 and poliovirus type II. HEp-2 cells were grown at 37 C on Auto-Pow MEM (Flow Laboratories, Rockville, Maryland) with 5% fetal calf serum and maintained on Auto-Pow MEM with 0.5% fetal calf serum. Unless specifically indicated all tissue culture media contained 100 units of penicillin and 100 ug of streptomycin per ml.

HEp-2 cells were subcultured by treating the cell monolayer with versene (buffered 0.02% EDTA). The cells were resuspended and dispensed in growth medium.

Preparation and assay of animal viruses

Adenovirus type 5 was obtained from the American Type Culture Collection. The virus which is 60 to 80 mu in diameter exhibits icosahedral symmetry. The protein coat of the virus is composed of 252 capsomeres or subunits (Fig. 1). When a stock of adenovirus 5 was prepared, the virus was released from cells showing advanced cytopathology by freeze-thawing the cells 3 to 5 times. Cell debris was removed by low speed centrifugation (650 x g for 10 min). Viral stock was stored at -78 C.

Poliovirus type II was obtained from the laboratory of Dr. T. G. Metcalf (University of New Hampshire). Preparation of poliovirus stocks was similar to that of adenovirus type 5.

Both viruses were titrated on HEp-2 cell monolayers in tubes. Six tubes were used per dilution and a 0.1 ml aliquot of the assayed dilutions was inoculated per tube. Viral dilutions were made in maintenance medium. The inoculated cultures along with uninoculated controls were incubated at 37 C. Eight days following inoculation all cell cultures were examined for cytopathic changes. The tissue culture infective does resulting in 50% infection was calculated according to the method of Reed and Muench (1938).

Bacterial host

The host for 41C was <u>Bacillus</u> <u>subtilis</u> 168 TM Sm^r which was isolated originally by Dr. I. L. Miller. The organism requires tryptophan and is resistant to streptomycin. The strain was supplied by Dr. Robert Zsigray, University of New Hampshire.

Bacterial virus

<u>Bacillus</u> <u>subtilis</u> phage 41C was obtained also from Dr. Robert Zsigray. The phage was isolated from soil and has been reported to be sensitive to streptomycin (Zsigray, 1968). Structurally the phage is composed of an icosahedral head which is approximately 500 $\overset{o}{A}$ in diameter and a tail structure which is 1400 $\overset{o}{A}$ long and 100 $\overset{o}{A}$ in width (Fig. 2).

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Figure 1. Adenovirus type 5 (Magnification 200,000 X)

Figure 2. <u>Bacillus</u> <u>subtilis</u> phage 41C (Magnification
40,000 X)
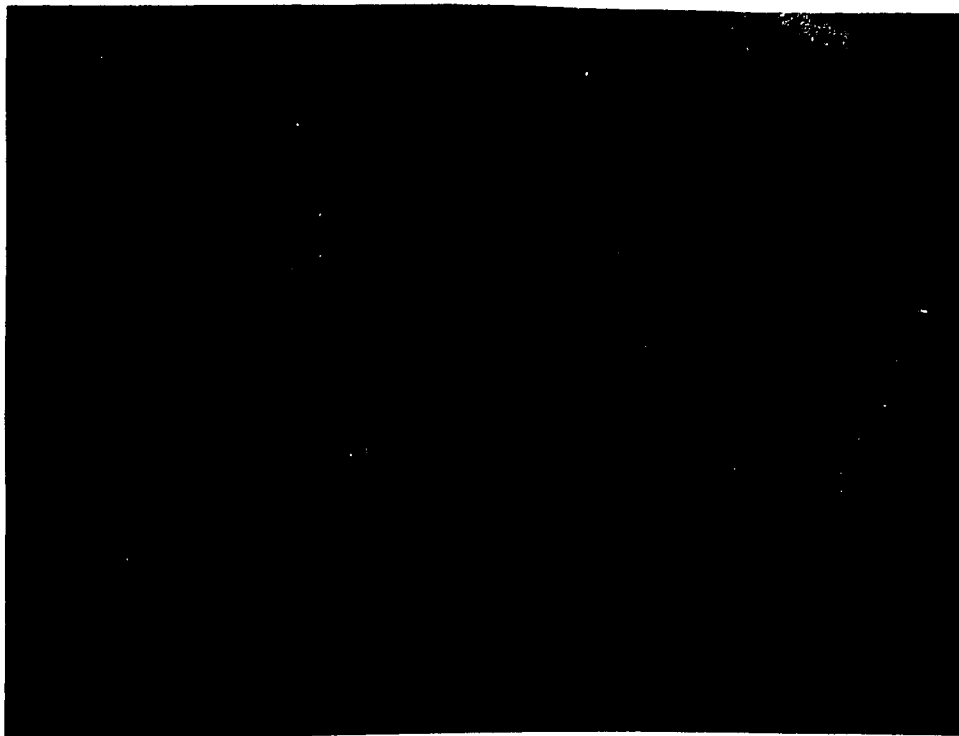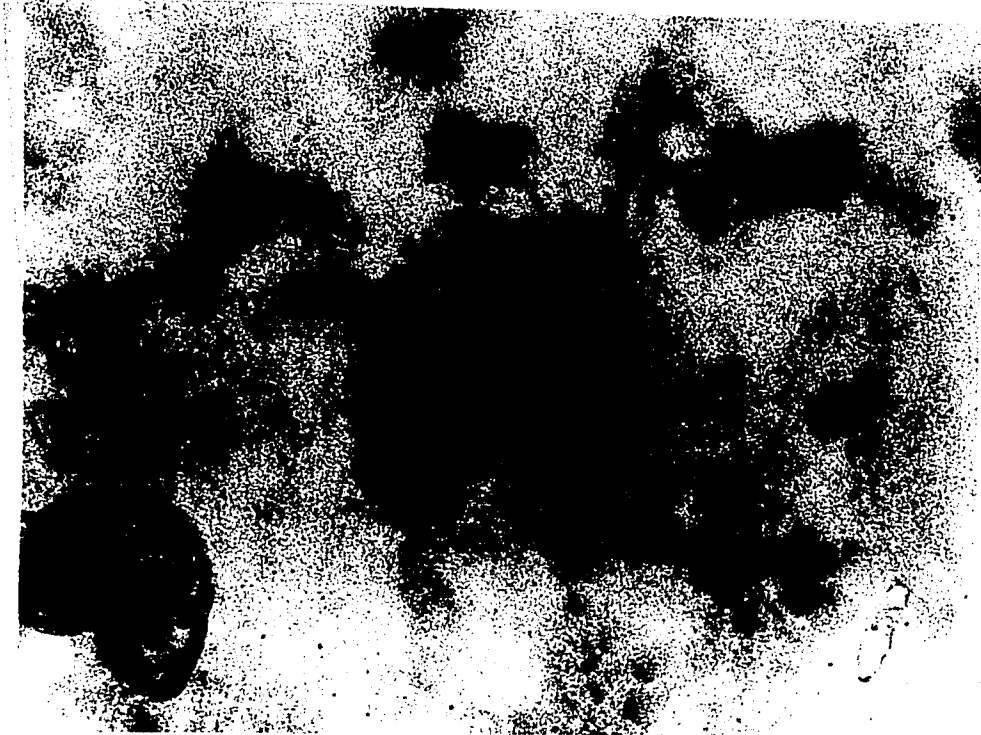
Bacteriophage titrations

Bacteriophage dilutions were made in TYS broth (see below) unless otherwise indicated; 0.1 ml aliquots of the assayed dilutions were added to TYS agar plates. Five ml of melted soft agar seeded with vegetative cells of B. subtilis 168 TM Sm^r were overlaid onto the agar plate and thoroughly mixed with the bacteriophage sample. All plates were incubated at 37 C for 24 hr. Following incubation plaque counts were made and recorded.

Media

TYS broth was composed of tryptone 10 g, yeast extract 5 g, NaCl 10 g, CaCl$_2$ 1 x 10^{-2} M and 1000 ml of distilled water. Solid media consisted of TYS broth with the addition of 1.5% agar (Difco): 0.7% agar was added to TYS broth for the soft agar overlay.

Culture conditions of bacterial host

Spores of B. subtilis 168 TM Sm^r were inoculated onto the surface of TYS agar plates and incubated at 37 C. Following germination the vegetative cells were washed from the surface of the agar plate and suspended in TYS broth. The bacterial suspension was adjusted to an optical density (O.D.) of 0.05 at 670 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. The inoculated broth culture was shaken at 37 C until the cell density reached an O.D. of 0.2 (approximately 1 x 10^7 colony forming units/ml). Cells at this stage were considered in the log phase and were used to propagate phage stocks, assay virus or prepare ^{32}P labeled phage.

Isolation and quantitation of marine bacteria

Marine bacteria were isolated on 0.45 micron membrane filters and cultured on 2216 Marine agar (Difco). Autoclaved estuarine water was used as the diluent. Plates were incubated at 18 C for 5 to 8 days. Colony counts were made and the results recorded.

Negative staining of 41C and adenovirus type 5

An aliquot of purified virus suspended in 1% ammonium acetate was mixed with an equal volume of 0.7% phosphotungstic acid (Brenner and Horne, 1959). A drop of the virus mixture was placed on a carbon-coated formvar grid and the excess moisture removed. The dried virus preparations were viewed with a Philips 200 electron microscope.

Scintillation cocktail and radiation assay

The scintillation cocktail used for counting the ^{32}P labeled samples has been described by Zsigray (1968). Briefly, the cocktail consisted of the following ingredients: Triton X-100 (Packard), 166 ml; 2,5 diphenyloxazole (Packard; PPO), 1.0 gm; 1,4 bis-2-(4-methyl-5-phenyloxazole)-benzene (Packard; dimethyl POPOP) 100 mg; deionized distilled water, 1000 ml. The mixture was stirred at room temperature for 30 minutes and then filtered through a Whatman #1 filter. The sample volume per vial was 1 ml and the scintillation cocktail volume was 15 ml. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3320. A gain setting of 7% and a window of 50 to 1000 were used. Samples were counted for 100 minutes or 10,000 counts whichever came first. In

comparing seawater with distilled water, no significant radiation quenching was observed in the system. The total sample count was adjusted to counts per min and corrected for background, dilution and isotope decay.

Procedure for ^{32}P labeling of bacteriophage 41C

The procedure described by Zsigray (1968) was used to insert ^{32}P into the DNA of the phage. Essentially the procedure consisted of growing up a log phase culture of B. subtilis 168 TM Sm^r to an O.D. of 0.2 in TYS broth. The culture was then centrifuged for 10 min at 12,000 x g in a Sorvall Model RC-2 at 5 C. The pellet was resuspended in TP medium (Kawakami and Landman, 1968). Phosphorus-32 was added to the TP medium at a concentration of 20 uc/ml. The culture was infected at a multiplicity of infection (MOI) of approximately 2 with 41C and the mixture was allowed to shake at room temperature for 150 min. The lysate was filtered through a 0.45 micron filter and the filtrate treated with 50 ug/ml of ribonuclease (RNase) and deoxyribonuclease (DNase) for 30 min at 37 C. Following enzymatic treatment the ^{32}P labeled phage suspension was loaded into an Oak Ridge polycarbonate ultracentrifuge tube (25.3 x 93 mm) and spun at 89,000 x g for 2¼ hr in a Spinco Model L ultracentrifuge. The pellet was washed 4 times and then resuspended in Auto-Pow MEM. It became necessary to resterilize the preparation by refiltration through a 0.45 micron filter prior to introducing the ^{32}P virus into the experimental systems.

Estuarine water

Except for an estuarine water sample collected off
Nanny Island at a depth of 7 ft with a bottle sampler, all
estuarine water samples were collected as grab samples in sterile
glass containers from surface water at Adams Point, Durham, New
Hampshire. Most samples were collected on the mid to high
tide. Salinity of the collected sample was determined hydro-
matically and was recorded along with temperature and pH.
Unless indicated, estuarine water samples were processed and
used within 24 hrs of their collection. The unused portion of
the samples was stored at 5 C.

Laboratory systems used to assay virus inactivation

Unless otherwise specified, virus inactivation studies
and the evaluation of factors considered to influence VIC were
carried out in Kimex milk dilution bottles. Estuarine water,
filtered estuarine water (0.22 micron Millipore) or autoclaved
estuarine water (16 psi for 15 min) were present initially at
a volume of 100 ml. Virus was added in a one ml volume sus-
pended in Auto-Pow MEM. In studies with 41C, phenol red,
antibiotics and serum were omitted from the suspending media.
When chloramphenicol was added at a concentration of 100 parts
per million (ppm), it increased the total volume of the system
by 2 ml.

Estuarine studies of adenovirus 5 survival

Survival studies with adenovirus 5 were carried out in
the Great Bay estuary at Adams Point, Durham, New Hampshire.
The virus was added at a 1:100 dilution to estuarine water

collected at Adams Point. After thorough mixing, a 10 ml

aliquot was removed and the remaining 90 ml transferred to a

seamless cellulose dialysis bag. The bag was secured in a rat

cage and immersed in the estuary. The procedure followed was

similar to that outlined by Metcalf and Stiles (1967, 1968).

Samples were removed and the dialysis bag changed in the

initial study every 7 days. However in subsequent studies it

was changed every 5 days. Temperature, salinity and pH of the

estuarine water at Adams Point were determined and recorded

on the sampling day. Prior to processing, the samples were

frozen at -78 C. Marine bacteria were inactivated by treating

equal volumes of the sample with ethyl ether for 18 hr at 5 C.

Ether was removed under vacuum and residual ether dispelled

from the sample in a Petri dish over a 2 hr period at room

temperature (Metcalf and Stiles, 1968). In the initial study,

no positive or negative virus control was used to verify the

potential of the estuarine water to inactivate virus. Essen-

tially the experiment was its own control. In a subsequent

study virus inoculated into estuarine and autoclaved estuarine

water maintained in glass bottles in the laboratory at a

temperature approaching that in the estuary served as positive

and negative viral controls. Later virus suspended in estuarine

or autoclaved estuarine water in seamless cellulose dialysis

bags and immersed in polypropylene tanks containing 8000 ml

of the respective estuarine water served as positive and

negative virus controls.

Method for studying effect of Cu++ on 41C

Initially a 0.1 ml virus inoculum in TYS broth was added to a 250 ml Erlenmeyer flask containing 100 ml of aged estuarine water with added copper at a concentration of 100 ppm. The flasks were placed on a rotary shaker at room temperature and allowed to shake for 4½ hr. At the end of the reaction period aliquots were removed and viral infectivity determined. Dilutions were made in TYS broth.

The system was later modified. Purified virus washed and resuspended in 0.85% NaCl served as the inoculum. One hundred ml of saline replaced the aged estuarine water. The contact time was reduced to 3 hr and saline replaced TYS broth as the diluent used for assaying viral infectivity. A spectrum of copper concentrations was considered (0.1 - 100 ug/ml).

DNase activity in estuarine water

B. subtilis 168 TM Sm^r DNA was added to estuarine water at a concentration of 135 ug/ml. DNase (40 ug/ml) in 10^{-4} M $MgCl_2$ was added to one aliquot of the estuarine water and the change in O.D. at 260 nm in a Bausch and Lomb Spectronic 600 Spectrophotometer was recorded.

Assay of DNase on ^{32}P labeled DNA in estuarine waters

A 1:10 dilution of ^{32}P labeled B. subtilis phage SP82G DNA (final concentration 5 ug/ml) which was obtained from John King (Department of Biochemistry, University of New Hampshire) was made in normal, filtered and autoclaved estuarine water. Following uniform distribution of the labeled DNA, the sample was divided into two 5 ml aliquots. One was

designated control and the other test. The test received 100

ug/ml of DNase and both test and control were incubated for

45 min at 37 C. Following incubation 1 ml was removed from

controls and tests and added to scintillation vials. These

represented the input of counts per ml of sample. Two ml

aliquots from both test and control were added to 14 x 100 mm

ignition tubes. Each tube received 0.5 ml of a 10% solution

of bovine serum albumin fraction V and 2.5 ml of cold 10%

trichloroacetic acid (TCA). The precipitate was pelleted in

a refrigerated (5 C) Sorvall Model RC-2 at 12000 x g for 15

min. One ml of the supernatant from each sample was added to

a scintillation vial. The precipitate was resolubilized by

the addition of 2 ml of the appropriate estuarine water plus

sufficient 1N NaOH to neutralize the precipitate. One ml of

the solubilized precipitate was added to scintillation vials.

Background radiation controls consisted of 1 ml of the specific

untreated estuarine water sample.

Procedure for ^{32}P labeled 41C estuarine water studies

One ml of the radioactive phage was added to 100 ml of

normal, filtered and autoclaved estuarine water. After shak-

ing the bottles to evenly distribute the phage inoculum, a

15 ml aliquot was removed from each bottle. The estuarine

water samples were incubated at 18 C and similar aliquots

removed on day 3 and 8. Ten ml of a given aliquot was pro-

cessed as previously outlined in the section dealing with

the assay of DNase on ^{32}P labeled DNA. Modifications in the

procedure were limited to the concentration and contact time

of the DNase (50 ug/ml for 30 min at 37 C). A portion of the
remaining 5 ml was used to determine the infectious titer of
the virus.

Structural alteration of 41C in estuarine water systems

Twenty-five ml of a stock phage suspension in TYS broth
which had been treated with 50 ug/ml of RNase and DNase was
centrifuged in Oak Ridge polycarbonate ultracentrifuge tubes
at 89,000 x g in a Spinco Model L ultracentrifuge for 2¼ hr
at 5 C. Initially the viral pellet was washed and resuspended
in 1 ml of Auto-Pow MEM. This was later modified and the
pellet was washed and resuspended in 1 ml of saline. Twenty
ml of estuarine or autoclaved estuarine water was added to the
suspended virus. Phage suspended in TYS broth served as initial
and terminal controls. Aliquots were removed at time zero and
at the termination of the study and assayed for viral infec-
tivity. All systems were incubated at 18 C.

After removing the final aliquots for infectivity
determinations, the systems (still in polycarbonate tubes) were
recentrifuged to pellet the phage. The pellet was washed once
and resuspended in 1 ml of 1% ammonium acetate. The washing
was required to remove residual marine salts which were shown
to interfere with the quality of the PTA stained preparations.
An aliquot of the sample was stained with 0.7% phosphotungstic
acid and the stained preparation viewed with a Philips 200
electron microscope to determine if any detectable structural
alteration had occurred to the phage particles as a result of
the observed inactivation.

CHAPTER IV

RESULTS

Survival of adenovirus type 5 in the estuary during the spring and summer of 1970

A survival study was conducted at Adams Point during April and May, 1970, to determine the survival potential of adenovirus 5 in the estuarine environment. During this period the salinity increased from 14.55 to 24.75 ppt and the temperature increased from 11 C to 13.5 C. The results of the study (Fig. 3) showed that over 90% of the initial viral input was inactivated within 7 days. However, complete viral inactivation was not observed until day 21. This observation suggests that the virus when present as a pollutant in the estuary, could survive for a sufficient period of time to represent a source of human infection. At the same time the data also illustrate the potential of estuarine water to inactivate virus.

Because adenovirus 5 survived for an appreciable time in the estuary, a second virus study was conducted during July of 1970. The objectives of this investigation which was conducted at a lower initial viral concentration, were to determine the reproducibility of the original observation and to evaluate the influence of changes which occur in the estuary during the summer. During July the salinity of the estuary increased from 29 to 31 ppt while the temperature increased from 18 C to 23 C. Under these conditions 99% of the initial viral infectivity was lost within 5 days (Fig. 4). However, complete viral inactivation was not recorded until day 15. The results obtained
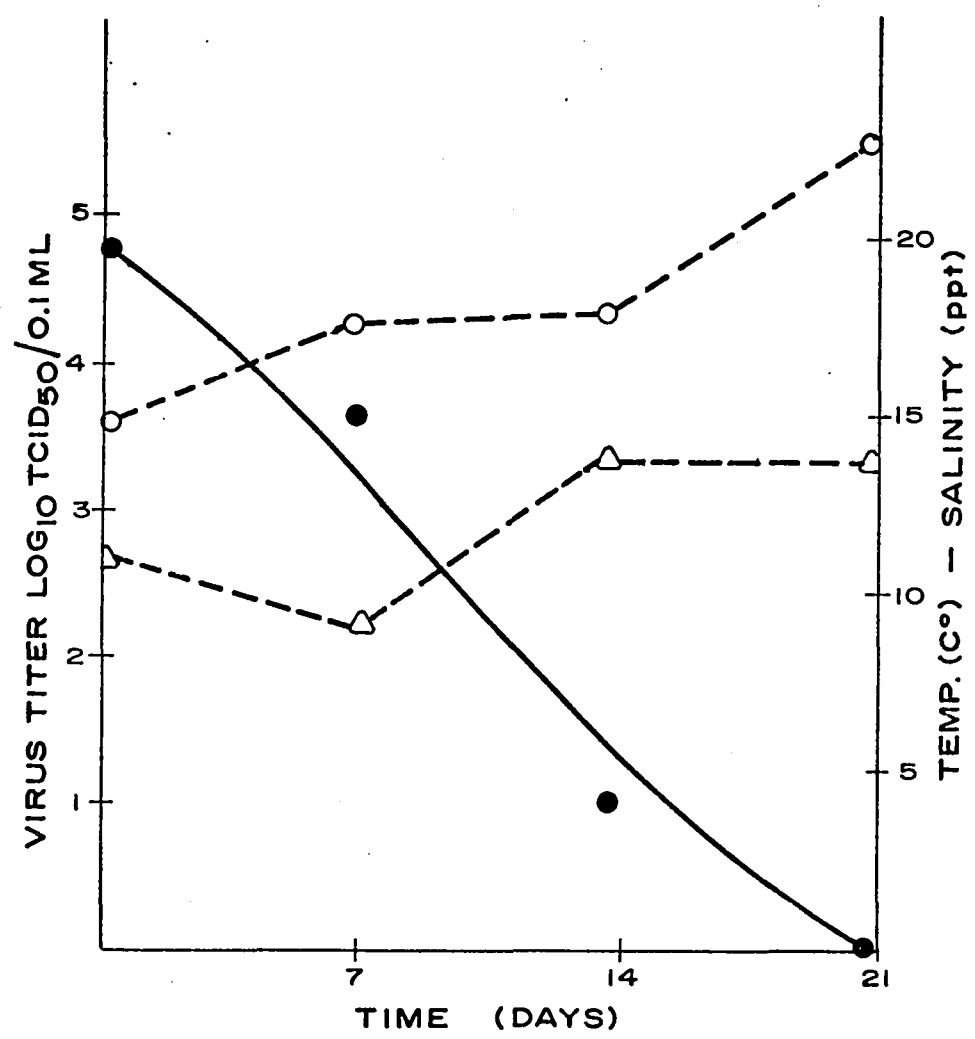
Figure 3. Inactivation of adenovirus 5 in the
estuary during April-May 1970.
Symbols: ● adenovirus 5; △, temperature; ○
salinity.
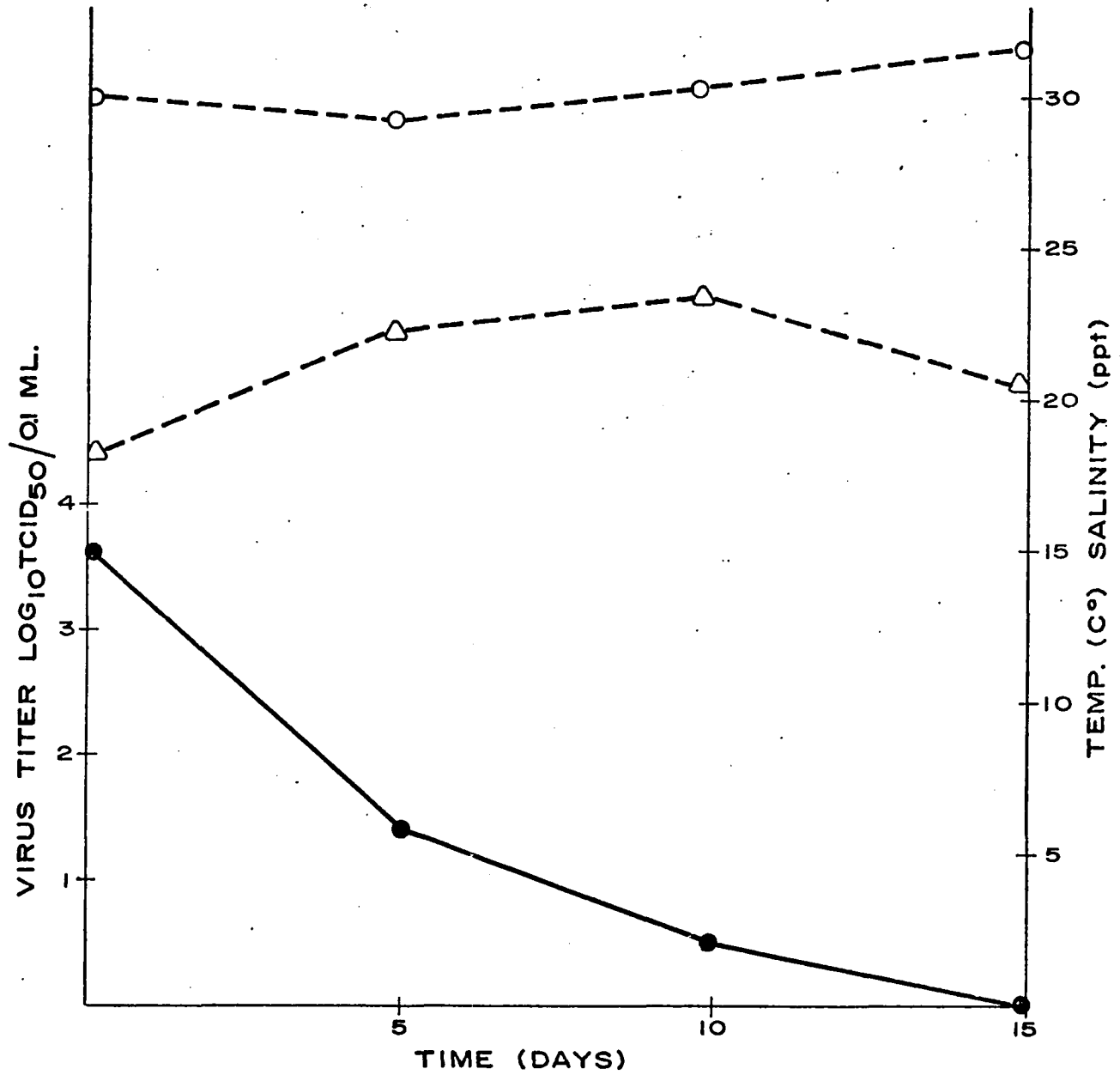
Figure 4. Inactivation of adenovirus 5 in the estuary
during July 1970. Symbols: ● adenovirus 5;
△, temperature; ○, salinity.

from this study remained consistent with those of the April-
May study.

After 7 days, the viral titer in the spring study
approached that initially used in the summer study. When both
studies were represented at approximately the same viral con-
centration (Fig. 5) the resulting graph showed that the viral
population in both systems displayed similar inactivation
profiles even though the loss in infectivity of virus in the
summer study occurred at a higher temperature and salinity.
This result suggested that the initial virus concentration may
influence the duration of the virus survival in the estuary.

Comparison of adenovirus inactivation in the estuary and under laboratory conditions

It was of interest to determine if the observed poten-
tial for adenovirus inactivation in the estuary could be
demonstrated under more controlled laboratory conditions. When
data from the July estuarine study was compared with those ob-
tained in the estuarine water maintained in glass bottles (at
18 C) in the laboratory, the inactivation was observed to
proceed at a faster rate in the environment than in the labora-
tory (Fig. 6). However, the laboratory result verified the
potential of estuarine water to inactivate the virus.

When estuarine water was autoclaved, its potential for
reducing the infectivity of the virus was greatly reduced (Fig.
6). This observation suggested that autoclaved estuarine water
might serve as a negative virus control.
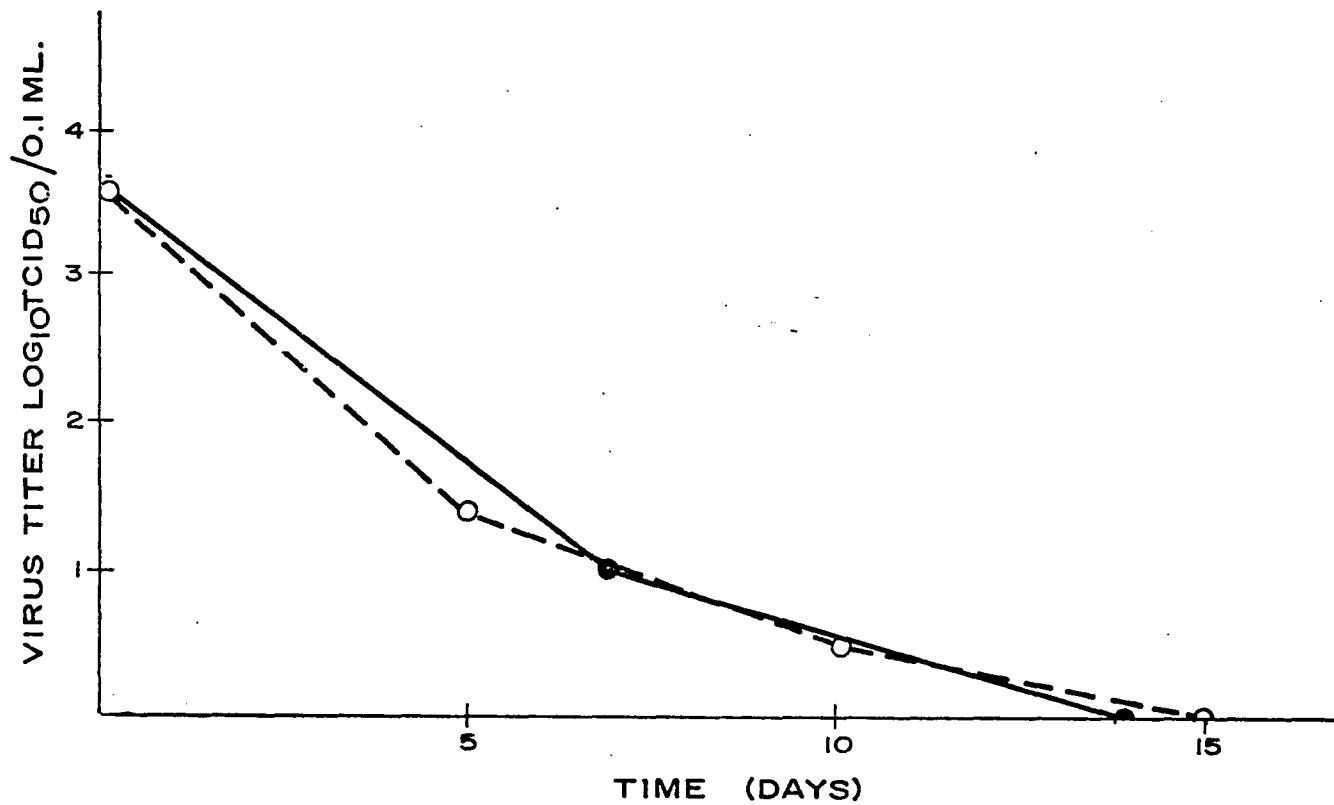
Figure 5. Comparison of the inactivation of adenovirus 5 during the spring and summer in the estuary. Symbols: ○ adenovirus 5 spring study; ●, adenovirus 5 summer study.
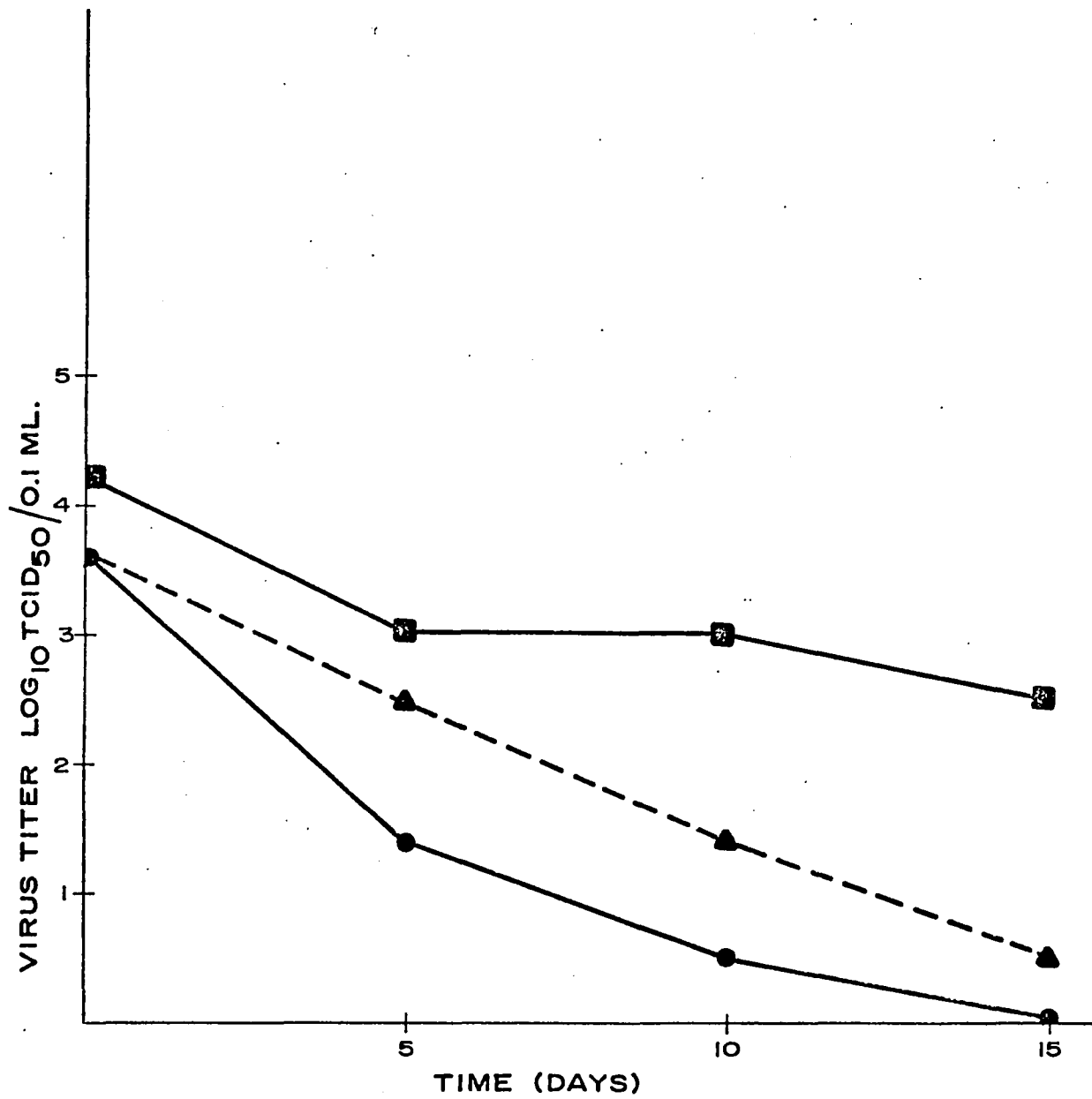
Figure 6. Comparison of adenovirus 5 inactivation in the estuary and under laboratory conditions. Symbols: ● adenovirus 5 estuary, ▲ adenovirus 5 laboratory estuarine water; ▣ adenovirus 5 autoclaved estuarine water control.

Adenovirus inactivation in estuarine water collected from different locations and depths in the estuary

The inactivation profile of adenovirus 5 was compared in estuarine water collected at the surface from Adams Point with that collected at a depth of 7 ft off Nanny Island to determine if the inactivating potential of estuarine water was restricted to a specific location or sampled depth within the estuary. The loss in viral infectivity recorded in both samples (Fig. 7) was similar and was considered to indicate that the viral inactivating potential of estuarine water was not re-stricted to a specific location or a specific sampled depth. This observation would be expected because a thorough mixing of the surface and bottom waters occurs in the estuary and thus eliminates most of the ecological niches found in unmixed bodies of water.

Inactivation of adenovirus 5 and poliovirus II in estuarine water

Since polioviruses have been used as the prototype enteric viruses in most studies which have considered marine or estuarine virus inactivation, a study was conducted to com-pare the inactivation of adenovirus 5 and poliovirus II. The investigation was carried out under laboratory conditions (18 C) in estuarine water collected off Nanny Island. The results from the study (Fig. 8) indicated that adenovirus 5 was more resistant to treatment with estuarine water than poliovirus type II. This observation emphasized the importance of con-sidering adenoviruses in addition to polioviruses in studies on viral inactivation.
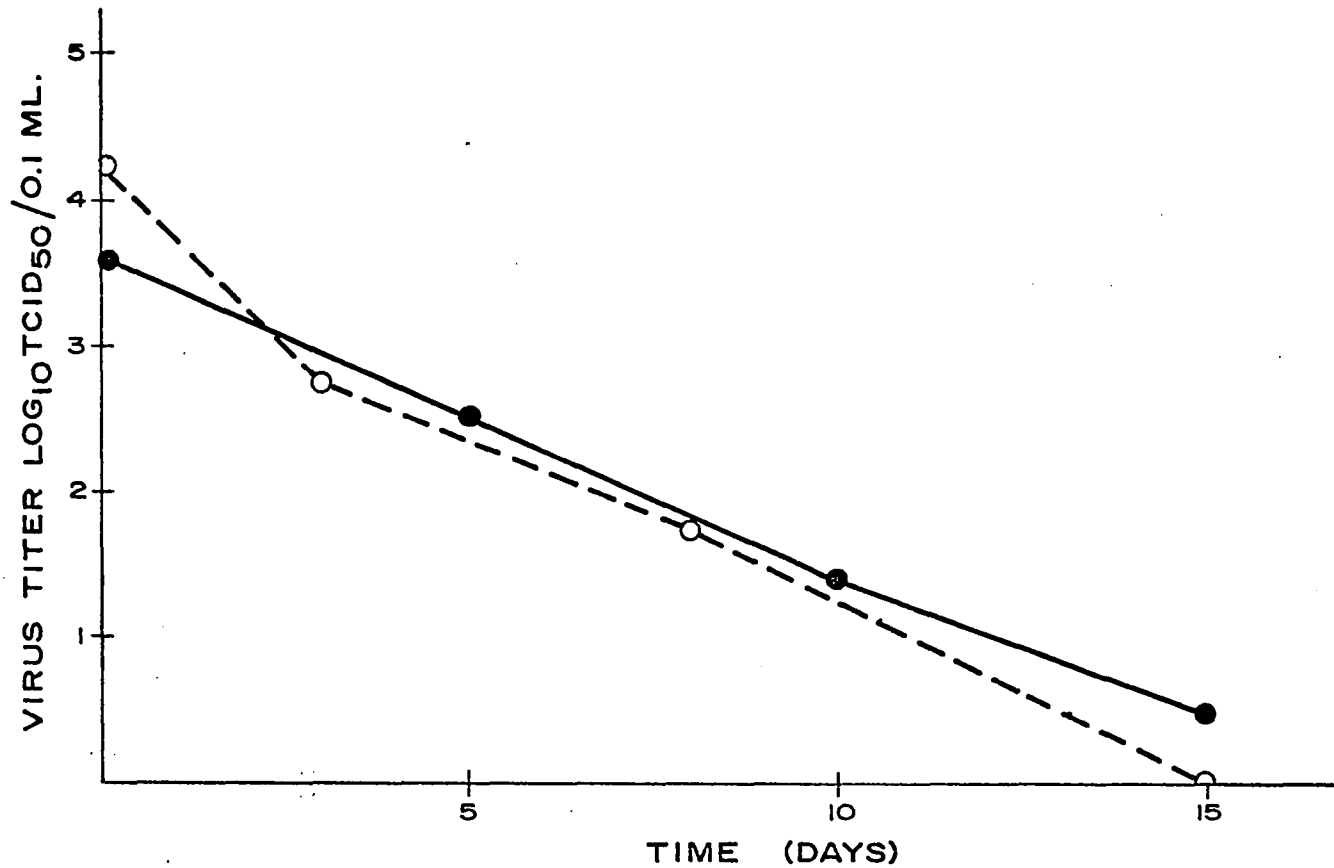
Figure 7. Comparison of adenovirus 5 inactivation in estuarine water collected from different locations and depths within the estuary. Symbols: ● estuarine water Adams Point; ○ estuarine water Nanny Island.
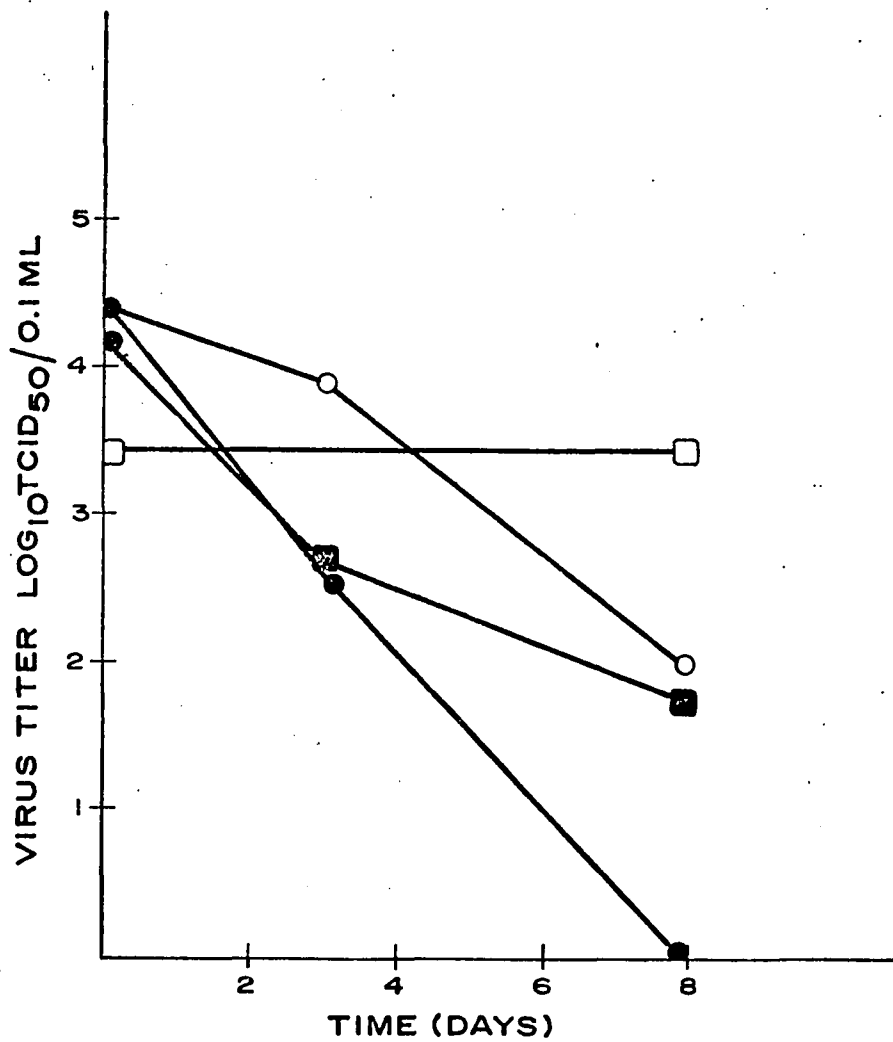
Figure 8.   Inactivation of adenovirus 5 and poliovirus
II in estuarine water. Symbols: O adenovirus
5 estuarine water; ☐ adenovirus autoclaved
estuarine water control; ● poliovirus
estuarine water; ▨ poliovirus autoclaved
estuarine water control.

Survival of adenovirus 5 in the estuary during the winter

The inactivation of adenovirus type 5 was studied during March of 1971 to determine the influence of winter conditions in the estuary on virus inactivation. During this study the reduction of infectivity of the virus was followed also in the laboratory in seamless cellulose dialysis bags immersed in 8000 ml of estuarine and autoclaved estuarine water. The temperature in the estuary during the winter study increased from 0.5 C to 2.5 C while the salinity decreased from 20 ppt to 14.5 ppt. The temperature in the laboratory was maintained at 6.2 C. The results indicated again (Fig. 9) that virus was inactivated at a more rapid rate in the estuary than under laboratory conditions. However, the duration of adeno-virus survival in both systems was extended and its observed rate of inactivation reduced greatly when compared to the summer study (Fig. 4). The concentration of adenovirus in the auto-claved system did not change over the course of the study.

A comparison of the infectivity data with the salinity ranges recorded during the winter, spring and summer studies suggests that salinity alone did not significantly influence adenovirus survival in the estuarine environment. However, temperature was considered to have a significant effect on virus survival in both the estuary and under laboratory con-ditions.

Effect of temperature on adenovirus inactivation in estuarine water

When virus inactivation was studied in estuarine water (collected in October, 1970) at 4 and 20 C, a more rapid
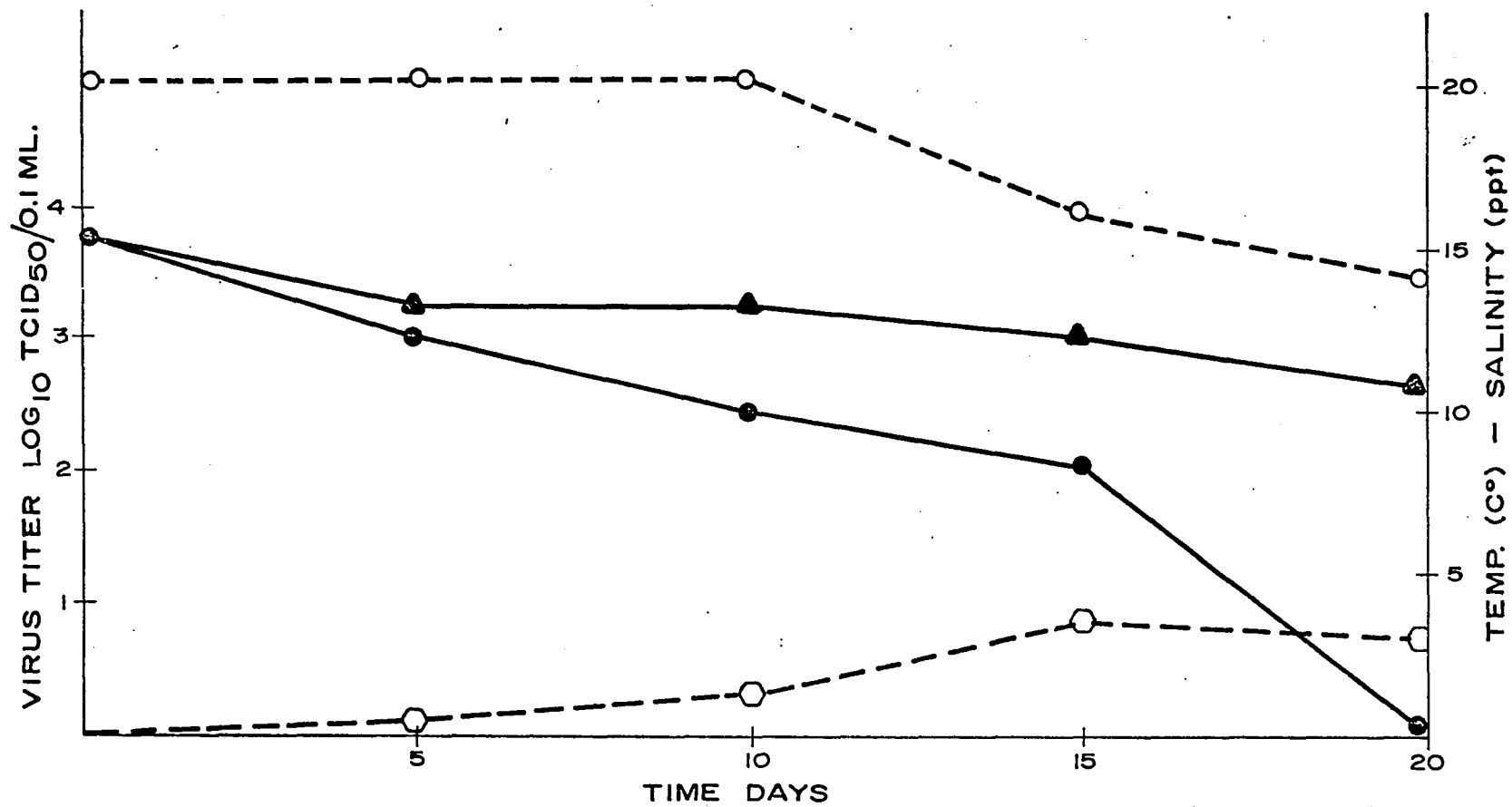
Figure 9. Comparison of adenovirus inactivation in the estuarine (March 1971) and in the laboratory. Symbols: ●, adenovirus 5 estuary; ▲, adenovirus 5 laboratory; O , salinity; ⬡ , temperature.

decrease in viral infectivity was noted at 20 C than at 4 C
(Fig. 10). The complete loss of detectable virus was recorded
at 20 C on day 31 while it remained present in the estuarine
water maintained at 4 C. This study reaffirmed the effect of
temperature observed in the winter study (Fig. 9) in influenc-
ing the inactivation rate of adenovirus in estuarine water.

The relationship of estuarine bacteria to adenovirus inactiva-
tion in the estuary

A survival study was conducted in the estuary (August,
1971) to determine if a detectable correlation exists between
estuarine bacteria and the loss in infectivity of the adeno-
virus. During the study the bacterial population was monitored
in the estuary and within the dialysis bag containing estuarine
water and virus. As can be seen from Fig. 11, the concentration
of bacteria in the estuary remained relatively constant over
the experimental period. However, the bacteria in the estuarine
water within the dialysis bag increased by more than 1½ orders
of magnitude. This increase in estuarine bacteria was corre-
lated with a decrease in the infectious titer of the virus.
The results from this study were interpreted to indicate that
virus inactivation may be related to estuarine microbial
activity and that temperature may be important to the extent
that it increases the physiological activity of the estuarine
microbes.

Relationship of estuarine bacteria to adenovirus inactivation
under laboratory conditions

When the estuarine bacterial population was monitored
along with adenovirus inactivation under laboratory conditions,
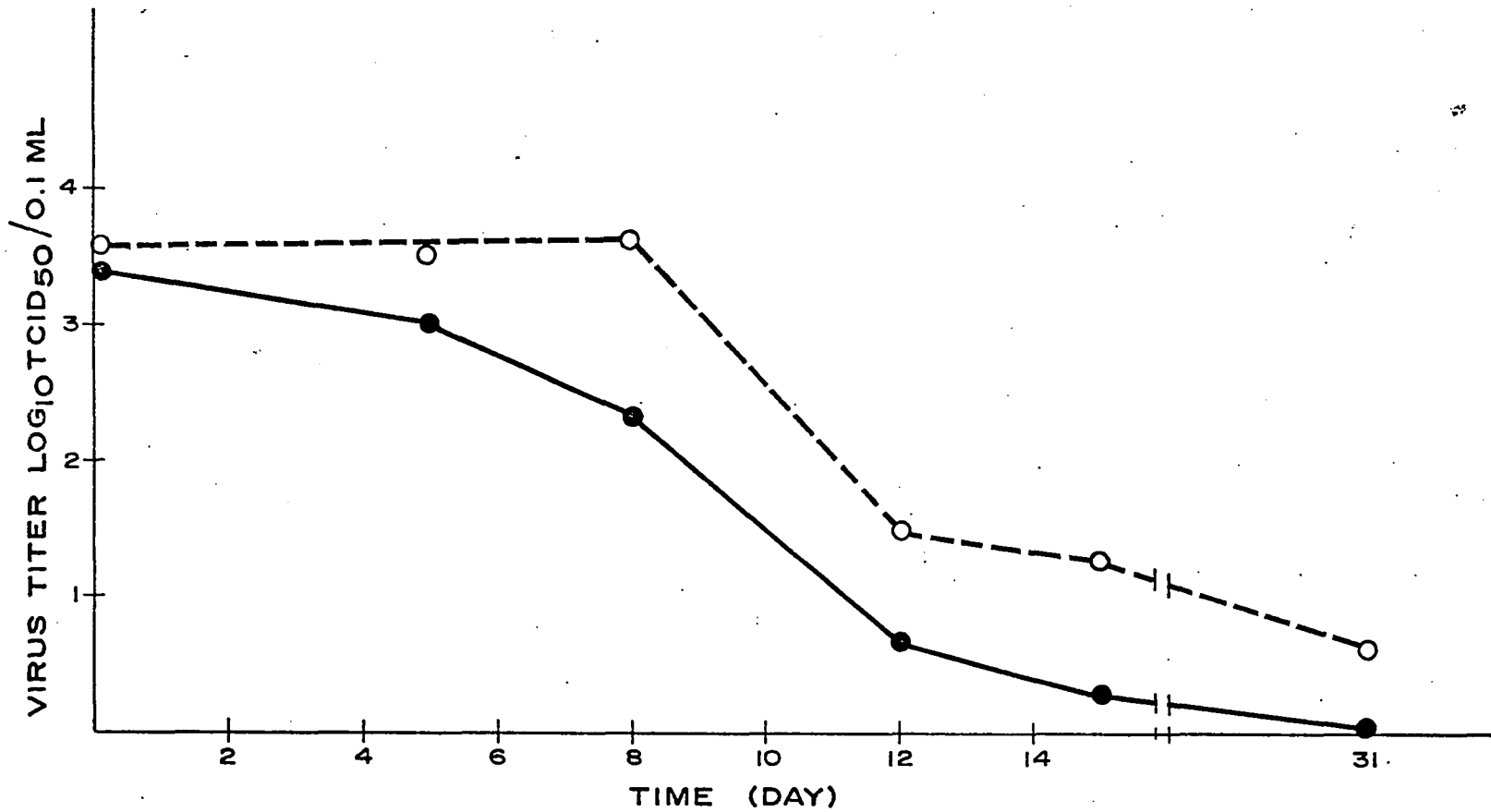
Figure 10. Effect of temperature on adenovirus 5 inactivation under laboratory conditions. Symbols: O , 4C; ● , 20C (salinity of estuarne water, 30 ppt).
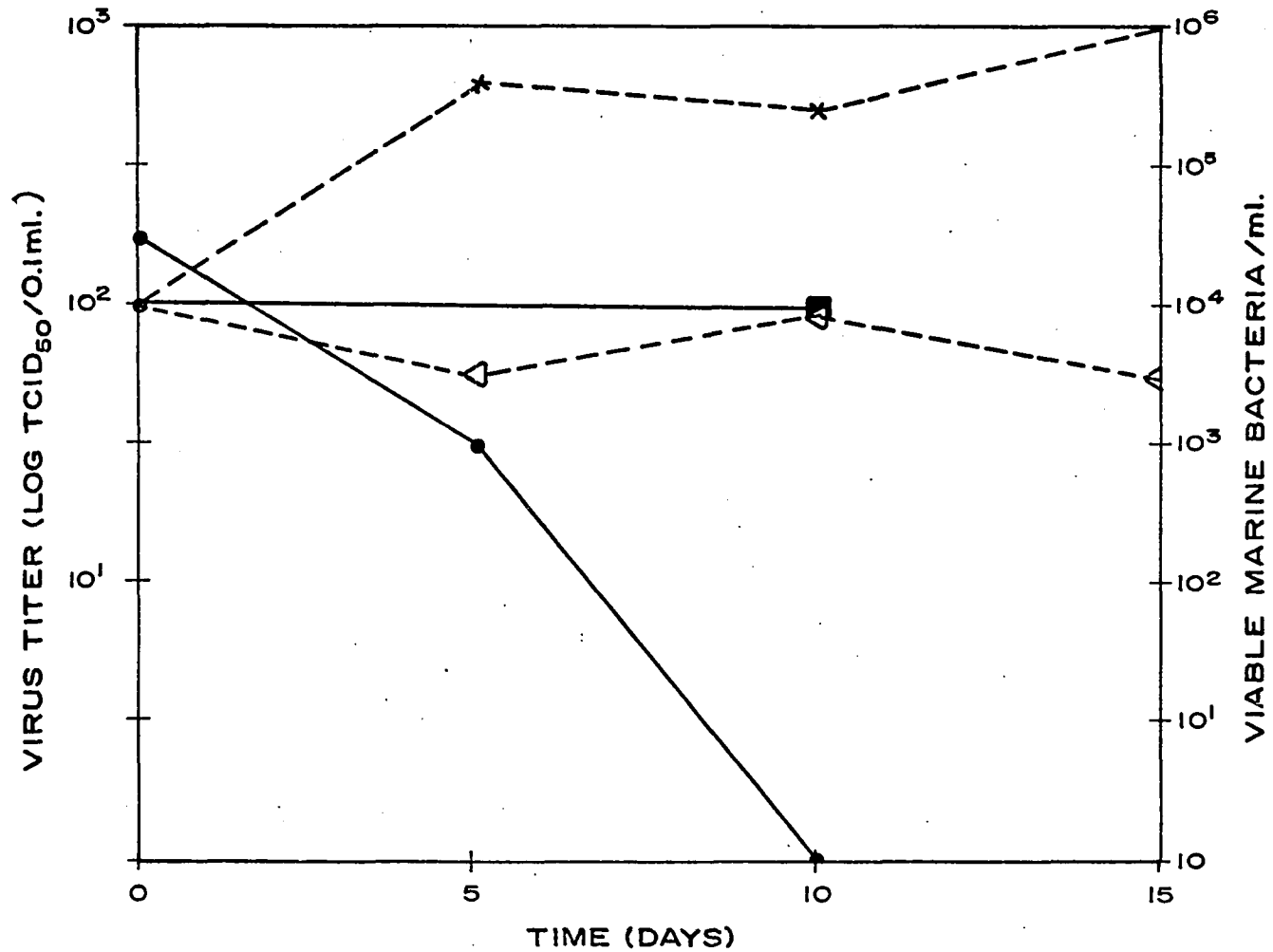
Figure 11. Relationship of estuarine bacteria to adenovirus 5 inactivation in the estuary (August 1971). Symbols: ● , adenovirus estuary (salinity 31 ppt); ▧ , adenovirus autoclaved estuarine water control; ◁ , estuarine bacteria in estuary; x, estuarine bacteria in dialysis bag.

the data (Fig. 12) were similar to those observed in the estuarine study. The increase in estuarine bacteria was correlated again with the loss in titer of adenovirus 5, suggesting that the mechanism by which viral inactivation was occurring may be the same in both the field and the laboratory.

Assay of radioactive virus in estuarine water

Several months were spent attempting to develop adequate radioisotope labeling and purification techniques in order to follow the molecular fate of the adenovirus particle in estuarine water. At the end of this period several critical problems both with the virus and cell line remained unresolved. A decision was therefore made to substitute a bacteriophage in place of the adenovirus. Biochemically both adenovirus 5 and B. subtilis phage 41C are composed essentially of protein and nucleic acid. The phage, 41C, was specifically chosen because the labeling and purification techniques were available for this virus.

The radioisotope assay procedure which was employed to determine the mechanism by which the virus was being inactivated in estuarine water appears in brief outline in Fig. 13. By following the outlined procedure and comparing the radiation levels in the various fractions (pellet, supernatant) as a function of time or with appropriate controls, it should be possible to determine if the loss in viral infectivity is accompanied by a disruption of the protein coat of the virus and the release of the viral DNA into the medium. It should also be possible to determine the molecular fate of any released viral DNA.
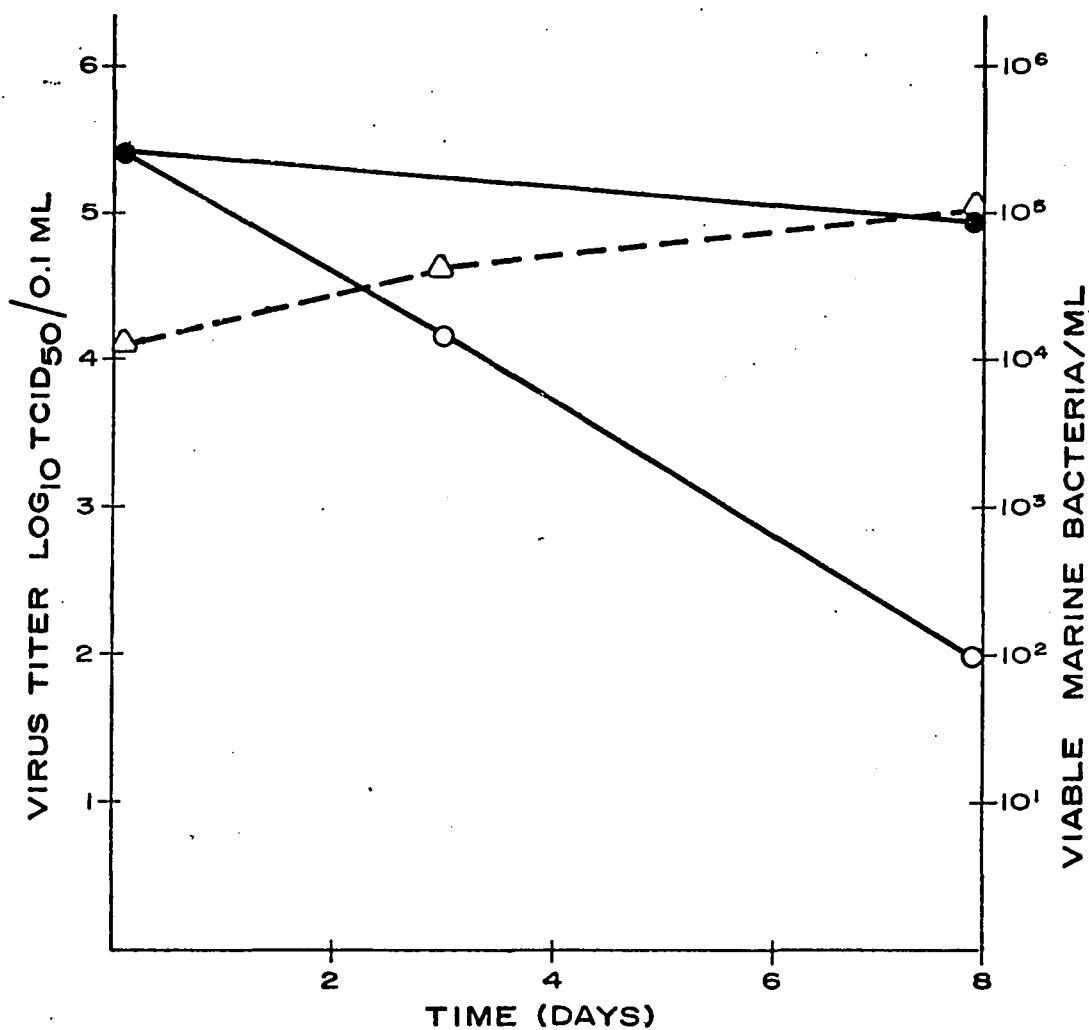
Figure 12. Relationship of estuarine bacteria to
adenovirus 5 inactivation under laboratory
conditions. Symbols: O , adenovirus 5
estuarine water (salinity 23 ppt); ● ,
adenovirus autoclaved estuarine water control;
△, estuarine bacteria.
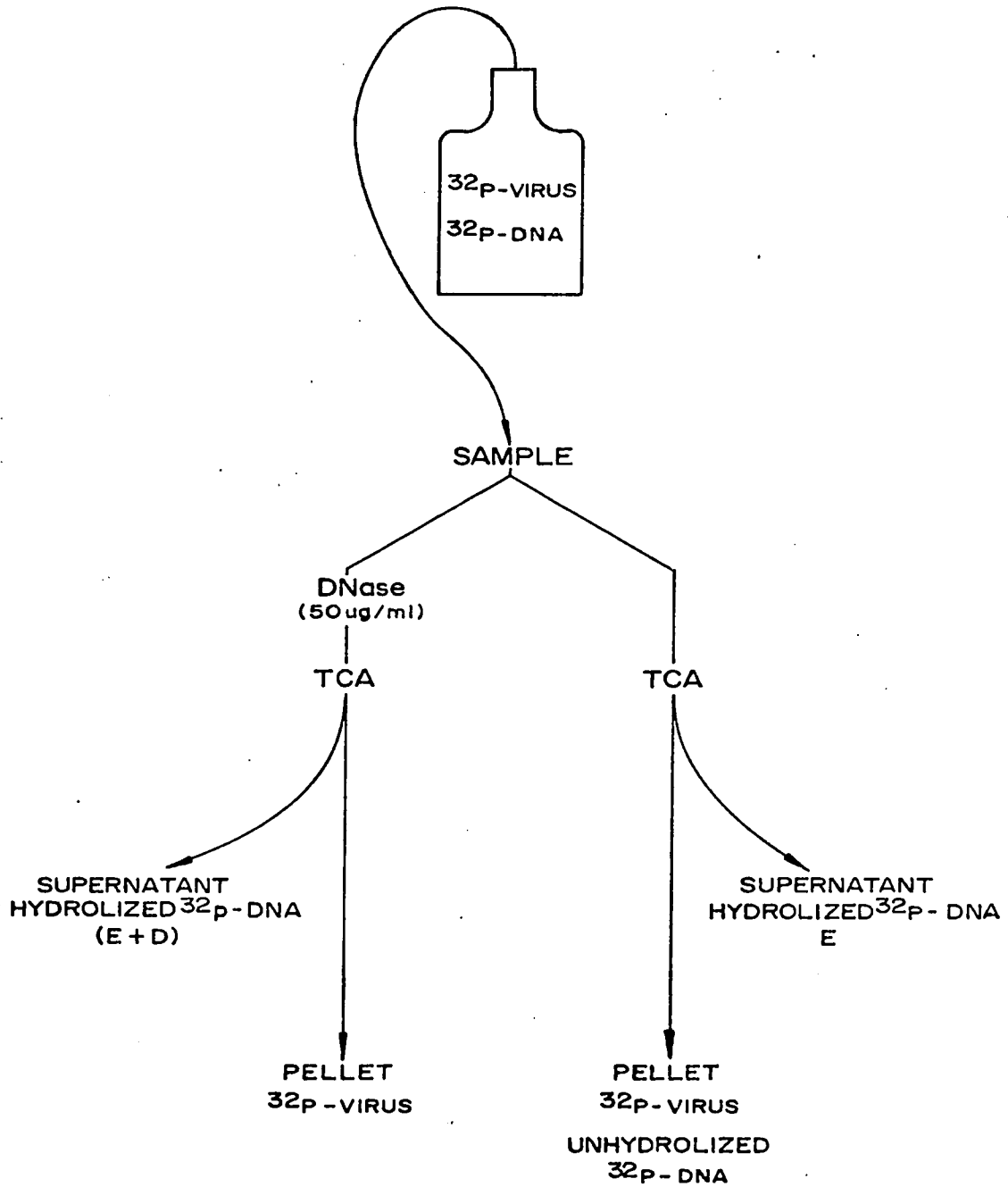
Figure 13. Assay for the released ^{32}P virus DNA.
Symbols: E, microbial enzymes; D,
deoxyribonuclease.

Activity of deoxyribonuclease on DNA in estuarine water

One of the first considerations in adapting radio-chemical labeling procedures to the environmental system was to determine whether deoxyribonuclease (DNase) could function in the estuarine environment. The results obtained from adding DNase (40 ug/ml) to two different estuarine water samples containing 135 ug/ml of B. subtilis DNA demonstrated that the enzyme appeared to function normally in the estuarine water (Fig. 14).

Effect of deoxyribonuclease on ^{32}P labeled DNA in various estuarine water systems

After establishing the enzymatic activity of deoxyribonuclease in estuarine water, it was necessary to determine the extent of DNA hydrolysis by the enzyme. Using ^{32}P labeled B. subtilis bacteriophage SP 82G DNA, the activity of the DNase was tested in normal, filtered and autoclaved estuarine water. The results from Table 1 indicated that the enzyme was able to hydrolyze 77% of the added DNA to non trichloroacetic acid (TCA) precipitable units. Even though the enzyme did not render the total DNA TCA soluble, it did hydrolyze the nucleic acid to the same degree ($\pm$ 1%) in all of the estuarine systems considered. Because the activity of the enzyme was constant in the three systems, its level of activity was not considered to interfere with the function of the radiation system or the interpretation of the results.

Specific labeling of phage 41C

The results of three separate labeling experiments involving 41C appear in Table 2. Ninety-two to 97% of the
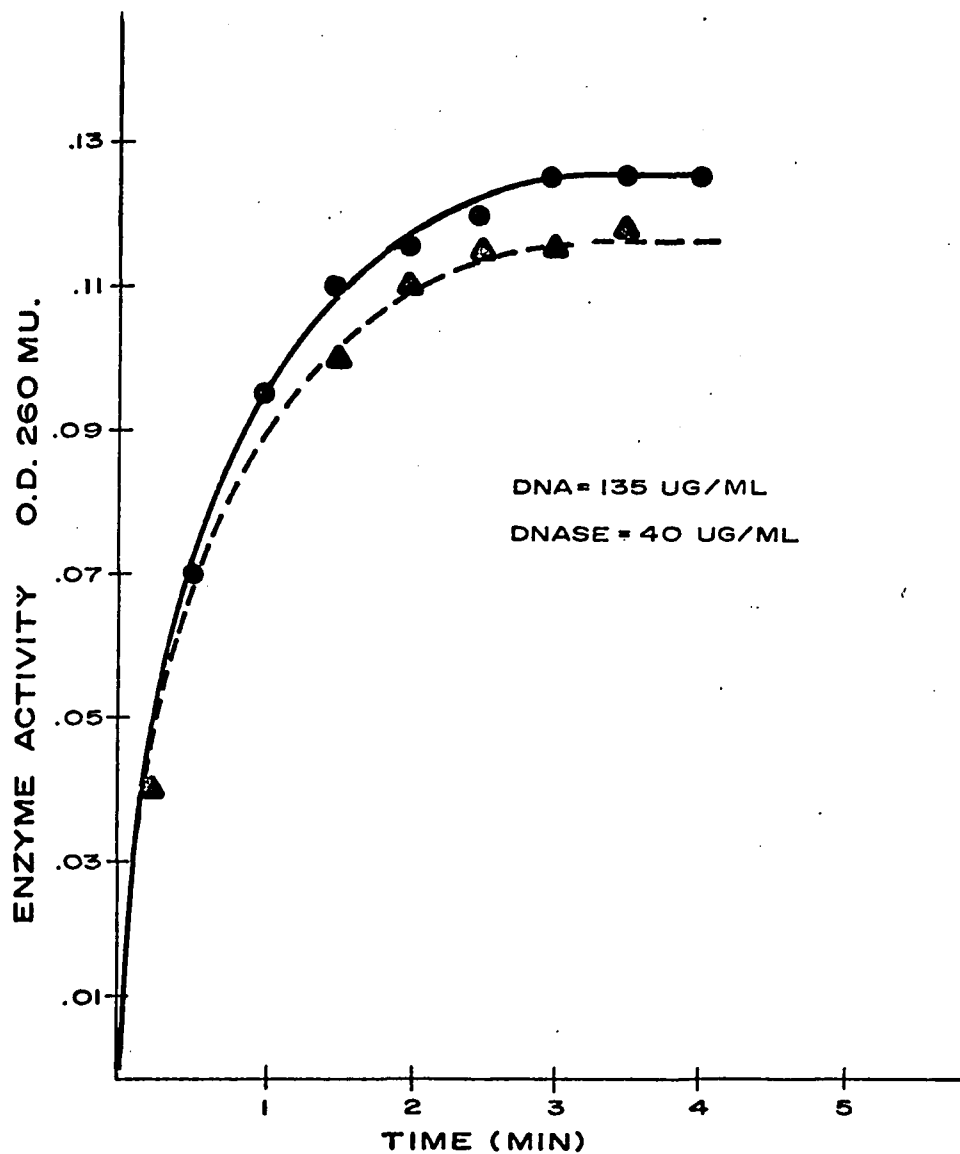
Figure 14. The functional ability of deoxyribonuclease in
estuarine water. Symbols: ● , estuarine water
Adams Point; ▲ , estuarine water Jackson
Estuarine Laboratory (salinity 24.5 ppt).

Table 1. Effect of DNase on ^{32}P labeled DNA in normal, filtered and autoclaved estuarine water

Sample	pH	Corrected CPM DNase-TCA Input	Treated Pellet	Super- natant	% Counts Released	Corrected CPM TCA Treated Input	Pellet	Super- natant	% Counts Released
EW[a] [b]	7.70	10,040	2,300	7290	76	10,222	8,083	155	1.9
FEW	7.15	9,938	2,130	7255	77	10,246	8,806	180	2
AEW	8.21	9,740	2,115	7355	78	10,116	8,405	150	1.7

[a]EW, estuarine water; FEW, filtered estuarine water; AEW, autoclaved estuarine water; TCA, trichloroacetic acid; CPM, counts per minute.

[b]Salinity of estuarine water 26.5 ppt.

Table 2. ^{32}P labeling of _Bacillus_ _subtilis_ phage 41C

Experiment	Phage titer PFU/ml	Counts/min		Super- natant	% non TCA precipitable counts	% TCA precipitable counts
		Input	Pellet			
1	5.2×10^{10}	3,302,300	3,181,250	83,250	2.6	97.4
2	9.0×10^{9}	207,700	194,650	8,000	3.9	96.1
3	2.5×10^{10}	289,300	249,875	27,370	8.2 (9.8)[a]	91.8

[a]DNase treated.

label was associated with the purified phage preparation fol-
lowing precipitation with TCA, indicating that the tracer was
in the form of high molecular weight compounds. When DNase
was added to an aliquot of the ^{32}P labeled phage, an insigni-
ficant increase in activity was noted in the supernatant
following TCA treatment. The amount of label incorporated
into the virus was sufficient for determing its fate in the
estuarine water system. However, the level of radiation intro-
duced was still low enough so as not to result in biological
suicide.

The effect of estuarine water on the molecular stability of 41C

The results of inoculating ^{32}P labeled phage into
estuarine water collected in July, 1971, are represented in
Fig. 15. As a function of time, there was a loss of infecti-
vity in the viral population exposed to the estuarine water.
This inactivation was correlated with the appearance of ^{32}P
labeled DNA in the TCA soluble fraction. As the percentage
of inactivation increased, there was a corresponding increase
in the percentage of labeled DNA released into the supernatant.
The radiation data further indicated that as fast as the viral
DNA became accessible to enzymatic attack, it was hydrolyzed
by microbial nucleases into non TCA precipitable units. This
observation was evidenced by the same level of radiation in
fractions of both DNase treated and untreated samples.

Several observations require consideration in inter-
preting the radiation data (Table 3) obtained from the normal
filtered and autoclaved estuarine water, during the study. At
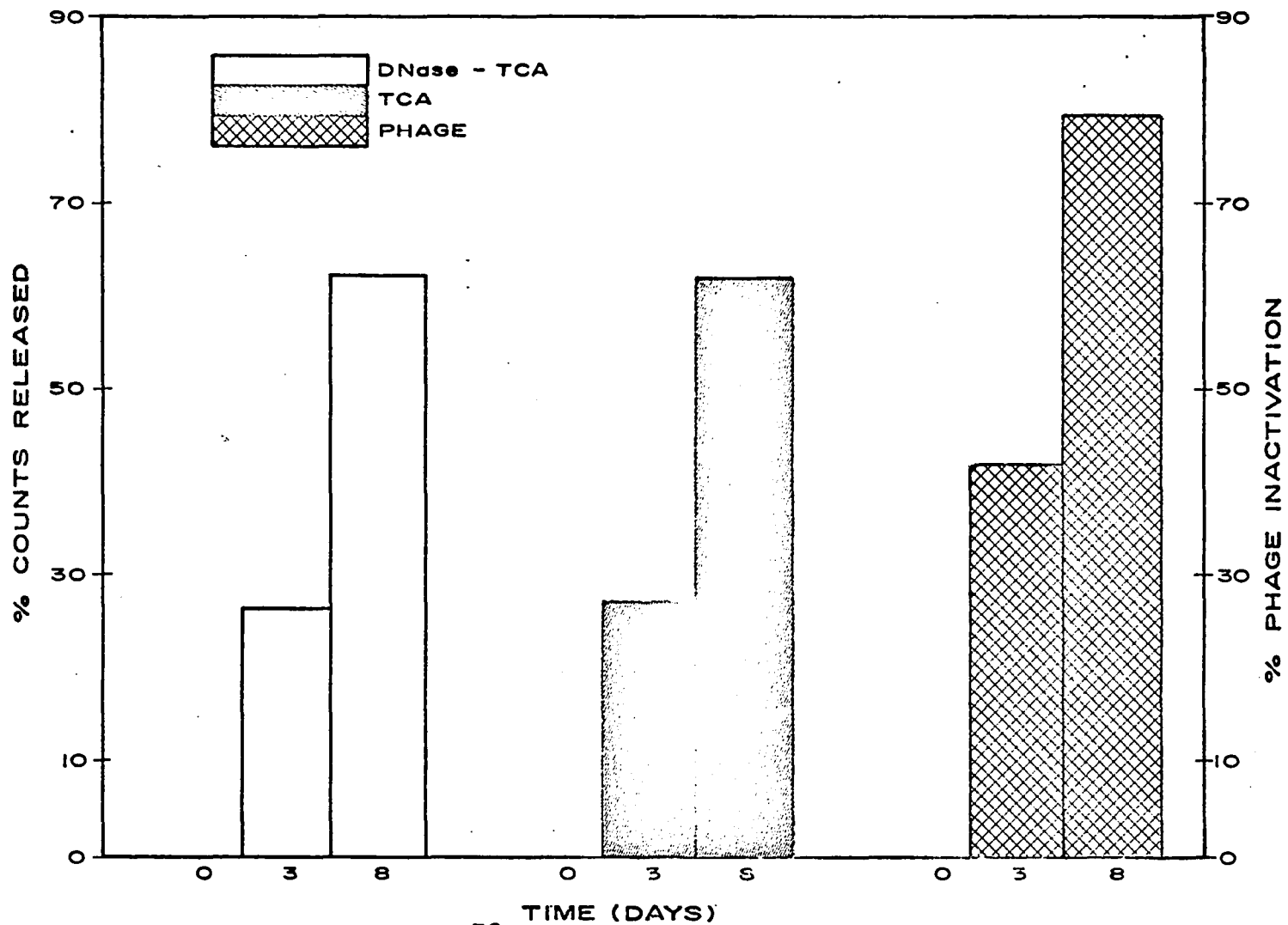
Figure 15. Release of ^{32}P labeled DNA during inactivation of Bacillus subtilis phage 41C in estuarine water (July).

Table 3. Effect of normal, filtered and autoclaved estuarine water on the molecular stability of ^{32}P labeled <u>Bacillus</u> <u>subtilis</u> phage 41C (July)

Sample Day	Corrected CPM DNase-TCA Treated Input	Pellet	Super-natant	% Counts Released	Corrected CPM TCA Treated Input	Pellet	Super-natant	% Counts Released	Phage titer (PFU)	% Phage Inactivation
E.W.[a][b]										
0	6,908	3,608	2,490	0	7,322	5,935	975	0	2.20×10^7	0
3	6,306	1,839	4,077	28	6,343	1,793	4,297	30	1.27×10^7	43
8	7,001	850	5,852	48	7,234	913	6,031	47	4.63×10^6	79
F.E.W.										
0	7,214	3,775	2,765	0	7,574	6,160	945	0	3.24×10^7	0
3	7,444	3,336	3,482	9	7,301	4,994	1,862	13.7	2.51×10^7	22
8	7,359	2,944	3,633	13	7,420	4,211	2,451	23.5	1.32×10^7	60
A.E.W.										
0	7,352	4,198	2,560	0	7,436	5,928	945	0	2.25×10^7	0
3	6,736	3,366	2,822	8	6,503	5,162	908	1.2	3.17×10^7	0
8	6,554	3,535	2,550	5	6,933	5,464	770	1.3	2.75×10^7	0

[a]Salinity of estuarine water 31 ppt.

[b]See Table 1, footnote a.

the outset of the experiment approximately 25% of the initially
introduced radiation was solubilized by the added DNase. This
indicated that a microbial contaminant was present in the puri-
fied phage stock and that prior to introducing the phage into
the experimental systems it had inactivated a portion of the
viral population. By comparing the radiation levels in the
DNase treated and untreated samples in all 3 systems on day 0,
it will be noted that the contaminating organism was not able
to hydrolyze effectively the accessible DNA to TCA soluble
units.

Also of significance was the activity of the added
DNase. By comparing the number of ^{32}P counts solubilized
following treatment with DNase on day 0, it will be observed
that the level of activity of the enzyme was the same in the
3 estuarine water systems.

The radiation data from the estuarine water system
demonstrated as a function of time, a shift of ^{32}P from the
insoluble (pellet) to the soluble (supernatant) state. This
radiation shift indicated a destruction of the phage genome
and was correlated with a loss in infectivity of the viral
population. In attempting to correlate the radiation shifts
observed in the estuarine water with that reported as percent
counts released, it should be noted that a discrepancy exists.
This inconsistency results from a correction of 2,400 counts
(released following DNase treatment on day 0) made in comput-
ing the percent counts released in estuarine water samples.
This correction was necessary to demonstrate that the total
counts released as a result of viral inactivation in the

estuarine water was the same in both the DNase treated and un-
treated sample.

The radiation counts in the DNase treated supernatant
increased from 2,490 to 4,077 on day 3 while in the untreated
supernatant the increase was from 975 to 4,297. This differ-
ence was a result of the hydrolysis by the estuarine organisms
of the DNA added initially with the phage in addition to that
released during viral inactivation. When all factors have
been considered the results from the radiation data are
identical with those illustrated in Fig. 15 and the interpre-
tation remains the same.

The radiation and infectivity data from the filtered
estuarine water system showed significant viral inactivation.
The recorded changes however were not as great as those ob-
served in the estuarine water. Since the organisms responsible
for the decrease in viral titer were not able to effectively
solubilize all of the available ^{32}P labeled DNA, a correction
could not be made in computing the percent counts released.
The organisms responsible for the viral inactivation in the
filtered estuarine water were not considered to be the same as
the organism responsible for the inactivation observed in the
phage stock.

One of the principal reasons for considering that this
experiment represents a valid picture of what was occurring
to the virus during exposure to estuarine water was that in
spite of the microbial contaminant introduced with the labeled
phage, the autoclaved control system showed no evidence of viral
disruption or inactivation. As a result of this observation

it was concluded that the release of ^{32}P labeled phage DNA and
the corresponding decrease in the infectivity of the phage
population were a result of the enzymatic activity of the
estuarine microorganisms. An assessment of what was occurring
in the filtered estuarine water to account for the inactivation
will be momentarily deferred.

The radiation study was repeated in estuarine water
collected during September, 1971. Prior to introducing the
labeled phage into the experimental systems it was resteri-
lized by passage through a 0.45 micron filter. The results
from the estuarine water system (Fig. 16) are similar to those
observed in the previous study and thus confirm (using a sterile
phage inoculum) the ability of estuarine microorganisms to
inactivate the phage by disrupting its coat protein and hydro-
lizing the viral genome.

A shift in radiation counts from the pellet to the
supernatant (Table 4) was again observed in the estuarine
water to coincide with a loss of infectivity in the phage
population. As the percentage of ^{32}P counts released into
the supernatant increased there was a corresponding inactiva-
tion of the virus. The phage in the filtered estuarine water
displayed a small but again significant drop in titer. This
result was attributed to the presence of a bacterial contaminant.

A progressive inactivation of the phage was also ob-
served in the autoclaved estuarine water system. This loss
of viral infectivity was accompanied by a corresponding loss
of radiation from the autoclaved water. The radiation data
suggested that the phage had been removed from suspension and

Figure 16. Release of ^{32}P labeled DNA during inactivation of Bacillus subtilis phage 41C in estuarine water (September).

Table 4. Effect of normal, filtered and autoclaved estuarine water on the molecular stability of ^{32}P labeled _Bacillus_ _subtilis_ phage 41C (September)

Sample Day	Corrected CPM DNase-TCA Treated Input	Treated Pellet	Super-natant	% Counts Released	Corrected CPM TCA Treated Input	Treated Pellet	Super-natant	% Counts Released	Phage titer (PFU)	% Phage Inactivation
E.W.[a][b]										
0	404	290	60	0	404	298	40	0	1.0×10^7	0
3	397	210	147	24	395	199	152	26	8.4×10^6	16
8	382	58	278	66	389	56	289	67	6.5×10^5	93.5
F.E.W.										
0	398	263	70	0	400	305	45	0	8.9×10^6	0
3	388	302	73	0	393	271	52	3.5	9.5×10^6	0
8	386	212	140	18.6	384	265	91	12.7	6.6×10^6	26
A.E.W.										
0	382	285	45	0	376	290	35	0	1.4×10^7	0
3	267	187	39	3.4	262	207	28	1	8.8×10^6	37.2
8	153	91	49	21.1	163	103	36	15.4	2.5×10^6	82.2

[a]Salinity of estuarine water 31 ppt.

[b]See Table 1, footnote a.

was attached to the sides of the glass container. The recorded viral inactivation was therefore interpreted to represent an artifact of the autoclaved system and was not considered to reflect any direct virus inactivation by the autoclaved estuarine water. Since the level of radiation in the supernatants did not change over the course of the study, it would appear that the protein coat of the phage was in part responsible for the observed adsorption.

At this point it should be emphasized that the estuarine water system was functioning optimally and that the results obtained with this system were in no way affected by the factors operating in the filtered or autoclaved estuarine water systems.

The radiation study was again repeated using estuarine water collected in November, 1971. The results from the inactivation of 41C (Fig. 17) in the estuarine water were again consistent with those of the July and September studies. The radiation data (Table 5) showed similar patterns to that previously described. The adsorption phenomenon of the phage in the autoclaved system was similar also to that observed during the September study.

There was no reduction in the titer of 41C recorded in the filtered estuarine water even though what appeared to be an estuarine bacterium was cultured from the water. The contaminating organism was a Gram negative vibrio like bacterium. Growth of this organism occurred on 2216 marine agar but not on blood or nutrient agar. The organism appeared to grow better at 20 C than at 37 C. The estuarine bacterium
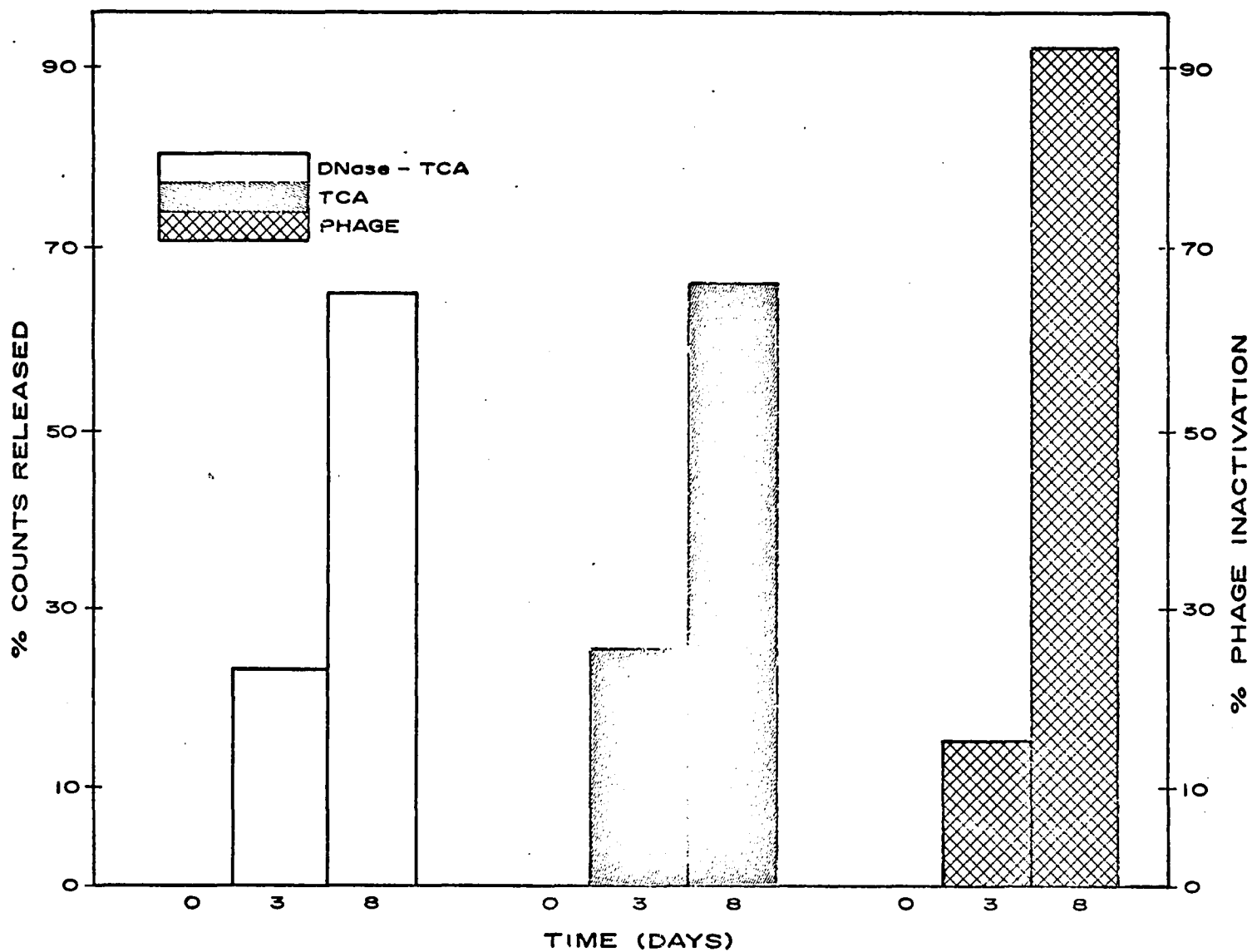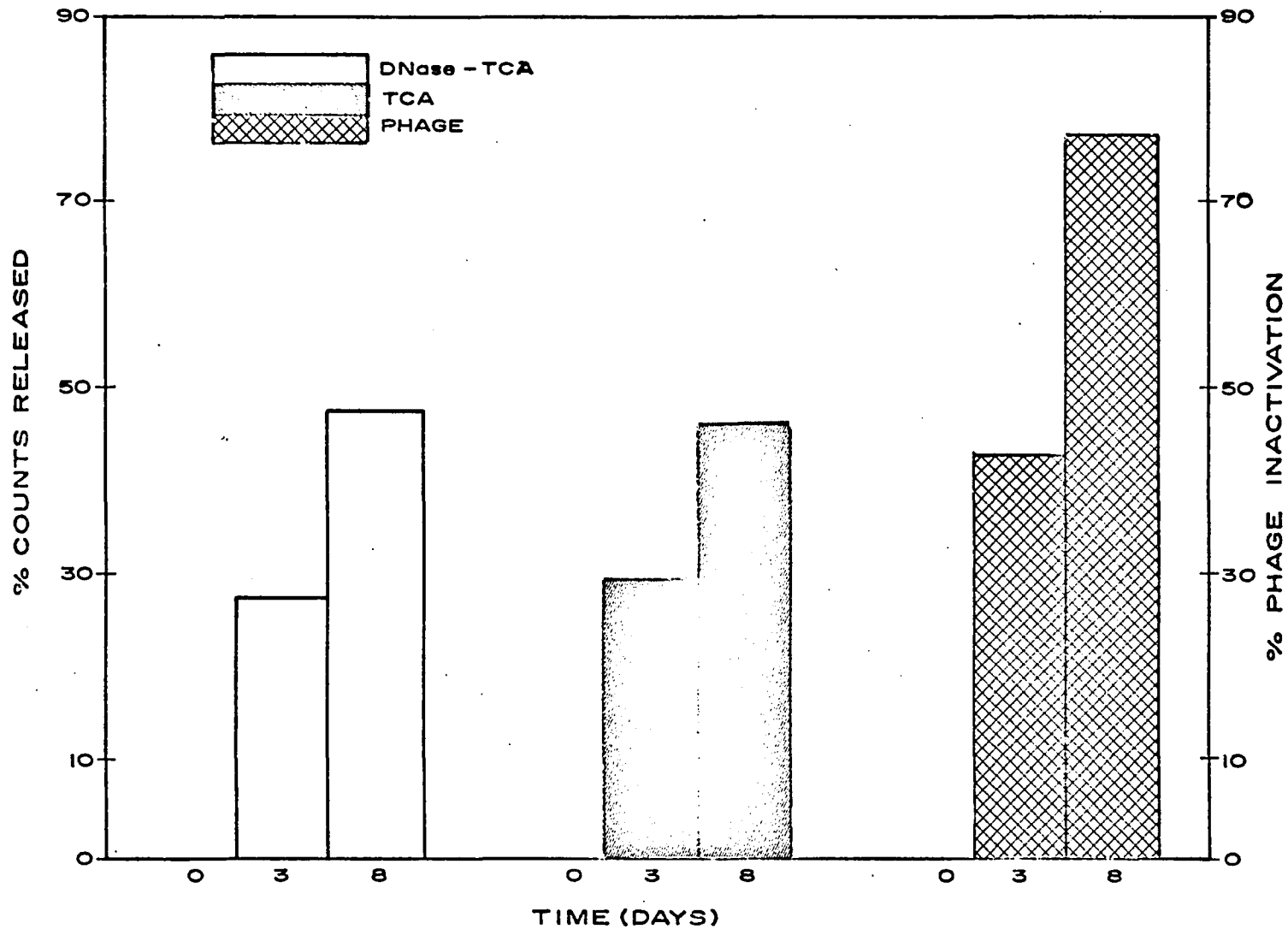
Figure 17. Release of ^{32}P labeled DNA during inactivation of Bacillus subtilis phage 41C in estuarine water (November).

Table 5. Effect of normal, filtered and autoclaved estuarine water on the molecular
stability of ^{32}P labeled <u>Bacillus</u> <u>subtilis</u> phage 41C (November)

Sample Day	Corrected CPM DNase-TCA Treated Input	Pellet	Super-natant	% Counts Released	Corrected CPM TCA Treated Input	Pellet	Super-natant	% Counts Released	Phage titer (PFU/ml)	% Phage Inactivation
E.W.[a] [b]										
0	2,032	1,758	166	0	2,002	1,796	120	0	1.2×10^8	0
3	2,050	1,170	700	28.8	1,950	1,160	670	28.0	6.9×10^7	42.5
8	1,920	480	1,210	63.0	1,880	490	1,210	62.6	2.5×10^7	79.2
F.E.W.										
0	2,018	1.760	156	0	2,026	1,808	129	0	1.3×10^8	0
3	2,100	1,770	250	3.9	2,030	1,660	240	6.0	1.0×10^8	0
8	2,020	1,590	300	7.9	2,170	1,740	370	10.9	1.4×10^8	0
A.E.W.										
0	1,992	1,710	143	0	2,000	1,735	124	0	1.3×10^8	0
3	1,440	1,140	170	5.3	1,390	1,440	120	2.4	6.4×10^7	50.8
8	850	590	130	10.8	890	760	110	6.3	4.3×10^7	67.0

[a]Salinity of estuarine water 29 ppt.

[b]See Table 1, footnote a.

following propagation under laboratory conditions was not
observed to pass through the filter initially used to sterilize
the estuarine water. Anderson and Heffernan (1965) have re-
ported that some marine bacteria which were initially able to
pass through a 0.45 micron filter lost this filtrability
following cultivation under laboratory conditions. Two stains
of sprilla were reported also to pass through a 0.3 micron
filter (Anderson and Heffernan, 1965).

Several studies were conducted to gain additional in-
formation concerning the adsorption phenomenon observed in the
autoclaved estuarine water. When ^{32}P labeled phage was added
to glass and plastic containers with normal and autoclaved
estuarine water and the radiation level monitored, the results
(Table 6) were consistent with the removal of phage from sus-
pension in the autoclaved system (ASG). The loss of radiation
from the estuarine water autoclaved in the glass bottle was
not reflected in the other systems considered. This would
appear to indicate that the preparation of the autoclaved
estuarine water as well as the container used for its storage
may influence the adsorption phenomenon. In adsorption studies
using unlabeled phage, a decrease in viral infectivity was
considered to indicate viral adsorption. The results from
studies using unlabeled virus in modified systems showed sig-
nificant variation. Therefore a definitive statement cannot
be made on the influence of the preparation of the autoclaved
estuarine water or its storage container on viral adsorption.

There were two further observations concerning viral
adsorption which are worth noting. The first concerns the

Table 6. Adsorption of ^{32}P labeled phage 41C to its activated glass container

Sample Day	CPM of ^{32}P labeled phage removed by different containers				
	SG[a]	ASG[a] [b]	ASTG[a]	SP[a]	ASP[a]
0	399	413	396	391	400
8	390	175	388	358	398

[a]SG, seawater glass container; ASG, autoclaved seawater glass container; ASTG, autoclaved seawater transferred to glass container; SP, seawater plastic container; AP autoclaved seawater plastic container.

[b]% loss of radioactivity in the ASG container, 58%.

results of a study undertaken to verify further the participation of the phage protein coat in viral adsorption. By introducing high molecular weight proteins in the form of 1 percent fetal calf serum into the autoclaved estuarine water immediately prior to adding the phage, it was considered that the added protein might be able to competitively block the adsorption of the phage to the sides of the glass container. The results from the study (Table 7) indicated that the added serum was effective in blocking the adsorption of 41C in the autoclaved system. This observation gave further evidence that viral adsorption was indeed occurring in the autoclaved system and that the coat protein of the phage was an essential element in this phenomenon.

Table 7. The effect of serum on the adsorption of 41C in autoclaved estuarine water at 18 C

Time (Day)	Virus titer (PFU/ml)		
	AEW[a]	AEWS[b]	FEW[c]
0	2.81×10^8	2.79×10^8	2.94×10^8
8	7.95×10^6	2.29×10^8	1.60×10^8

[a]AEW, Autoclaved Estuarine Water
[b]AEWS, Autoclaved Estuarine Water with Serum
[c]FEW, Filtered Estuarine Water

The second observation concerns the treatment of the surfaces of a glass bottle which had contained phage in autoclaved estuarine water with versene and the recovery of more phage per ml in the versene (4.0×10^5 PFU/ml) than in the removed estuarine water (1.4×10^4 PFU/ml). This preliminary

result would suggest that ionic species may play a role in viral adsorption.

In summary, the results from the radiation studies with 41C demonstrate that viral inactivation or the loss of infectivity of the virus in estuarine water was accompanied by a disruption of the protein coat of the phage and the hydrolysis of the phage genome by microbial nucleases to non TCA precipitable units. Furthermore the observed potential for virus inactivation was present in the estuarine water during July, September and November, 1971 and therefore was not considered to be of sporadic occurrence in the estuary.

The results from the filtered estuarine water appear to indicate that in the absence of microbial contamination filtered estuarine water did not significantly influence the inactivation of the virus.

The results obtained with the autoclaved systems were considered highly significant in that they allowed a differentiation between what normally would have been reported as chemical inactivation and what was considered to represent an artifact of the system.

<u>Structural alteration of 41C in estuarine water</u>

Because the radiation data suggested that the inactivation of 41C was accompanied by a disruption of its coat protein, it was of interest to determine if any structural alteration of the phage particle could be noted following exposure of the virus to estuarine water. The results recorded in Tables 8 and 9 reflect the decrease in infectivity of the phage that were exposed to estuarine water. Auto-Pow MEM which

Table 8. Infectivity data from initial electron microscopy study on structural alterations of <u>Bacillus</u> <u>subtilis</u> phage 41C in estuarine water

Sample	Time		% Inactivation
	Day 0	Day 3	
TYS Broth[a]	7.8×10^{10}	7.3×10^{10}	6.5
AEW	8.4×10^{10}	8.0×10^{10}	4.8
EW[b]	8.7×10^{10}	1.1×10^{10}	87.4

[a]TYS Broth, tryptone-Yeast Extract-Salt Broth; AEW, autoclaved estuarine water; EW, estuarine water.

[b]Salinity of estuarine water 26 ppt.

Table 9. Infectivity data from modified electron microscopy study on structural alterations of <u>Bacillus</u> <u>subtilis</u> phage 41C in estuarine water

Sample	Time			% Inactivation (Day 4)
	Day 0	Day 3	Day 4	
AEW[a]	3.3×10^{10}	4.1×10^{10}	3.1×10^{10}	6.1
EW[b]	6.6×10^{10}	4.4×10^{9}	3.3×10^{6}	99.9

[a]See Table 8, footnote a.

[b]Salinity of estuarine water 25 ppt.

was initially used to resuspend the purified virus was later
changed to saline (NaCl 0.85%) to limit the marine bacterial
population to a level sufficient to allow the viewing of the
remaining phage population. Fig. 18 is a representative elec-
tron micrograph which depicts the state of the viral particles
initially and on day 3 in TYS broth. Fig. 19 is a representa-
tive micrograph of the viral population after 3 or 4 days in
the autoclaved estuarine water. As can be seen in both
electron micrographs the phage particles are numerous and
intact. Figs. 20 and 21 are representative electron micrographs
of the viral particles following exposure to estuarine water.
A dramatic reduction in the phage population was observed and
this was correlated with a decrease in infectious virus. An
increase in the structural components of the phage was also
noted to accompany the reduction in intact virus. This obser-
vation is illustrated in Fig. 20. Numerous phage tails and
empty phage heads can be seen along with phage whose heads
appear to be partially hydrolyzed. Thus the results of the
electron microscopic studies on the structural alteration of
41C confirmed the hypothesis that the disruption of the phage
particle occurs in estuarine water and further verify the
interpretation of radiation data.

Effect of chloramphenicol on 41C inactivation in estuarine water

Because the data obtained from the radiation and elec-
tron microscopic studies verified that the mechanism by which
41C was being inactivated was biological and resulted from
enzymatic degradation, a study was conducted to determine

Figure 18. State of the viral particles in TYS broth
(Magnification 12,500 X)

Figure 19. State of the viral particles in autoclaved estuarine
water (Magnification 12,500 X)
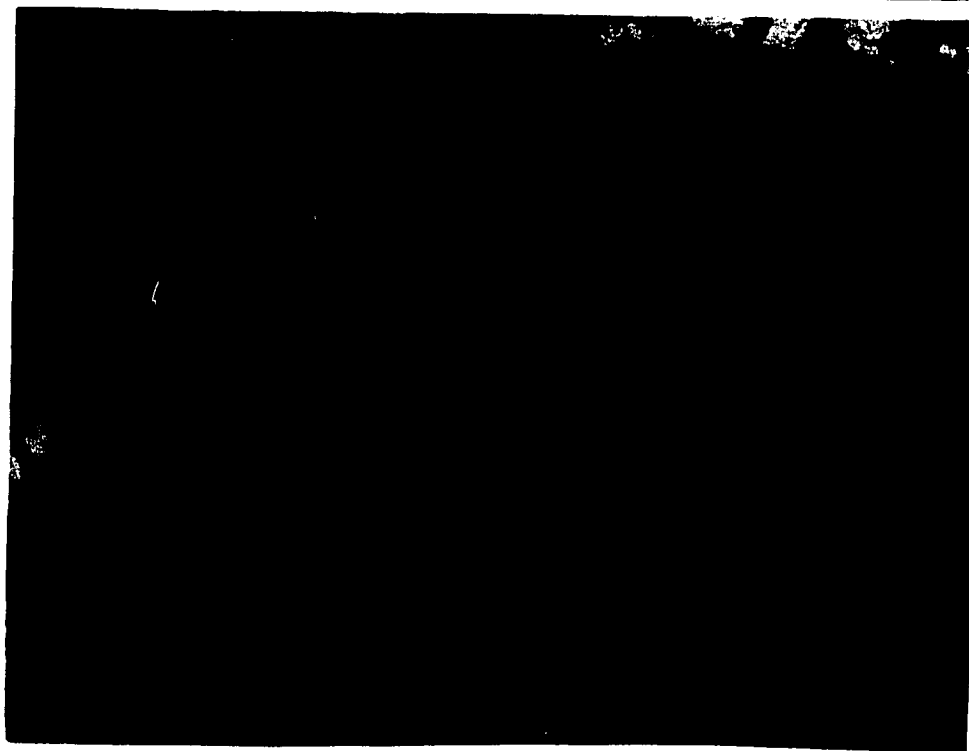
Figure 20. State of the viral population following exposure
to estuarine water (P, intact phage; H, empty phage
head; PH, partially hydrolyzed phage; T, phage tail;
F, bacterial flagellum; Magnification 25,000 X)

Figure 21. State of the phage population following exposure
to estuarine water (Magnification 12,500 X)
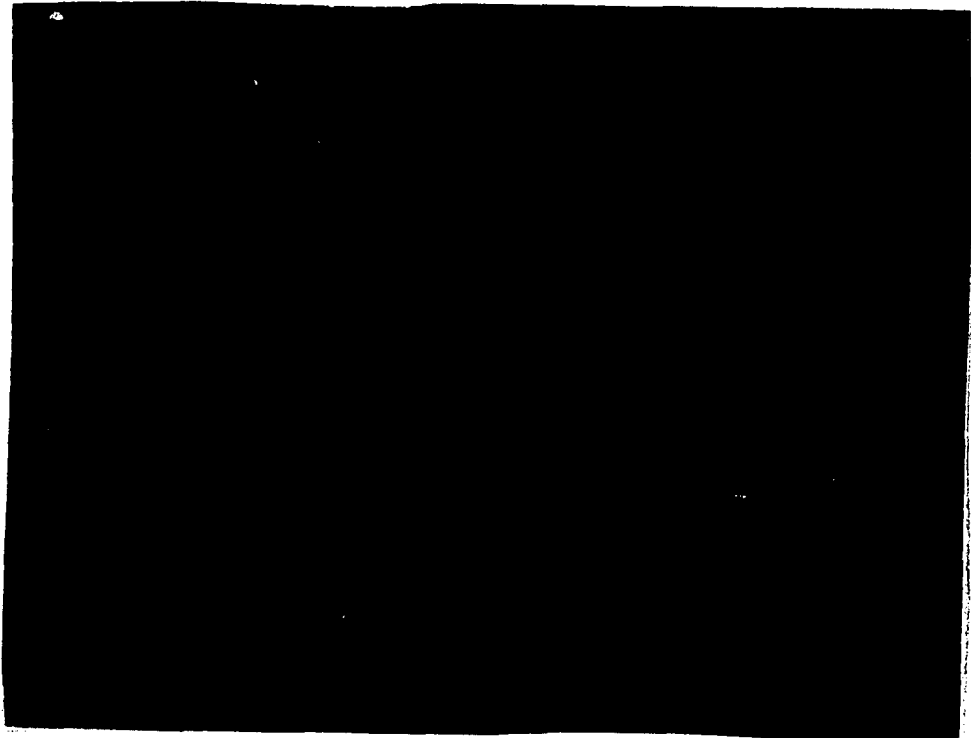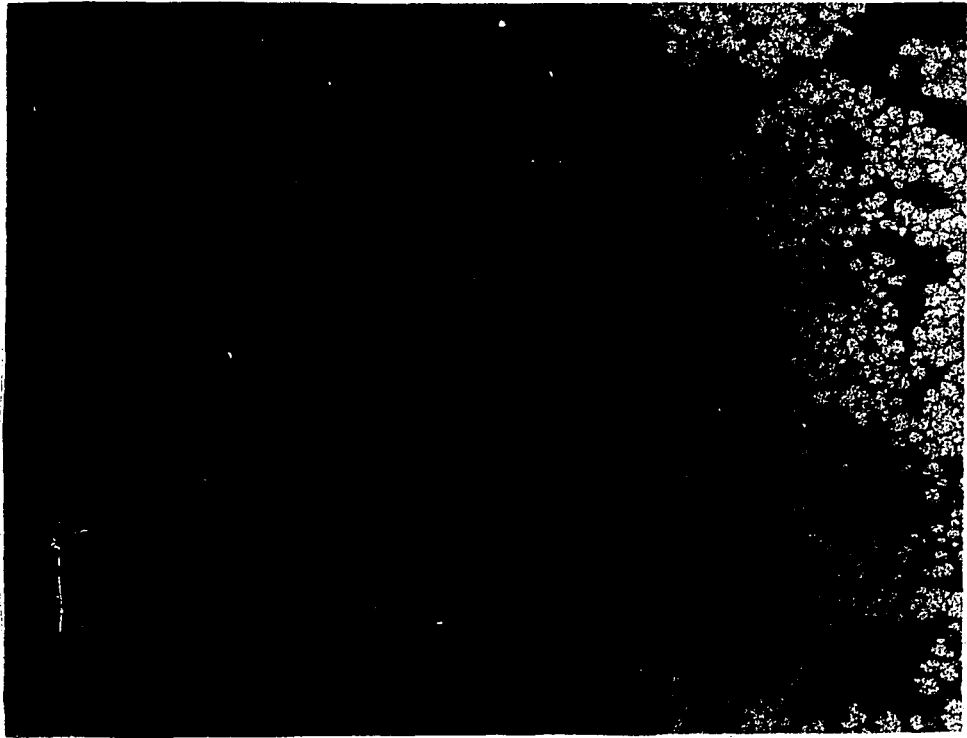
whether this inactivation process could be arrested by intro-
ducing an inhibitor of protein synthesis into the estuarine
water. Carlucci and Pramer (1960) in studies on the inactivation
of E. coli in seawater screened several antibiotic compounds
to determine the stability and effectiveness of these substances
against marine bacteria. Some of the compounds (although ef-
fective for a short time) were considered unstable for long
term studies. The compound which was chosen for use in this
study was chloramphenicol. This substance which inhibits pro-
tein synthesis by blocking the formation of the peptide bond
at the level of the ribosome has been shown to be both stable
and effective against marine bacteria (Carlucci and Pramer,
1960).

The level of chloramphenicol, 100 ppm (100 ug/ml)
used was that concentration reported by Carlucci and Pramer
to reduce the marine bacterial colony forming units by 98.6%.
Table 10 represents the results of two studies conducted to
determine whether the presence of chloramphenicol could alter
the inactivation profile of 41C in estuarine water. As illus-
trated from the data, the antibiotic was able to arrest the
inactivation of the phage. Thus continued protein (enzyme)
synthesis was required for the continued inactivation of the
virus. Any inhibition of protein synthesis would therefore
be reflected in a complete or reduced rate of viral inactiva-
tion. The results from this study are consistent with this
interpretation.

When the effect of chloramphenicol was tested using
adenovirus type 5 (Table 11) the results were not as conclusive

Table 10.   The effect of chloramphenicol on the inactivation
of <u>Bacillus</u> <u>subtilis</u> phage 41C in estuarine water

Study	Time	EW[a] [b]	EWC[a]	AEWC[a]
1	Day 0	4.4×10^8	4.8×10^8	5.1×10^8
	Day 3	5.3×10^7	4.2×10^8	3.7×10^8
	Day 8	3.2×10^6	2.9×10^8	1.9×10^8
2	Day 0	3.0×10^8	2.4×10^8	2.2×10^8
	Day 3	1.7×10^6	2.8×10^8	2.3×10^8
	Day 8	7.2×10^5	1.2×10^8	1.5×10^7

[a]EW, Estuarine water; EWC, estuarine water with 100 ppm
chloramphenicol; AEWC, autoclaved estuarine water with 100
ppm chloramphenicol.
[b]Salinity of estuarine water 25 ppt.

Table 11.   The effect of chloramphenicol on the inactivation
of adenovirus type 5 in estuarine water

Sample	Virus titer ($TCID_{50}$/0.1 ml)	
	Day 0	Day 8
EW[a]	$10^{5.33}$	$10^{2.50}$
EWC	$10^{5.75}$	$10^{3.75}$
AEWC	$10^{5.25}$	$10^{4.25}$

[a]See Table 10, footnotes a and b.

as those obtained with the phage. They do however suggest
that chloramphenicol was able to alter the inactivation pro-
file of the adenovirus and this is consistent with an enzymatic
mechanism of adenovirus inactivation.

Effect of chloramphenicol on the structural alteration of 41C in estuarine water

To verify the effectiveness of chloramphenicol in
arresting the structural alteration of 41C in estuarine water,
an electron microscopic study similar to those reported pre-
viously was carried out using estuarine water with and without
chloramphenicol. The infectivity data from the study (Table
12) once again confirmed the effectiveness of the antibiotic
as an inhibitor of viral inactivation. The electron micrograph
which appears in Fig. 22 represents the structural state of
the phage particles following exposure to estuarine water in
the presence of chloramphenicol. As can be seen the phage are
numerous and intact. The phage population exposed to estuarine
water without chloramphenicol (Fig. 23) showed a significant
reduction in the number of intact virus. The results from this
study reaffirm the potential of chloramphenicol to inhibit
viral inactivation and thereby maintain the structural integ-
rity of the viral particle in the estuarine water.

The effect of copper on the inactivation of 41C in estuarine water

One of the possible mechanisms suggested for the inac-
tivation of ØX174 in filtered seawater was that of heavy
metal ion inactivation (Mitchell and Jannasch, 1969). Since

Table 12. Infectivity data from the inactivation of
Bacillus subtilis phage 41C in estuarine
water with chloramphenicol subjected to
electron microscopic examination

Sample	Phage titer (PFU/ml)	
	Day 0	Day 4
EW[a]	1.7×10^{10}	4.1×10^{6}
EWC	1.4×10^{10}	7.0×10^{9}

[a]See Table 10, footnotes a and b.

Figure 22. State of the viral population following exposure
to estuarine water with chloramphenicol (Magnification
25,000 X)

Figure 23. State of the viral population following exposure to
estuarine water without chloramphenicol (Magnification
25,000 X)
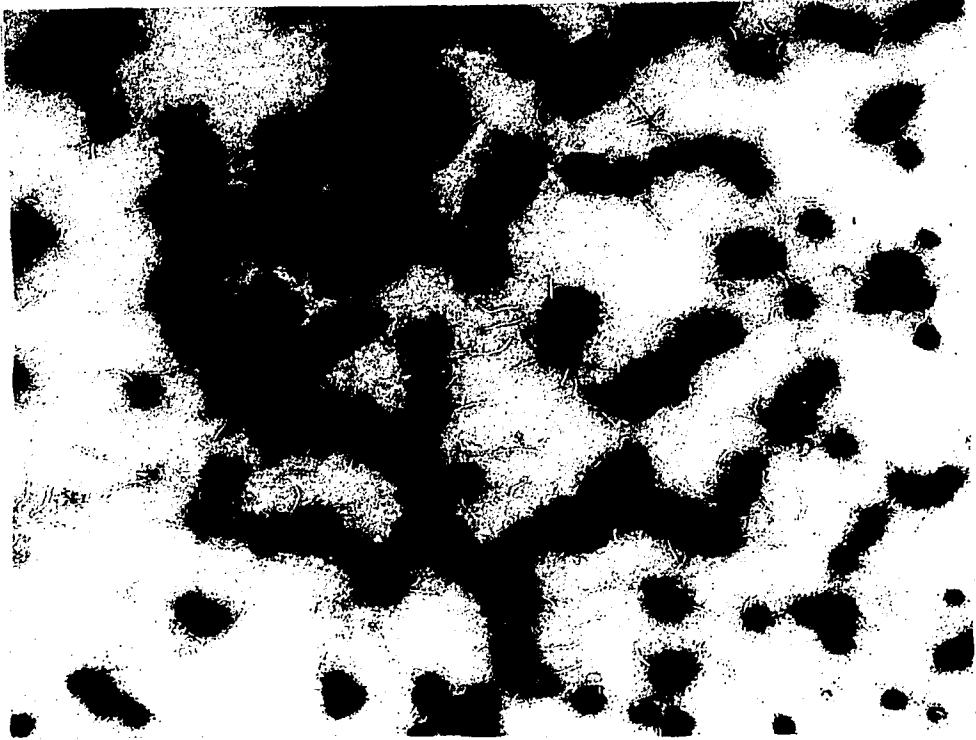
41C requires the divalent cation calcium (Ca^{++}) at some stage in its replicative cycle, it was of interest to determine whether 41C could be inactivated by copper (Cu^{++}). The results of the exposure of the phage to various copper concentrations appear in Tables 13 and 14. The initial results at a copper concentration of 100 ppm (100 ug/ml) in aged estuarine water or normal saline (NaCl 0.85%) gave evidence of phage inactivation. However, in the case of the aged estuarine water the observed inactivation was accompanied by a visible precipitate. The inactivation therefore was considered to result from precipitation and not a virus-ion-host cell interaction. The result of using saline in place of the estuarine water also showed a similar inactivation in the absence of a noticeable precipitate. Because the organic matter between the two suspending media varied, the results were again interpreted to reflect a possible precipitation of the phage. Since 100 ppm copper represents 100,000 times the concentration found in seawater (Goldberg, 1965; ZoBell, 1946), reduced copper concentrations were considered in the presence of purified phage suspended in saline. The addition of purified virus was implemented to reduce any nonspecific copper ion interaction which might mask the ion's effect on the phage. The results which appear in Table 14 gave no evidence of phage inactivation even at copper concentrations as high as 10,000 times that found in seawater. It was therefore concluded with respect to 41C that heavy metals do not play a direct role in the inactivation of the virus.

Table 13.   Effect of 100 ppm copper on the infectivity of
Bacillus subtilis phage 41C

Sample	PFU/ml	% Inactivation
Control EW[a]	2.93×10^7	0
Copper EW	6.06×10^6	79[c]
Control NaCl[b]	1.7×10^7	0
Copper NaCl	2.3×10^6	86

[a]See Table 10, footnote a.
[b]NaCl concentration 0.85%.
[c]Precipitate noted in system.

Table 14.   Effect of copper concentration on the infectivity
of Bacillus subtilis phage 41C in saline

Copper Concentration[a] ug/ml	Phage titer PFU/ml	% Inactivation
0	2.1×10^6	0
10	2.4×10^6	0
1	4.2×10^6	0
0.1	4.0×10^6	0

[a]Copper concentration in seawater 0.001 ug/ml.

CHAPTER V

DISCUSSION

Research efforts in the field of environmental virology
have focused on the significance of human viruses in water.
Interest in this area can be traced to the early nineteen
hundreds and the work of Kling who advocated that all polio-
myelitic epidemics originated from contaminated surface water
(Kling, 1929). Continued isolation of human viruses from river,
estuary, and coastal seawater as well as shellfish in areas
receiving domestic wastes have caused increased concern over
the potential hazard that these viruses may represent to
public health.

Current research is directed toward the development
of methods for concentrating and quantitatively enumerating
virus from water and sewage (Hill et al., 1972). In addition
to determining the types of viruses present and their relative
concentrations, these methods are necessary also for evaluat-
ing the effectiveness of disinfectants in eliminating the
passage of infectious human virus into receiving waters. Accu-
rate data in these areas are not as yet available (Hill et al.,
1972; Berg, 1970b); therefore, any critical evaluation of the
significance of human viruses in water must await their
acquisition.

Research dealing with the effects of receiving water
on viral pollutants have received only limited consideration.
In studies conducted by Clark and coworkers (1956) river water
was shown to have an adverse effect on coxsackievirus A2.

Hedstrom and Lycke (1964) reported similar effects with poliovirus type III in seawater as did Metcalf and Stiles (1967) whose studies included poliovirus type I, coxsackievirus B3 and echovirus 6 in estuarine water.

This investigation was prompted by the potential significance of infectious human virus in receiving water and by the limited amount of information available concerning the fate of these viral pollutants. The New Hampshire estuarine environment was chosen specifically for study because of the continued isolation of human viruses from its water and shellfish (Metcalf and Stiles, 1968). Adenovirus 5 was used in the study because of the recent association of adenoviruses with cases of infectious hepatitis (Hartwell et al., 1966; Hatch and Siem, 1966; Kohler and Springer, 1967) and the limited consideration afforded these enteric viruses in previous environmental studies.

The effect of estuarine water on adenovirus 5 was studied in the estuary during spring and summer of 1970. The results (Figs. 3 and 4) demonstrated that the virus was able to survive for a significant period of time in the estuary (10 to 14 days) and thus represented a potential source of human infection. A similar observation concerning the survival potential of other enteric viruses in the estuary was made by Metcalf and Stiles (1967).

Of further interest was the potential exhibited by the estuarine water to reduce the infectious titer of the virus. An analysis of the data obtained from two inactivation studies conducted during April–May and July, 1970 (Fig. 5),

indicated that the survival of the virus in both studies was approximately the same during a 14 to 15 day exposure to estuarine water. Neither increased temperatures nor salinities (recorded during the July study) were effective in increasing the rate of viral inactivation. This observation indicated that within the salinity range recorded, increases in the salt content of estuarine water did not exert a significant influence on virus infectivity.

Similar observations were reported by other investigators. In studies on seawater ranging in osmolarity from 196 to 644 mOm/l, Magnusson and coworkers (1966) were not able to detect a direct correlation between virus inactivating capacity (VIC) of the seawater sample and its osmolarity or ion concentration. It was further reported that the VIC of seawater was relatively stable between 0.15 M and 1 M NaCl. However, significant decreases in the viral inactivating capacity of seawater were noted at salinities above and below this range (Magnusson et al., 1966). The significance of salinity in viral inactivation may rest more with the influence it appears to exert on other factors in seawater than on any direct effect on the virus itself. This interpretation is also consistent with the data obtained in the present study. It is not, however, in complete agreement with all reported studies (Matossian and Garabedian, 1967). In addition to salinity, the rise in temperature was not considered to alter significantly the inactivation profile of the virus.

The overall difference in survival time recorded between the two studies was interpreted to reflect the higher

initial concentration of virus used during the spring study.
The influence of viral concentration on survival was substan-
tiated in a more direct manner by Plessieurs and Terre (1961)
using poliovirus in a seawater system. The increased sur-
vival potential observed by Metcalf and Stiles (1967) for
other representative enteric viruses was considered also to
reflect the influence of a higher initial viral concentration
($10^{8.5}$ to $^{9.5}$/specimen).

To further verify the inactivating potential of estua-
rine water, adenovirus 5 was studied simultaneously in the
estuary and in the laboratory. Although viral inactivation
proceeded at a faster rate in the estuary than in the laboratory
(Fig. 4), the results did confirm the viral inactivating
potential of the water. Similar parallel studies were not
available in the literature for comparison.

Most investigators have previously noted that heating
river or seawater resulted in a significant reduction in the
virus inactivity capacity of the water (Lycke et al., 1965;
Clark et al., 1956). When autoclaved estuarine water was
included in the laboratory study (Fig. 4) the rate of viral
inactivation was reduced similarly. This observation suggested
that the autoclaved water might serve as a negative viral
control. The results from the autoclaved estuarine water
further suggested that the major ions present in the estuarine
water were not exerting a significant influence on reducing
viral infectivity.

Because poliovirus has served as the prototype of
enteric viruses, an inactivation profile of this virus was

compared with adenovirus 5 in estuarine water. The inactiva-
tion rate of poliovirus II was greater than that for adeno-
virus 5 (Fig. 8). This result emphasized the significance of
considering adenoviruses along with other representative
enteric viruses in studies on viral inactivation. Comparative
data on adenovirus and poliovirus survivals have not been
established for estuarine water. In comparing the viral
inactivating capacity of seawater for different viruses,
Magnusson and coworkers (1966) noted that a greater loss in
infectivity was recorded for polioviruses II and III than for
adenovirus 7. Poliovirus I was the most resistant to inactiva-
tion when compared to the other viruses. Although the results
were obtained using a seawater system, the slower rate of
adenovirus inactivation as compared to poliovirus II was similar
to that observed in the estuarine water.

Previous investigators (Shuval et al., 1970, Magnusson
et al., 1966) have reported that the virus inactivating capacity
of seawater was not restricted to a specific location or sample
depth (up to 190 meters) in the marine environment. In assess-
ing whether these observations were also reflective of the
estuarine environment, the loss in infectivity of adenovirus
5 was compared in surface water obtained from Adams Point
with estuarine water collected at a depth of 7 ft off Nanny
Island (Fig. 7). The results were similar to those obtained
in the marine environment. It was also of interest to note
the similarities in the inactivation profiles of adenovirus
in the two estuarine water samples.

Data from a winter survival study conducted in the
estuary during March of 1971, again demonstrated that a more
rapid loss of virus occurred in the estuary than in the
laboratory. However, the results from both systems reflected
a decreased rate of viral inactivation (Fig. 9) when compared
to the summer study (Fig. 4). On further analysis of the data,
temperature appeared to be influencing viral inactivation.
When the loss in viral infectivity was studied in the laboratory
at 4 C and 20 C, the data (Fig. 10) again demonstrated a slower
rate of viral inactivation at the lower temperature (4 C).
When these results are interpreted in light of those previously
discussed, it would appear that during the spring and summer
studies the temperatures recorded in the estuary were within
a range considered optimum for the functional activity of the
viral inactivating factors. During the winter the lower
temperature reduced the level of activity of these factors
and this was reflected in a decreased rate of inactivation.
Thus it would appear that the significance of temperature,
like salinity, rests more with its effect on other factors in
the estuarine water rather than on the virus itself. Metcalf
and Stiles (1967) were able to demonstrate a similar effect
of temperature on other enteric viruses both in the estuary
and laboratory.

The temperature data suggested that biological systems
might be involved in the loss in viral infectivity; therefore
research was conducted to determine if any correlation existed
between estuarine bacteria and viral inactivation. During a
survival study conducted in August, 1971, the bacterial

population was monitored in the estuary and within the dialysis
bag containing the estuarine water and adenovirus. The bacterial
population in the estuary remained relatively constant through-
out the study. However, the estuarine bacteria within the
dialysis bag showed a significant increase in numbers and this
was related directly to a reduction in virus titer (Fig. 11).
Similar data were also obtained in the laboratory (Fig. 12).
These observations suggested that estuarine bacteria might be
responsible for the VIC of estuarine water.

In research with marine systems other investigators
(Shuval et al., 1970, Mitchell and Jannasch, 1969) have
reported a similar correlation between marine bacteria and
viral inactivation. Studies with individual marine isolates
revealed a spectrum of viral inactivating potential (Magnusson
et al., 1967) indicating that the VIC was associated with
marine bacteria and was not restricted to a single marine
species. Magnusson and coworkers (1967) selected a bacterial
isolate displaying a high VIC for further investigation. When
the organism Cl Vibrio parahaemolyticus was compared with sea-
water for its ability to inactivate poliovirus II both systems
displayed a significant VIC. Each system responded similarly
to treatment with ether, glycine, heat and sonication. These
observations suggested that the VIC reactions occurring with
V. parahaemolyticus could be the same as those responsible for
the loss in infectivity of the virus in seawater. However,
the investigators agreed additional evidence was necessary
before any definite statement could be made.

To date, the VIC potential of marine bacteria appears
to be an unstable characteristic. When V. parahaemolyticus
was passaged at 23 C it eventually lost its VIC. However,
following subculture at 4 C it regained this capacity (Magnusson,
1967). Following publication of the study, the organism was
reported to have lost its ability to inactivate virus (Shuval,
personal communication).

Shuval and coworkers (1970) confirmed most of the
observations reported by Magnusson and his colleagues. When
these results were presented, Shuval reported that 8 marine
isolates which had displayed virus inactivating capacity had
lost this ability. Passage of the organisms at 4 C did not
restore their VIC.

Because of the unstable and elusive nature of the
viral inactivating potential exhibited by marine bacteria, an
attempt was made to elucidate the mechanism of viral inacti-
vation while still maintaining the integrity of the estuarine
water system. If the mechanism could be ascertained using
an intact system, it might reflect more closely that of the
estuary. Previous investigators have attempted to elucidate
the mechanism of viral inactivation by considering the additive
effect of isolated components of the seawater system on viral
infectivity. This approach was not considered ideal, since
it cannot be inferred that the isolated components, when
combined in the intact system exhibit the same viral inactivat-
ing potential as was observed when tested alone.

The difficulty in obtaining meaningful data from a
complex and constantly changing environmental system has led

many investigators to follow the latter approach. In some instances system separation may represent the only avenue to researching a particular phenomenon. This approach was not considered necessary in elucidating the mechanism of viral inactivation in estuarine water.

The concepts involved in researching this question were relatively simple and the procedures involved proved to be adaptable to the estuarine environmental system. The first procedure consisted of introducing ^{32}P into the DNA of the virus at a level which would retain biological function and at the same time allow for the monitoring of the virus particle in an estuarine environment. Unfortunately several unresolved technical problems arose and it was not possible to use the adenovirus for this phase of the investigation. Consequently, B. subtilis phage 41C which is biochemically similar to the human virus was used for the radioisotope studies. The bacterial virus did have several advantages over the adenovirus both in handling and assaying. A single assay point in the human viral studies required 10 days for determination while a bacterial viral titration could be performed within 24 hours at a fraction of the total cost and man hours.

Because of the biochemical similarity between the two viruses, the results obtained with 41C might reflect also the mechanism of adenovirus inactivation as well as other bacterial and animal viruses of similar composition. This interpretation is based on the assumption that a unique mechanism for the in-activation of biochemically similar viruses does not exist in the estuarine environment.

The ^{32}P labeled phage was introduced into normal,
filtered and autoclaved estuarine water systems and its in-
fectivity and the location of the isotope were monitored. The
data from 3 separate studies (Tables 3, 4 and 5) conducted over
a 5 month period demonstrated loss of viral infectivity in
estuarine water to be correlated with the release of ^{32}P
labeled DNA into the suspending medium. The DNA appearing in
the medium was not precipitable by TCA indicating that it was
in a low molecular state. Virus in the autoclaved estuarine
water showed no evidence of molecular alteration. The data
obtained suggested that viral inactivation proceeded via a
biologically mediated system involving enzymatic disruption
of the protein coat of the virus and hydrolysis of the viral
DNA. The data further indicated that at least 2 enzymes were
involved in the molecular alteration of the virus, a proteinase
and a nuclease.

The failure of other investigators to demonstrate
enzymatic activity in viral inactivation was considered to
reflect in part a limitation in the nature of their approach.
Their results, however, suggested to the present investigator
that the enzymes responsible for viral degradation might be
located at the cell surface and therefore, would not be demon-
strable in the medium. In summary, the mechanism of viral
inactivation of B. subtilis phage 41C (in an intact estuarine
water system) is enzymatic and involves the molecular altera-
tion and partial destruction of the virus.

The radiation and infectivity data from the filtered
systems were intended to complement the results obtained in

the estuarine water. Difficulties encountered with bacterial contaminants however made this impossible. The loss of viral infectivity in the filtered systems was considered due to the presence of bacterial contaminants and not the filtered estuarine water. This interpretation does not include a possible influence exerted on the system by heavy metals.

The spectrum of viral degradation observed in the filtered systems (Tables 3, 4 and 5) suggest the possibility that some estuarine bacteria may not contain surface enzymes for viral inactivation while others may contain one or more of the enzymes involved.

Because of the short time interval between filtration and use of filtered water, a determination of the sterility of the filtered system could not be made prior to introducing the virus. Whether the cultured organisms were indeed passing through the filter or whether the filters were defective was not ascertained. The filters, however, when used for sterilizing other solutions yielded sterile filtrates. An attempt to recover one of the contaminant organisms in the filtrate following cultivation under laboratory conditions was unsuccessful.

The data from the autoclaved estuarine water system were complementary to that obtained from the estuarine water. However the results did require clarification of what initially appeared to be a significant amount of viral inactivation. If infectivity had been the only parameter monitored in the autoclaved system, the observed reduction in viral titer would have been interpreted to represent a type of chemical

inactivation. When ^{32}P labeled virus was monitored for infectivity and radiation levels, it was possible to discriminate between chemical inactivation and adsorption of virus to the sides of the glass container. Because virus was being removed from suspension and was not available for sampling, the accompanying reduction in viral titer was considered to represent an artifact of the system and not a direct effect of autoclaved estuarine water on the virus.

It should be noted that data were gathered to verify that the loss of virus did not result from an aggregation phenomenon. If this had occurred, the radiation would have been unevenly distributed in the autoclaved water. Samples from the autoclaved system showed that the tracer was evenly distributed throughout the water and thus verified that the loss of virus was external to the sampled water.

Unlabeled phage provided evidence which suggested that both the container and the preparation of the autoclaved water may influence the adsorption of virus. Because of the variation in results among several experiments exploring the influence of modifications in the container and the preparation of the water, no concise statement could be made concerning either of these factors.

On analyzing the infectivity and radiation data, it appeared that the protein coat of the virus was actually participating in the adsorption phenomenon. The hypothesis was confirmed by blocking effectively viral adsorption with the addition of 1% fetal calf serum (Table 7).

Evidence was presented which suggested that cationic species may play a role in the adsorption phenomenon. However the results using versene were only preliminary and therefore the importance of the observation is not yet known.

In earlier studies on viral inactivation, reduction in viral infectivity were noted in heated or autoclaved water (Clark et al., 1956, Magnusson et al., 1966). When autoclaved seawater was filtered through a 0.22 micron filter (Mitchell and Jannasch, 1969) or centrifuged at 16,000 RPM for 2 hr, however, a dramatic increase in the VIC of the water occurred. The increased viral inactivation, which in one instance was considered to be chemical in nature, was based solely on the infectivity data alone. These results raise the question of whether the observed loss in viral infectivity actually represented chemical inactivation or resulted from an adsorption phenomenon similar to that encountered in this investigation. The question is considered pertinent in view of the fact that both filtration and centrifugation would tend to remove organic material which in the case of serum is effective in blocking viral adsorption. By reducing the concentration of these blocking substances, it would be expected that an increased viral adsorption would occur and that this would be reflected in an increased rate of viral inactivation. This interpretation of the data from these studies (while worthy of mention) should not be considered to reflect what was actually occurring in their systems. Apart from its influence on the results of this investigation, the importance of the adsorption phenomenon lies

in the potential impact it may be exerting on the results of other studies.

Because the radiation data suggested a molecular alteration of the virus, studies were conducted to determine if any structural alteration of the virus could be detected following exposure to estuarine water. By employing electron microscopic techniques, it was possible to correlate the loss of viral infectivity with a dramatic decrease in intact viral particles. This decrease in the viral concentration was accompanied by a corresponding increase in the number of viral components. A spectrum of virus degradation following exposure to estuarine water was shown in the electron micrographs (Figs. 20 and 21). A comparison of the radiation and electron microscopic data indicated that the molecular alterations observed in the isotope studies reflected structurally an extensive enzymatic degradation of the viral particle by the estuarine bacteria.

The virus suspended in TYS broth or autoclaved estuarine water showed little evidence of viral inactivation. This result was also reflected clearly in the electron micrographs of their respective viral populations. The data obtained from these electron microscopic studies therefore verified further the interpreted results of the radiation studies.

Since radiation and electron microscopic data both indicated that an enzymatic mechanism was involved in viral inactivation, an attempt was made to arrest the inactivation of 41C in estuarine water by a protein inhibitor. The antibiotic chosen was chloramphenicol which has been reported

stable and affective against marine bacteria. The antibiotic

does not effect the activity of protein already synthesized

but is capable of blocking synthesis of new proteins at the

level of the ribosome by preventing peptide bond formation.

The results obtained from the studies demonstrated that chlor-

amphenicol was able to arrest effectively viral inactivation.

This observation indicated that continued protein or enzyme

synthesis was required for the continued inactivation of B.

subtilis phage 41C. The loss in virus infectivity which did

occur in the presence of chloramphenicol might have resulted

in part from enzymes present at the time the antibiotic and

virus were introduced into the system. It might also reflect

the VIC of bacteria resistant to the action of chloramphenicol.

The data, however, did complement the results obtained with

the radioisotope and the electron microscope and, therefore,

reinforce the concept of enzymatic degradation of virus as

the principal mechanism of viral inactivation in estuarine

waters. The electron microscope was used also to establish

the effect of chloramphenicol in arresting the structural

degradation of the virus (Fig. 22).

The effect of chloramphenicol in arresting the inac-

tivation of adenovirus 5 in estuarine water was studied.

Although the antibiotic was not as effective in inhibiting

the inactivation of adenovirus 5 as it was with 41C, the

results were still consistent with an enzymatic mechanism of

inactivation.

None of the investigators who have studied viral inac-

tivation have reported attempts to arrest it by inhibiting

protein synthesis. In studies with the Cl _Vibrio_ _parahaemoly-_
ticus isolate, an antibiotic sensitivity series was performed
on the organism (Gundersen _et_ _al._, 1967). However, the effect
of protein inhibitors on the virus inactivating capacity of
the isolate was not reported.

In studying viral inactivation in seawater, Mitchell
and Jannasch (1969) reported that seawater filtered through
a 0.22 micron filter displayed a high VIC. They suggested
inactivation of $\emptyset$X174 in the filtered water might be related
to the relatively high concentration of heavy metals present.
In the reported data, however, there was no evidence to suggest
that the virus was sensitive to heavy metal ion inactivation.

Because heavy metals might influence viral inactiva-
tion, studies with _B._ _subtilis_ phage 41C were conducted using
various concentrations of copper. It was postulated that the
cation might bond to the surface of the viral particle changing
its surface charge sufficiently to prevent adsorption of the
virus to the host cell. It might also oxidize a sulfhydryl
group(s) which might be essential in the reduced state for
viral attachment.

In the initial studies conducted at a copper concentra-
tion of 100 ppm (100 ug/ml) in estuarine water a 79% reduction
in viral infectivity (Table 13) was recorded. The reduction
in virus titer was however accompanied by a visible precipitate
and therefore was considered to represent viral precipitation
and not a virus-ion-host cell interaction. The reduction in
viral infectivity (86%) obtained when saline was substituted
for the estuarine water was again considered (in the absence

of a visible precipitate) to reflect a possible precipitation
of the virus. Since 100 ppm copper represents approximately
100,000x the concentration of the metal found in seawater
(Goldberg, 1965), reduced copper concentrations were considered.
These concentrations (Table 14) were tested under conditions
which minimized any non-specific copper ion interactions. The
results demonstrated that even at a copper concentration
10,000x that of seawater (10 ug/ml) no inactivation of the
virus was observed. Therefore the data indicated that copper
did not play a direct role in the inactivation of 41C. Fair
and coworkers (1948) in studying the effect of silver on
Theiler's mouse encephalomyelitis virus reported that 14.1 mg/l
silver ion failed to inactivate the virus.

In conclusion, an analysis of the data from the environ-
ment and laboratory indicates that the principle mechanism of
viral inactivation in estuarine water is biological and results
from the enzymatic degradation of the viral particle. Other
environmental factors (temperature, salinity, heavy metal ion
concentration) were considered to influence viral inactivation
indirectly. Ecologically, enzymatic degradation of virus is
considered to represent a response of estuarine bacteria to
a source of nutrient and energy in the form of the viral par-
ticle.

Further investigation will be performed to determine
the mechanism of viral inactivation of other human and bacterial
viruses. Individual estuarine and fresh water bacteria dis-
playing VIC will be monitored to determine whether any relation-
ship exists between the virus inactivating capacity of the

bacteria and the nutrient level of the water. In addition there is interest in locating and isolating the enzymes involved in viral inactivation.

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