ENTEROVIRUS INACTIVATION IN SOIL AND STRUCTURAL CHANGES ASSOCIATED WITH THE INACTIVATION OF SOIL-BOUND VIRUSES

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ABSTRACT

The inactivation of polioviruses in soil was examined and reported upon in two chapters. The results presented in Chapter II entitled "Enterovirus Inactivation in Soil" showed that the inactivation of radioactively labeled poliovirus type-1 and coxsackievirus B-1 in soils saturated with surface water, groundwater and septic tank liquor was directly proportional to temperature. Virus persistence was also related to soil type and the liquid amendment in which viruses were suspended. At 37°C, no infectivity was recovered from saturated soil after 12 days, and at 4°C viruses persisted for at least 155 days. No infectivity was recovered from dried soil regardless of temperature, soil type, or liquid amendment. Additional experiments showed that evaporation was largely responsible for the decreased recovery of infectivity from drying soil, however, increased rates of inactivation at low soil moisture levels may have amplified the virucidal effects of soil evaporation.

The experimental results in Chapter III entitled "Structural Changes Associated with Poliovirus Inactivation in Soil" indicated that the loss of infectivity of poliovirus in moist and dried soils was a result of irreversible damage to the virus particles. The damage included dissociation of viral genomes and capsids, and degredation of viral RNA in the soil environment. Under drying conditions, capsid components could not be recovered from the soils. Further studies in sterile soils indicated that, under moist conditions, the viral RNA

was probably damaged prior to dissociation from the capsid. However, in sterile dried soil RNA genomes were recovered largely intact from the soil. These results suggest that polioviruses are inactivated by different mechanisms in moist and drying soils.

Taken together, the findings in Chapters II and III suggest that any virus hazard associated with the application of wastewater or sewage sludge to agricultural land can be greatly reduced or eliminated by allowing the soil to dry after waste application.

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CHAPTER I

GENERAL INTRODUCTION

Heavy demands are being placed on the world's limited water supplies by an expanding population that has increased the domestic, industrial, agricultural, and recreational need for this resource. These demands have led to a search for alternative sources of water and more efficient use of existing water supplies (49, 52). In arid regions of the world, waterwater has been used extensively for irrigation, and in some areas, treated wastewater is being directly recycled into potable supplies (41). In the United States, over 1,000 communities use wastewater or sludge for agricultural purposes (51). As a result of recent environmental legislation, it is estimated that the annual production of sewage sludge will double by the early 1980's (2). This legislation, along with a heightened national awareness of the need to conserve natural resources, has led to the development of new technologies for the treatment of sewage and sewage solids. These treatment methods will enable more efficient use of the nutrients and organics contained in the sewage (26, 43, 47, 48, 62). The implementation of these new technologies should reduce the pressures on existing water supplies and raw materials that are used in the production of synthetic fertilizers (51).

The public health hazards associated with the use of wastewater and sludge for agricultural purposes has not been fully evaluated. Wastewater is known to contain pathogenic bacteria, viruses, and parasites that may not be completely removed by sewage or sludge

treatment processes (7, 23). Over 100 types of enteric viruses have been isolated from raw sewage, and viruses are routinely recovered from the effluents and sludges that result from sewage treatment (5, 23, 25, 34). These viruses pose a potential danger to human health if the effluents and sludges are used in agriculture. The real nature of this hazard is illustrated by the increases in respiratory and enteric illness that have been documented in an agricultural commune in Israel where wastewater is used for spray irrigation (29). The hazard associated with the entry of viruses into the food-chain from crops treated with sewage or sludge has not been fully investigated, but viruses have been shown to become associated with the surface and interior parts of plants treated with municipal wastes (1, 32, 39). These vegetable-associated viruses were shown to persist for over two weeks in one study (32), and for a period of time exceeding that required for crop maturation in another investigation (1).

In addition to the possible health hazard accompanying virus association with crops, it is possible that viruses from wastewater and sludge could percolate through the soil mantle and contaminate underlying groundwater (15, 50). An outbreak of hepatitis A has been traced to contamination of groundwater by a septic tank that discharged effluent to soil with a shallow water table (54). Additional outbreaks of poliomyelitis and non-bacterial gastroenteritis have been tentatively associated with sewage contamination of groundwater supplies (8).

If viruses applied to soil in wastewater and sludges are to become contaminants of groundwater or crop surfaces, the viruses must be able to withstand a variable period of association with soil. The viral contaminants must be capable of persistence until the physical conditions in the soil allow movement of the virions to crop surfaces or through the soil to groundwater. The virus-soil interaction is very complex, and soil-associated viruses will be subjected to a variety of physical and chemical stresses during their residence in the soil mileu (3, 22). Viruses are electrically charged colloidal particles with a negative charge at the pH's encountered in most soils (3, 22). The behavior of viruses in soil has been shown to be similar to that of other colloidal particles of similar size (17). Adsorption of viruses to soil particles is thought to be the primary mode of virus association with soils (3, 22). Even though the virus-soil interaction is complex, it is not necessarily destructive for the viruses. Soil-bound viruses and viruses eluted from soil have been shown to retain infectivity (20, 45).

The ability of viruses to persist in the soil environment has been demonstrated under field and laboratory conditions; however, the distinction between physical removal and inactivation of the viruses has not been made. In studies of secondary sewage effluent applied to a Florida cypress dome, Wellings et al. (60, 61) isolated viruses 28 d after effluent was applied to soil. Virus survival in the soil environment is also indicated in the results of Vaughn et al. (53) who isolated naturally occurring sewage viruses from groundwater

up to 30 feet below recharge basins on Long Island. Similarly, Moore et al. (38) recovered infective viruses from soil one month after sludge application. Additional field studies with sludge and sewage amended soils showed that seeded poliovirus persisted up to 96 d in cold weather, and from 8 to 11 d in warmer weather (52). Bagdasar'yan (1) recovered infective viruses from sewage-treated soils 180 d after soil amendment.

In contrast to experimental results indicating extended virus persistence in the soil environment, field and laboratory studies at the Flushing Meadows Project in Arizona indicated excellent removal of viruses from wastewater by soil treatment (19, 24, 31). In the field studies, no viruses were detected in monitoring wells at the site even though the wastewater applied over the 10-year duration of the project had completely replaced the native groundwater (24).

While laboratory studies on virus persistence in soils do not duplicate conditions in the natural environment, they can provide some estimate of virus persistence and a more controlled evaluation of the factors that influence virus survival in soil. Lefler and Kott (33) recovered infectious poliovirus after 90 d of incubation in moist sand and after 77 d in dried sand. In studies with soil columns, Duboise et al. (16) noted less than 90% reduction of seeded poliovirus after 84 d at 4°C. During this same period, greater than 5 logs of infectivity were lost from soil incubated at 20°C. Duboise et al. (16) also found that soil drying greatly reduced the number of recoverable viruses. Similarly, Ward and Ashley (58)

found that drying greatly reduced virus infectivity in sewage sludge. Studies with insect and plant viruses have also demonstrated extended persistence of non-enteric viruses in soil. Miyamoto (36) recovered infective wheat yellow mosaic virus after two years of incubation in soil. The nuclear polyhedrosis virus of the cabbage looper persisted for 231 weeks in soil without apparent reduction in infectivity (28).

Studies on the persistence of soil-associated viruses are incomplete and highly variable. The results of the field and laboratory studies just cited show that certain factors influencing virus stability in soil have been elucidated. Virus type, temperature, and soil moisture probably play a major role in determining virus persistence in the soil-associated state. Persistence is also influenced to a lesser extent by factors such as soil type, soil pH, ionic composition, and organic content (3, 22). Additionally, studies have suggested that virus persistence is greater in sterile soils than in non-sterile soils, implying that biological activity may influence virus stability in soils (1).

While limited attention has been given to the study of the kinetics of virus inactivation in soil and the environmental factors influencing inactivation, virtually no research has been directed toward uncovering the molecular mechanisms of virus inactivation in soils systems. Elucidation of the molecular events associated with the loss of virus infectivity in soil may help to identify the causative agent(s) of inactivation and eliminate or reduce the need for empirical inactivation studies in every soil destined for sewage or sludge application.

Investigations into molecular mechanisms of virus inactivation have mainly been confined to studies in water, sewage sludge, and aerosols. O'Brien and Newman (42) showed that poliovirus RNA was altered upon virus inactivation in surface water. The viral RNA was degraded into small fragments; however, no major capsid alterations or changes in adsorption to HeLa cells were seen. Extensive studies of virus inactivation in sewage sludge by Ward and Ashley (56) showed that poliovirus inactivation in digested sludge was accompanied by changes in two capsid proteins and nicking of the RNA genome. Further investigation revealed that ammonia (NH_3) in the digested sludge was the virucidal agent (57). Later, Ward (55) demonstrated that ammonia was only effective against poliovirus RNA while it was associated with the viral capsid. In studies on the inactivation of viruses in sludge dried by evaporation, Ward and Ashley (58) observed that the inactivation of polioviruses in drying sludge was accompanied by the release of RNA which was recovered from the sludge in a degraded form. Murray et al. (40) found that poliovirus RNA was altered as a consequence of virus adsorption to inorganic materials.

The molecular events associated with picornavirus inactivation in aerosols have been extensively studied by deJong and coworkers (10, 11, 12). Generally, picornaviruses are rapidly inactivated in aerosols at low relative humidities (10). This aerosol mediated inacitvation was accompanied by conformational changes in the virus capsids and the release of infectious RNA (11). The infectious RNA was very stable against the effects of heat and dessication.

Similarly enteroviruses have been shown to release their RNA when inactivated by physical means such as elevated temperature, intense ultraviolet irradiation, and acidic pH's in certain buffers (4).

The loss of viral RNA under these conditions is often accompanied by the loss of viral-protein 4, a change in antigenic character, and the formation of intact, empty capsids (4). Several enteroviruses have been shown to have two resonating conformational states associated with two distinct isoelectric points (35). Virus inactivation by the physical processes described above often results in the irreversible stabilization of the viruses in the more acidic conformational state (35), suggesting changes in the capsid that would likely affect infectivity and antigenicity.

The studies reported here are presented in two parts. Chapter II entitled "Enterovirus Inactivation in Soil" addresses the inactivation of poliovirus and coxsackievirus B-1 in soils from the Mesilla Valley in southern New Mexico. The experiments in this part also examine some of the environmental factors that could influence the persistence of viruses in these soils. As previously mentioned, prior investigations into virus inactivation in soils did not differentiate between virus inactivation and the inability to recover viruses from the soil-bound state. The experiments discussed in Chapter II were done with radioactively labeled virions in order to distinguish between irreversible adsorption and inactivation.

The experiments presented in Chapter III entitled "Structural Changes Associated with Poliovirus Inactivation in Soil" were designed to explore the molecular events associated with the inactivation of

polioviruses in Mesilla Valley soils. These studies were also done with radioactively labeled virions in the hope that some general concepts relative to virus inactivation in soils could be delimited.

CHAPTER II

ENTEROVIRUS INACTIVATION IN SOIL

Introduction

Previous field and laboratory studies on virus persistence in the soil environment have yielded contradictory results. Several investigations have indicated extended persistence and movement of wastewater viruses in soils (60, 61). Conversely, other investigators have shown efficient virus removal, and presumably destruction, by soil treatment of virus-contaminated wastewaters (19, 24, 31). One of the greatest problems in resolving the inconsistencies found in these experiments has been the inability to distinguish between virus inactivation in soil and the incomplete recovery of soil-bound virions. Recovery techniques have been developed; however, interpretation of recovery efficiency has been hampered by the lack of methods to quantitatively measure any changes in virus recovery as experimental conditions differ from the optimal conditions used in the development of recovery methods. Poor recovery efficiency due to irreversible binding of viruses to soil, or a lack of detection due to masking of viral infectivity could be erroneously interpreted as virus inactivation.

These difficulties can be resolved by the use of purified, radioactively labeled virus preparations in soil inactivation experiments. This not only permits the calculation of virus recovery efficiency, but also determination of specific infectivity (plaque-forming units/cpm) which provides a measure of the virus inactivation

that has occurred in the soil. The experiments reported here characterizing virus inactivation in southern New Mexico soils have made use of radioactively labeled virions. They conclusively show that under the conditions examined, virus inactivation was responsible for the observed decreases in the number of viruses recovered from soil under different conditions of soil moisture, temperature, and virus-suspending medium.

Materials and Methods

Viruses and Cells. The poliovirus type-1 (Mahoney) and coxsackievirus B-1 used in soil inactivation experiments were propagated and assayed on HeLa cell monolayers as described by O'Brien and Newman (42). Radioactively labeled virus stocks were prepared using [^{3}H] uridine and ^{14}C protein hydrolysate as previously described (56). Labeled virions were purified by centrifugation in 15-30% glycerol gradients, and capsid labeled stocks were further purified by equilibrium density centrifugation in cesium chloride (56).

Virus Inactivation in Soil. Virus inactivation experiments were done in a sandy soil from the bed of the Rio Grande and a sandy loam soil from an adjacent agricultural field. Physical and chemical analyses of the soil samples were done by the New Mexico State University Soil Testing Laboratory. To determine virus adsorption to soil, approximately 1 x 10^{8} plaque-forming units (pfu) of [^{3}H] uridine-labeled poliovirus were suspended in either groundwater from a 3-m deep well, Rio Grande water, or septic tank liquor. Two-ml aliquots were then added to 1-g soil samples, and the soil-virus slurry was mixed gently on a shaker for 1 h. The mixtures were then centrifuged at 8,000 x g to sediment soil particles, and virus adsorption was determined by assaying the pfu and radioactivity in the supernatants. Virus numbers in subsequent experiments were always kept below the virus adsorption capacity for each soil-suspending medium combination.

For studies on the inactivation of soil-associated viruses, aliquots containing approximately 1 x 10^{7} pfu of appropriately labeled

viruses suspended in river water, groundwater, or septic tank liquor were adsorbed at 22°C to 5 g of soil in screw-cap tubes. The virus-soil mixtures were incubated in the sealed screw-cap tubes at 4°C, 22°C, or 37°C. Viruses were eluted at predetermined intervals by vigorously mixing the soil samples with two 15-ml aliquots of pH 11.5 glycine (0.1 M) EDTA (0.01 M), calf serum buffer (1%) (45). The eluates were centrifuged to remove suspended soil particles and then adjusted to pH 7 with 0.1 N HCl. Neutralized eluates were assayed for pfu on HeLa cell monolayers and for radioactivity by liquid scintillation spectrometry. The measurement of recovered radioactivity in the eluates allowed the calculation of recovery efficiency, and pfu recoveries were adjusted accordingly. Sampling was carried out until no pfu were recovered in two successive samples.

The persistence of viruses in dry soils was examined by allowing open tubes containing the virus-soil mixtures to dry (in dessicators) at the appropriate temperatures. When the samples were gravimetrically dry, viruses were eluted from the dried soils and saturated soil control samples and assayed as outlined above. Soil-associated inactivation of viruses at specific soil moistures was studied by suspending [^{3}H] uridine-labeled poliovirus in groundwater, and adding the virus suspension to soil samples until the soil was saturated. Approximately 35 g of the saturated soil were placed in 12 x 50 mm petri dishes. The petri dishes were placed in a dessicator until the desired soil moisture level was reached. Initial 5-g samples were taken and the dishes were sealed to prevent additional moisture loss. The sealed samples were incubated at 22°C, and samples were taken at intervals

over the next 10 d. Viruses were then eluted with two 2.5-ml aliquots of pH 11.5 buffer followed by determination of pfu and radioactivity as described above. Results were calculated as specific infectivity/ g dry weight of soil.

Results

Virus Adsorption Capacity. Virus particles behave as colloids in solution, and virus adsorption to soil is dependent in part upon such soil characteristics as pH, cation exchange capacity, organic matter content, and clay content (3). These characteristics were determined for the two soil types used in these experiments. It is clear from the data presented in Table 1 that the most significant difference in the two soils was the clay content which was 19% for the sandy loam compared to 0.08% for the sandy soil. This difference in the clay content was reflected in the difference in the cation exchange capacities of the two soils as shown in Table 1.

To ensure that the numbers of viruses applied to the soils did not exceed the virus adsorbing capacity of the soil, the adsorption of poliovirus to the soils was determined with the viruses suspended in river water, groundwater and septic tank liquor. Virus adsorption ranged from 1.1×10^7 pfu/g adsorbed by sandy soil from river water to 1.7×10^7 pfu/g adsorbed by the sand loam soil from groundwater and septic tank liquor (Table 2). In agreement with published reports (3, 22) the sandy loam soil adsorbed a greater number of viruses from all suspending media than did the sandy soil. In subsequent experiments, the number of viruses applied to the soils was always less than the adsorption capacity determined for each soil-suspending medium combination. This was done to enhance the probability that the added viruses were adsorbed to the soil rather than trapped in the liquid in the interparticulate spaces in the soil.

Table 1. Some properties of the soils used in virus inactivation experiments.

Soil Property	Soil Type	
	Sand	Sandy Loam
pH	8.0	7.7
Cation Exchange Capacity[a]	0.35	17.9
Organic Matter[b]	<0.03	0.9
Clay[b]	0.8	19.0

[a]Meq/100 g soil

[b]Percent w/w

Table 2. Adsorption of poliovirus to Rio Grande Valley soils[a].

Suspending Medium and Soil Type	Total pfu Added	pfu in Supernate	pfu Absorbed/g Soil
River Water +			
Sand	1.3×10^7	1.7×10^6	1.1×10^7
Sandy Loam	1.3×10^7	2.8×10^5	1.2×10^7
Ground Water +			
Sand	1.7×10^7	1.4×10^6	1.6×10^7
Sandy Loam	1.7×10^7	4.4×10^5	1.6×10^7
Septic Liquor +			
Sand	1.7×10^7	1.7×10^6	1.5×10^7
Sandy Loam	1.7×10^7	1.0×10^5	1.6×10^7

[a] 1 g of soil was suspended in 2 ml of liquid contining about 1×10^7 pfu of [^{3}H] uridine-labeled viruses. The soil-virus suspension was mixed for 1 h and centrifuged to remove soil particles. Virus adsorption capacity was determined by subtracting pfu remaining in the supernate from pfu added to the soil. A control sample of viruses in liquid amendment showed no detectable virus inactivation during the 1 h adsorption period.

Effect of Soil Elution Procedure on Virus Infectivity. Viruses were eluted from the soil samples with pH 11.5 buffer. Since exposure to high pH's for extended periods has been shown to be virucidal (21). the effect of the elution procedure on virus infectivity was determined. The specific infectivity of a [^{3}H] uridine-labeled virus loading suspension was compared with that of the same viruses eluted from the soil with the high pH buffer. The results showed that no detectable change in specific infectivity occurred as a result of the elution process.

Inactivation of Viruses in Saturated Soil. The effect of incubation temperature on the inactivation of viruses in saturated soils was studied by applying radioactively labeled viruses in the three liquid amendments to soil samples in sealed tubes. Viruses were eluted at intervals, and the eluates were assayed for recovered pfu and radioactivity. As shown in Table 3, at 37°C no pfu were recovered after 12 d, whereas at 4°C pfu were recovered from the soil for at least 155 d. Thus, the persistence of poliovirus in saturated soils was temperature dependent regardless of soil type or liquid amendment.

The effects of soil type and aqueous amendment on the inactivation of viruses in saturated soils were then determined by comparing poliovirus inactivation in the amendments with virus inactivation in liquid amended soils. These results, summarized in Table 4, show that under the conditions used in these experiments, poliovirus was consistently inactivated more slowly when suspended in septic tank liquor than in river water or groundwater. Also, adsorption to sandy loam soil appeared to be protective of the viruses while adsorption to sand had little effect on the rate of poliovirus inactivation.

Table 3. Effect of soil temperature on inactivation of poliovirus in saturated soils[a].

Soil Type	Suspending Medium	Soil Temperature					
		4°C[b]		22°C		37°C	
		Sample Day[c]	% Initial pfu Recovered	Sample Day	% Initial pfu Recovered	Sample Day	% Initial pfu Recovered
Sandy loam	River water	158	3.1	19	0.140	6	0.400
Sand	River water	158	0.5	13	0.530	12	0.200
Sandy loam	Groundwater	155	4.3	39	0.010	7	0.003
Sand	Groundwater	155	0.3	21	0.030	11	0.020
Sandy loam	Septic liquor	180	0.8	92	0.004	11	0.007
Sand	Septic liquor	180	5.4	36	0.500	11	0.010

a. Approximately 1 x 10^7 pfu of [^{3}H] uridine-labeled poliovirus were applied to soil samples in sealed tubes. Viruses were eluted periodically and assayed for radioactivity and pfu. Sampling was continued until no pfu were recovered from two consecutive samples. Results were corrected for recovery efficiency.

b. Temperature at which soils were incubated.

c. Day last pfu were recovered.

Table 4. Effects of soil type and amendment on inactivation of poliovirus at 22°C[a].

Inactivation in:	Days Required for 90% Reduction in Infectivity
River water	3.4
River water + Sandy Loam	7.0
River water + Sand	4.6
Groundwater	5.4
Groundwater + Sandy Loam	6.3
Groundwater + Sand	5.3
Septic liquor	12.0
Septic liquor + Sandy loam	21.1
Septic liquor + Sand	8.4

a. Approximately 1×10^7 pfu of [^{3}H] uridine-labeled viruses were added to liquid medium or soil in sealed tubes and assayed directly or after elution from soil. Viruses in liquid amendments were assayed for pfu after dilution, and soil-associated infectivity was assayed after elution.

Effect of Soil Moisture on Virus Inactivation. Recent reports have indicated that the dewatering by evaporation of sewage sludges containing viruses resulted in the inactivation of the sludge-bound viruses (58). However, earlier experiments in this laboratory with viruses percolating through soil columns suggested that a reduction in soil moisture by evaporation may lead to irreversible binding of viruses to soil particles rather than virus inactivation. In order to determine which of these two possibilities would occur in the soils and amendments used in the previous experiments, [^{3}H] uridine-labeled polioviruses in groundwater, river water, or septic tank liquor were absorbed to soil samples in tubes which were then sealed or left open. The tubes were incubated at 4°C, 22°C, and 37°C. When the open tubes were dry, viruses were eluted from the dry soil and a corresponding saturated soil control. The results of these experiments are summarized in Table 5. It can be seen that regardless of temperature, soil type or liquid amendment, no infective virions were recovered from the dried soil samples. However, pfu were recovered in all cases from saturated soil controls and at least 80% of the added radioactivity was recovered. In addition to the influence of temperature on virus inactivation rates noted in the previous experiments, it can be seen that the temperature in these experiments greatly influenced the times required to dry the soil samples. The efficient recovery of radioactivity from the soils along with the decrease in the specific infectivities of these eluted virions suggest that the virus were inactivated rather than irreversibly bound to soil particles.

Table 5. Effect of soil drying on inactivation of poliovirus[a].

Soil type	Suspending Medium	4°C[b] Specific infectivity[c]			22°C Specific infectivity			37°C Specific infectivity		
		Sample Day[d]	Saturated Soil	Dry Soil	Sample Day	Saturated Soil	Dry Soil	Sample Day	Saturated Soil	Dry Soil
Sandy loam	river water	30	1.3×10^{3}	$<2.5\times10^{-2}$[e]	16	1.25×10^{1}	$<2.5\times10^{-2}$	3	5.0×10^{0}	$<2.5\times10^{-2}$
Sand	river water	30	1.0×10^{3}	$<2.5\times10^{-2}$	12	7.5×10^{0}	$<2.5\times10^{-2}$	3	3.2×10^{2}	$<2.5\times10^{-2}$
Sandy loam	ground water	30	1.4×10^{3}	$<2.5\times10^{-2}$	16	7.5×10^{0}	$<2.5\times10^{-2}$	3	7.5×10^{0}	$<2.5\times10^{-2}$
Sand	ground water	30	9.0×10^{2}	$<2.5\times10^{-2}$	12	1.5×10^{1}	$<2.5\times10^{-2}$	3	2.9×10^{2}	$<2.5\times10^{-2}$
Sandy loam	septic liquor	30	1.3×10^{3}	$<2.5\times10^{-2}$	16	1.1×10^{2}	$<2.5\times10^{-2}$	3	2.1×10^{2}	$<2.5\times10^{-2}$
Sand	septic liquor	30	1.7×10^{3}	$<2.5\times10^{-2}$	12	3.8×10^{1}	$<2.5\times10^{-2}$	3	2.1×10^{2}	$<2.5\times10^{-2}$

[a] Soils were initially seeded with approximately 1×10^{7} pfu of [^{3}H] uridine-labeled viruses. Soils maintained at saturation were in sealed tubes. Detection limit of assay procedure was 1×10^{2} pfu/5g sample.

[b] Temperature at which soils were incubated

[c] Specific infectivity (puf/cpm) was initially 2.5×10^{3}

[d] Day on which exposed soil sample was dry

[e] No pfu were recovered from dried soils. Under these conditions specific infectivity is reported as $<2.5\times10^{-2}$.

While the results of the previous experiment showed that drying was virucidal, the possibility remained that this virucidal activity was unique to poliovirus type-1, the two soil types used in the experiments, or that the results were only characteristic of laboratory conditions.

Inactivation of Coxsackievirus B-1 in Soil. To determine if soil dewatering by evaporation was virucidal for viruses other than poliovirus, soil samples at 22°C were seeded with [^{3}H] uridine-labeled coxsackievirus B-1 in groundwater. As before, viruses were eluted from the soils when the open samples were dry. The recovery efficiencies (cpm eluted/cpm adsorbed x 100) in Table 6 show that the labeled coxsackieviruses were not irreversibly bound to the soil. Also, the changes in the specific infectivities of the eluted viruses make it clear that coxsackievirus was inactivated in soil, and, as with poliovirus, soil drying was virucidal.

Effect of Soil Type on Virus Inactivation by Soil Dewatering.

To examine the relationship between soil type and virus inactivation by dewatering, six soil samples were obtained in a transect of the Mesilla Valley in southern New Mexico. Labeled poliovirus suspended in groundwater was adsorbed to duplicate samples of the six soils as well as to samples of bentonite clay and a commercial potting soil containing 70% organic matter. The results in Table 7 show that, although the drying times differed for the soils, no infectivity was recovered from the dried soils whereas pfu were recovered from the corresponding saturated soil samples.

Table 6. Specific infectivity (pfu/cpm) of [^{3}H] uridine-labeled coxsackievirus B-1 eluted from saturated and dry soils.[a]

Soil type	Specific Infectivity		
	Day 0, Saturated	Day 6, Saturated	Day 6, Dry
Sandy loam	1.2×10^3 (98)[b]	2.9×10^1 (93)	<0.06[c] (70)
Sand	1.3×10^3 (99)	8.6×10^1 (90)	<0.06 (86)

[a] Approximately 5×10^6 pfu of [^{3}H] uridine-labeled coxsackievirus B-1 in ground water were adsorbed to soil samples at 22°C. Viruses were eluted from a saturated soil sample after 1 h and from the remaining samples when the samples in the open tubes were dry. Eluates were assayed for pfu and radioactivity.

[b] Number in parenthesis is the percent of adsorbed radioactivity recovered by elution.

[c] No pfu were recovered. Under these circumstances, specific infectivity is reported as <0.06.

Table 7. Effect of drying on the inactivation of poliovirus in selected Mesilla Valley soils at 37°C.

Sample Number	Soil Type	Sample Day	% pfu recovered	
			Saturated Soil	Dry Soil[b]
1	Sand	4	4.600	<0.0005
2	Sandy loam	4	0.560	<0.0005
3	Sand	4	0.500	<0.0005
4	Sand	4	1.200	<0.0005
5	Sand	4	0.900	<0.0005
6	Sand	4	0.300	<0.0005
Bentonite	Clay	9	0.010	<0.0005
Potting Soil	--	9	0.008	<0.0005

[a]Soil samples 1-6 were obtained from a transect of the Mesilla Valley. Viruses were suspended in groundwater, and about 1×10^7 pfu of [^{3}H] uridine-labeled viruses were added to 1 g soil samples. Sealed and open samples were placed in a dessicator and eluted when open samples were dry.

[b]No pfu were recovered from dry soils, however, the lower limit of detection under these conditions was 0.0005% of the original virus load.

Inactivation of Poliovirus in Soil under Simulated Field Conditions. Questions are often raised as to the applicability of laboratory results to actual field conditions. While the results of the preceeding experiments suggest that soil drying would be virucidal under field conditions as well as in the laboratory, this hypothesis was examined by adsorbing approximately 5×10^8 pfu of poliovirus in 360 ml of groundwater to 3 kg of sandy soil. The soil and viruses were thoroughly mixed, and the resulting mixture was equally divided into three containers. One sealed container and two open containers were placed in an outside shaded area where the temperature ranged from 4°C to 10°C during the experiment. After 10 d, samples were removed for determination of soil moisture, and three 5-g samples were taken from each container for virus elution and assay.

The results summarized in Table 8 show that the pfu in the moist sample (15% soil moisture) decreased by 69.0% during the 10-d period. The soil dried to 1.6% soil moisture showed a 99.7% decrease in pfu, and less than 0.009% of the applied pfu were recovered from the soil dried to 0.6% soil moisture. This result shows that the virucidal effects of soil drying seen in the laboratory experiments were representative of what occurs under these simulated field conditions.

Effect of Dewatering and Reduced Soil Moisture on Poliovirus Inactivation. The results of the previous experiments indicated that recoverable infectivity decreased as the soil moisture content was reduced by evaporation. The increased virus inactivation could be due to virucidal effects of the evaporative process or increased rates of viral inactivation at reduced soil moisture levels. To

Table 8. Inactivation of poliovirus in soil under simluated field conditions.[a]

Sample #	Sample Day	$\%H_2O$	pfu recovered/g dry wt. of soil	% of initial pfu recovered
1	0	12	1.5×10^6	100
1	10	12	4.7×10^5	31.3
2	10	1.6	3.8×10^3	0.3
3	10	0.6	ND[b]	0

[a]About 5×10^8 pfu of poliovirus type-1 in groundwater were added to 3 kg of sandy soil. After thorough mixing, 1 kg portions were placed in sealed or open containers which were then placed outside in a shaded area. After 10 d, samples were taken for soil-moisture determination and elution of virus.

[b]None detected. Lower limit of detection under these conditions was 150 pfu/sample which was 0.09% of the adsorbed viruses.

examine the influence of evaporation and reduced soil moisture on virus inactivation, approximately 2 x 10^9 pfu of [^{3}H] uridine-labeled poliovirus were added to 35-g samples of sandy soil in 12 x 55-mm petri dishes. One dish was sealed to maintain the original 18% soil moisture, and the remaining samples were dried to various moisture levels over the next 4 h. After dewatering, an initial sample was eluted from each moisture level to determine the virucidal effects of evaporation, and the remaining samples were sealed and incubated at 22°C over a 10-d period. To determine inactivation rates at each soil moisture level, samples were eluted at specified times during this period and assayed for pfu and radioactivity. The effects of evaporation on virus recovery during the 4-h period are shown in Figure 1. Virus recovery decreased as soil moisture was decreased from 18.0% to 2.9% by evaporation. However, a marked decrease in recovered infectivity was noted in the samples dewatered to 1.2% and 0.6% soil moisture. Thus, there appeared to be a critical soil moisture below which virucidal effects of evaporation were amplified.

The effects of soil moisture on virus inactivation were determined in soils held at various moisture levels. As shown in Figure 2, viruses were inactivated at essentially the same rates in the soils at 18.0% to 2.9% moisture content. However, the rates of virus inactivation increased markedly in the soils held at 1.2% and 0.6% moisture.

The results presented in Figures 1 and 2 suggest that the decreased recovery of viruses in dried soils may have been due not

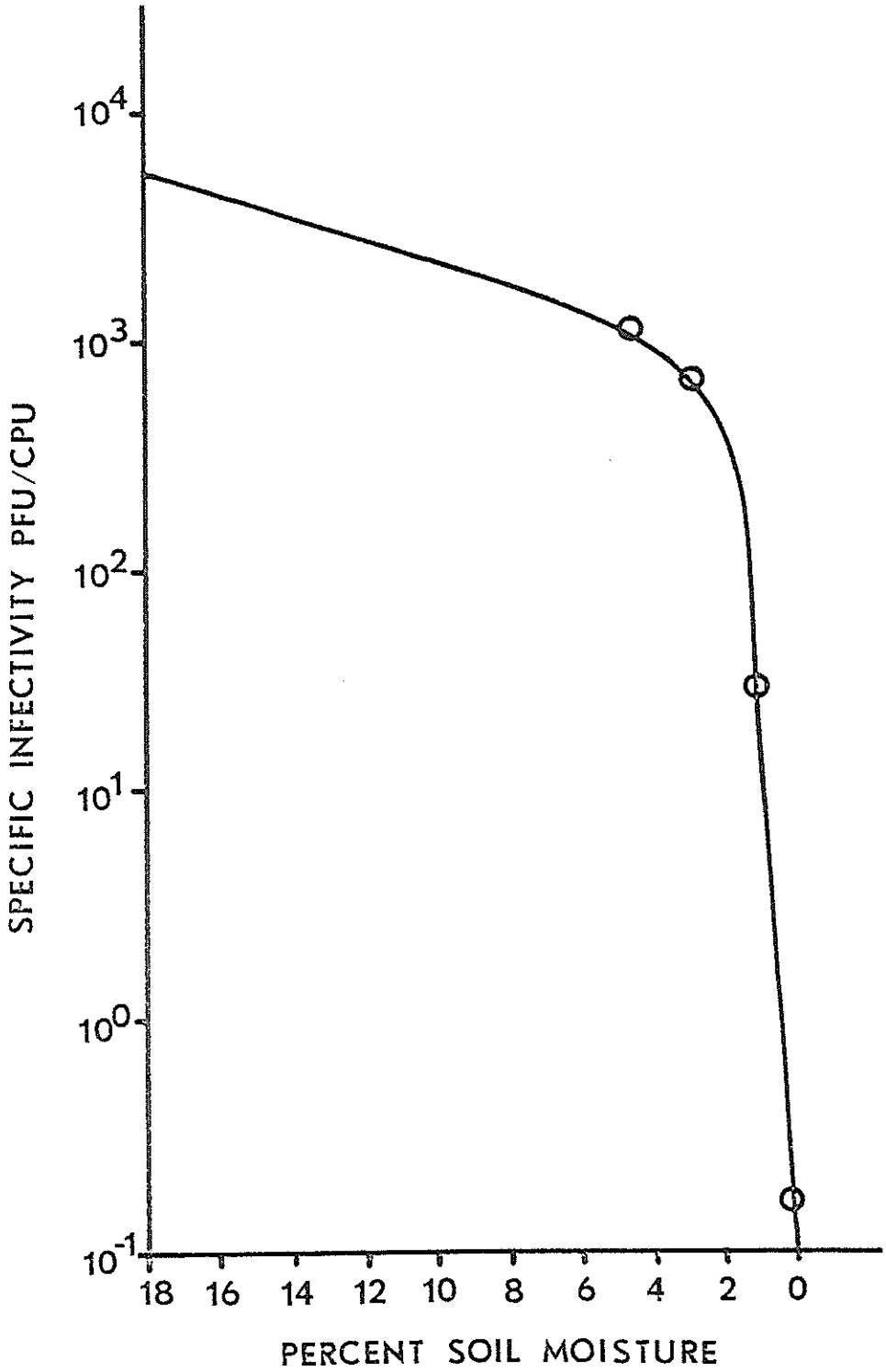

Figure 1. The effect of soil dewatering on the specific infectivity (pfu/cpm) of poliovirus type-1. Approximately 2 x 10^4 pfu of [^{3}H] uridine labeled poliovirus type-1 in groundwater was applied to soil samples at 22°C. Soil samples were dried to 18.0%, 4.7%, 2.9%, 1.2% and 0.6% soil moisture in a dessicator and sealed to prevent further moisture loss. Viruses were eluted from all samples when the final sample reached 0.6% soil moisture. Eluates were assayed for pfu and recovered radioactivity.

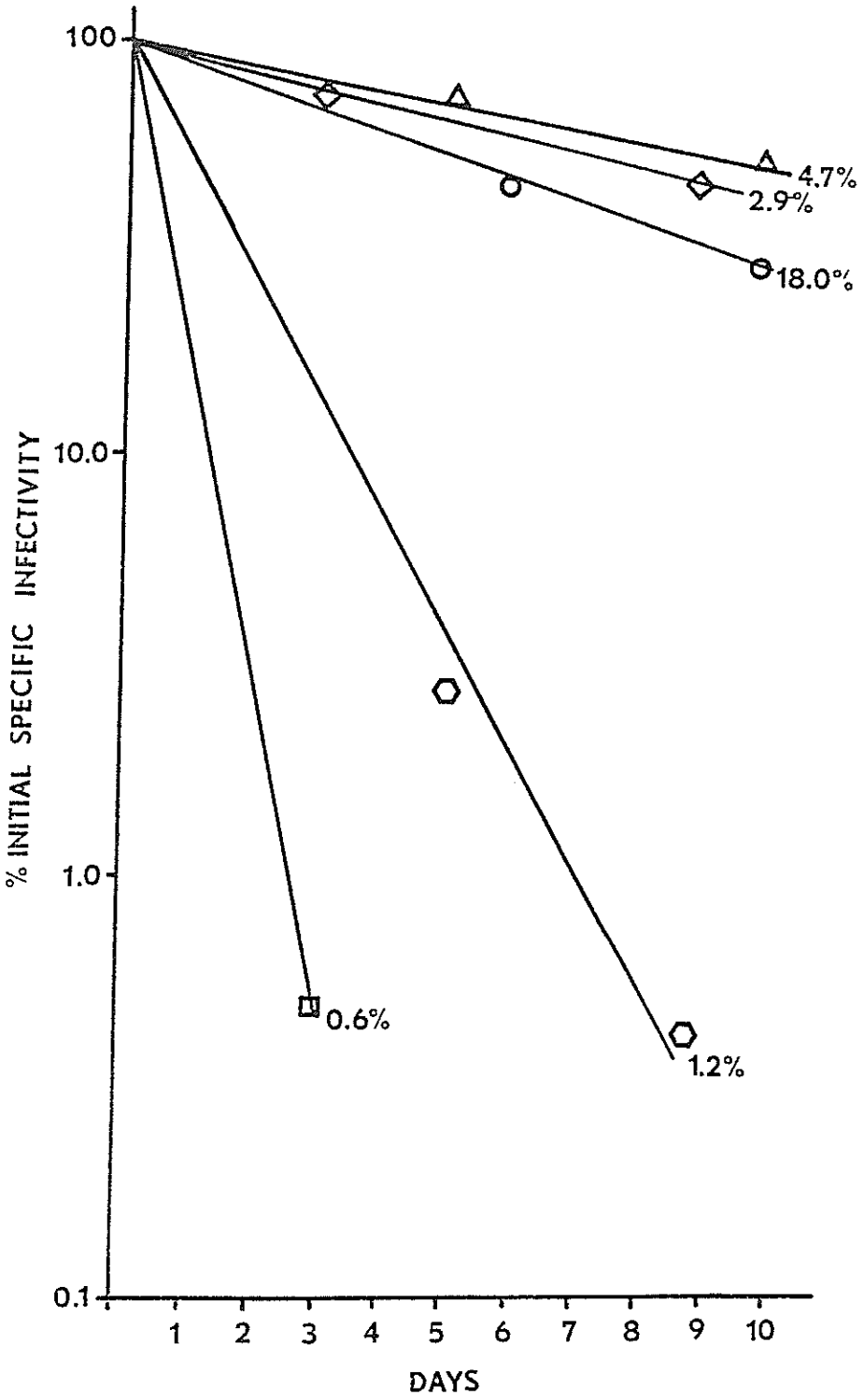

Figure 2. Inactivation of poliovirus type-1 at various soil moisture levels. Virus-seeded soil samples were dried in dessicators as in Figure 1. During the next 10 days, viruses were eluted from the soil samples held at the moisture levels indicated above. Eluates were assayed for pfu and radioactivity, and results are plotted as percent of the initial specific infectivity (pfu/cpm) for each soil sample.

only to evaporation, but also to the increased rates of virus inactivation at low soil moisture levels.

While studying the influence of the surface area to volume ratio on virus inactivation in liquids, Ward and Ashley (58) noted that virus inactivation rates increased when small liquid volumes were used. They suggested that this observation may have resulted from continuous evaporation within the confines of the sealed container as the small amount of liquid and the overlying air reached a saturation equilibrium. This possiblity cannot be ruled out in these experiments, and the increases in virus inactivation rates at very low soil moisture levels may have been due to continuous evaporation and redistribution of moisture within the sealed container. If this were the case, then virus inactivation in drying soils may have been entirely due to evaporation rather than increased inactivation rates in dewatered soil. The relative effects of evaporation and low soil moisture may be impossible to resolve with the techniques used here. However, in another experiment no poliovirus infectivity could be recovered from virus seeded soil which was dried in less than 1 h by a stream of sterile, dried air. This complete inactivation of viruses in a short period of time that would allow virtually no time for moisture equilibrium suggests that evaporation may be the primary factor responsible for virus inactivation in drying soil and supports the notion that continuous evaporation may have been responsible for the increased inactivation rates seen at low soil moisture levels.

Discussion

In agreement with the results of others (9, 16, 46) the findings presented here clearly show that the rate of virus inactivation in saturated soils is temperature dependent. The extended persistence of viruses in wastewater saturated soils at low temperatures indicates that viruses deposited in soils by septic tank effluents or by sewage application to land in cold seasons may remain infectious for long periods. Subsequent rainfall, irrigation, or effluent application could create conditions in the soil that would permit virus migration to underlying groundwaters. While temperature appeared to be the most important factor determining virus inactivation in these experiments, virus persistence was also influenced by soil type and the aqueous medium in which the viruses were applied to the soil. The effects of soil type and liquid medium on virus inactivation were most apparent at 22°C, a temperature typical of that found at a depth of three to 20 m for most of the year in southern New Mexico. Under these conditions saturated sandy loam was a less hostile environment for viruses than sandy soil. Additionally, viruses in soil saturated by septic tank liquor persisted longer than viruses in soils saturated with groundwater or Rio Grande water. The extended survival of viruses in septic tank liquor saturated soil at moderate temperatures is significant in that septic tank liquor is a medium in which viruses are likely to be discharged to the soil throughout the year. Wastewater and sewage sludges have also been shown to protect some viruses against inactivation under a variety of conditions (7, 44, 59).

While the influence of temperature on virus inactivation in saturated soils was apparent, perhaps more important was the relationship between temperature and the time required for soil dewatering by evaporation. Because no infectivity was recovered from dried soil, and because the rate of soil drying was temperature dependent, the main effect of temperature on virus survival under field conditions may be its influence on evaporation rates. It should be noted that although septic tank liquor protected viruses against inactivation under saturated soil conditions, this liquid afforded no protection against inactivation in drying soils. Although it was impossible to differentiate between virus inactivation due to evaporation and reduced soil moisture levels in these experiments, the results suggest that evaporation may be the primary cause of viral inactivation in drying soils and that a critical moisture threshold may exist. The enhancement of virus inactivation by evaporative dewatering was demonstrated by Ward and Ashley (58) in experiments with sewage sludge. They observed a large decrease in virus infectivity as the sludge solids content was increased from 65% to 83% by evaporation. Similar results were observed by Hurst et al. (52) with sewage sludge applied to land. Moore and Sagik (37) also noted that virus recovery from soil decreased as the soil moisture content was lowered by evaporation. However, they did not differentiate between decreased recovery efficiency and inactivation. The virucidal activity of drying may partially explain the reduced recovery of infectious viruses seen by others when soil was allowed to partially dry between virus applications (16, 24, 31). With the

exception of Ward and Ashley's (58) studies with sewage sludge, none of these studies made use of labeled viruses to provide a measure of virus recovery efficiency. Because of this, it would be difficult to differentiate between virus inactivation and irreversible soil binding in assessing the decrease in the recovery of infectious viruses. The studies reported here made use of RNA labeled viruses, and the results showed that RNA label was readily eluted from the soil. However, the changes in the specific infectivities of the eluates indicated that viruses were inactivated. While it is not always possible or even desirable to use labeled viruses in many experiments with soil or sewage sludge, the use of labeled viruses in laboratory or small-scale field experiments may provide important insights into the events associated with virus inactivation under similar field conditions.

Although solids-bound viruses have been shown to retain their infectivity (44), the results presented here do not eliminate the possibility that some soluble soil component eluted from the soil was masking the infectivity of the recovered viruses. The results in Chapter III of this thesis show conclusively that the viruses were inactivated due to irreversible damage in the soil environment.

CHAPTER III

STRUCTURAL CHANGES ASSOCIATED WITH POLIOVIRUS INACTIVATION IN SOIL

Introduction

The studies presented in Chapter II on the inactivation of enteroviruses in soil showed that the loss of virus infectivity in moist soil was dependent on soil temperature and, to a lesser extent, on soil type and the medium in which viruses were applied to the soil. Studies on virus inactivation in soils that were allowed to dry showed that no infectivity could be recovered from the soil regardless of soil temperature, soil type, or liquid amendment. Radioactively labeled viruses were used in these experiments, and the recovery of radioactivity from the soils suggested that viruses were being inactivated rather than irreversibly bound to the soil particles.

The possibility remained that the inability to recover virus infectivity was due to the masking of viral infectivity by some component eluted from the soil along with the viruses. This question can only be answered by examining the state of the viruses after they are inactivated in the soil environment. The studies reported here used capsid and RNA labeled viruses to examine the viral changes associated with poliovirus inactivation in soil under moist and dry conditions.

Materials and Methods

Viruses and cells. Poliovirus type-1 (Mahoney) was used throughout these studies. The viruses were propagated and assayed for infectivity as previously described (42). Virus preparations labeled with either [^{3}H] uridine or ^{14}C reconstituted protein hydrolysate were grown and purified as described elsewhere (56).

Inactivation of poliovirus in soil. For studies in moist soil, purified, radioactively labeled polioviruses were diluted and adsorbed to soil samples in sealed tubes. The tubes were incubated at 37°C and viruses were eluted at intervals with pH 11.5 glycine (0.1 M), EDTA (0.01 M), calf serum (1%) buffer. The eluates were neutralized with 0.1 N HCl prior to assay for infectivity and total radioactivity.

Poliovirus inactivation in dried soils was examined by adsorbing labeled viruses to soil in tubes as described above. Samples were placed in dessicators until an open sample was gravimetrically dry. Viruses were then eluted from the soils and assayed as before.

To prevent virus degradation by naturally occurring proteases or nucleases in the soil, a series of inactivation studies were done in washed, heat sterilized, sandy soil. Virus preparations were adsorbed to the soil as before except that viruses were eluted with sterile 0.1 M tris(hydroxymethyl)amino methane, 0.01 M EDTA (TE) buffer adjusted to pH 8.0.

Analysis of inactivated viruses and viral components. Ultracentrifugation analyses of viruses were done in 15 to 30% linear glycerol gradients containing 0.1 M NaCl, 0.01 M tris(hydroxymethyl)-amino methane, and 0.001 M EDTA (56). Virus samples were layered on the gradients which were then centrifuged at 30,000 rpm in an SW41 rotor for 3 h at 4°C. Radioactively labeled RNA was further analyzed by sedimentation in 5 to 30% glycerol gradients prepared as outlined above. These gradients were centrifuged in an SW65 rotor at 65,000 rpm for 1.5 h at 4°C. Fractions were collected from the bottom of all gradients and analyzed for radioactivity by scintillation spectrometry. The position of unaltered 156S viral particles and 35S RNA was determined by sedimentation in separate but identical gradients.

The conformation of the polioviruses was also analyzed by isoelectric-focusing according to the method of Korant and Lonberg-Holm (30). Approximately 75 µl of eluted virus was adjusted to 20% sucrose with a 40% sucrose solution containing 1% ampholite (BIORAD, pH 3 to 10) and layered onto 10 to 40% gradients (with 1% ampholite) at the position corresponding to 20% sucrose. Gradients were generated in 20 x 0.6 cm siliconized glass tubes sealed at one end with dialysis tubing. For electorphoresis, the lower reservoir contained 40% sucrose in 1% (vol/vol) sulfuric acid, and the top reservoir contained 2% (vol/vol) ethanolamine. Electrophoresis was carried out at a constant voltage of 250 V for approximately 5 h. Voltage was then increased in 50 V increments for periods of 15 min until a final voltage of 500 V was reached for the same period. Control samples of unaltered

and heated (56°C for 5 min) polioviruses were focused in separate gradients. Fractions were collected from the bottom of the tubes and aliquots were assayed for radioactivity. The pH gradients were determined after the addition of glass-distilled water to each fraction.

Results

Comparative recoveries of viral nucleic acids and proteins from polioviruses inactivated in moist and dried soils. Before attempting to look for possible alterations of virion components associated with loss of viral infectivity in soil, it was first necessary to be able to recover both viral protein and RNA from polioviruses "inactivated" in the soil environment. Previous studies showed efficient recovery of radioactivity from RNA-labeled viruses after incubation in moist and dry soils. Therefore, experiments were done to see if similar recovery could be obtained with viruses labeled in the protein capsids.

Capsid and RNA-labeled viruses were adsorbed to soil in sealed and open tubes which were incubated at 37°C. Viruses were eluted when the open sample had dried, and eluates were assayed for infectivity and radioactivity.

During the course of the experiment, the specific infectivity of the viruses recovered from the moist soil decreased about 3 orders-of-magnitude. The specific infectivity of the viruses in the dried soil decreased by more than 5 orders-of-magnitude during this same period. Upon measurement of the radioactivity recovered from the moist and dried soils, it was determined that both the RNA and protein portions of virions were recovered from moist soils (Table 9). However, only the RNA label was recovered efficiently from the dried soils. This result indicated that viral RNA and protein were dissociated after inactivation in dried soils. If this was the

Table 9. Recovery of RNA and capsid label from viruses incubated in saturated and dried soils[a].

Soil Type	% radioactivity recovered after 6 days			
	RNA label		Capsid Label	
	Moisture Content		Moisture Content	
	Saturated	Dried	Saturated	Dried
Sandy Loam	83.7	71.0	72.3	0.6
Sand	99.0	86.5	83.6	1.2

[a] Approximately 2 x 10^7 pfu (8,000 cpm) of labeled viruses were adsorbed to soil in sealed and open tubes. Tubes were incubated at 37°C and viruses were eluted when the open sample was dry.

case, and if the released RNA was inactivated, then inactivation in dried soil, at least, was irreversible. To test this hypothesis more detailed studies of virions inactivated in moist and dried soils were made.

Inactivation of poliovirus in moist soils. Because efficient recovery of both the capsid and RNA portions of polioviruses was obtained after inactivation in moist soils, the state of virion components after inactivation was first examined under that condition. For this study, RNA and capsid labeled virus suspensions were adsorbed to soil samples in sealed tubes. The samples were incubated at 37°C and viruses were eluted periodically for determination of infectivity and radioactivity. The specific infectivities of the eluted viruses (Fig. 3) decreased nearly 5 orders-of-magnitude during 15 days of this experiment.

Aliquots of the eluted viruses were subjected to sedimentation analysis in 15 to 30% glycerol gradients. The gradient profiles of the eluted RNA-labeled viruses in Fig. 4A show that RNA was released from the virions as they were incubated in the soil. After 15 d of incubation almost all of the [^{3}H] uridine label was released from the virions and was no longer sedimentable under these conditions. In Fig. 4B it can be seen that, concomitant with the loss of RNA from the virions, the capsid-associated radioactivity was sedimented as particles with sedimentation coefficients of about 80S which corresponds to the sedimentation coefficient of empty poliovirus capsids (4).

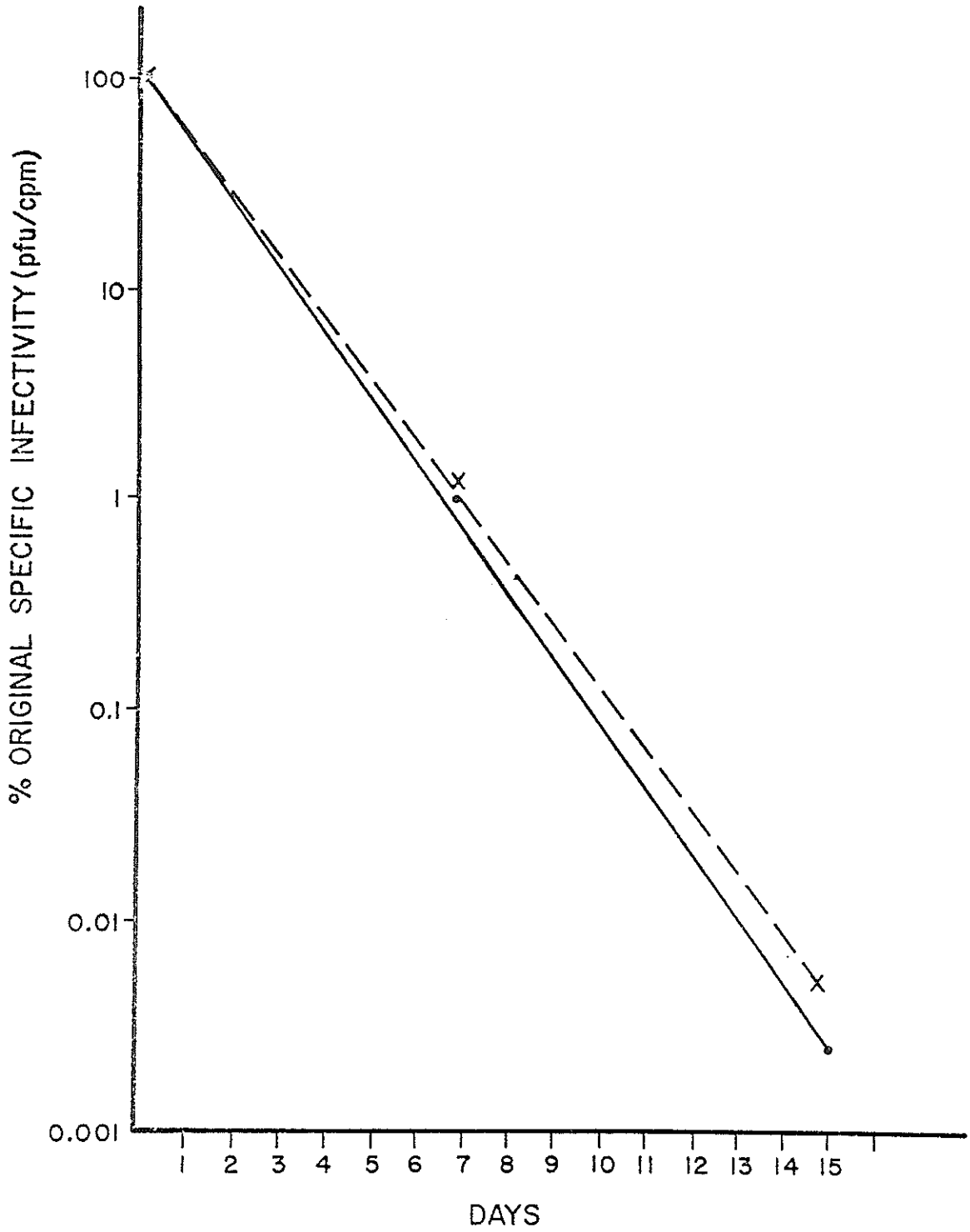

Figure 3. Inactivation of poliovirus in saturated soil at 37°C. Purified capsid-labeled (×) and RNA-labeled (●) suspensions of poliovirus type-1 were adsorbed to soil in sealed tubes. The viruses were eluted periodically for determination of recovered pfu and radioactivity from which the specific infectivity of the samples was calculated.

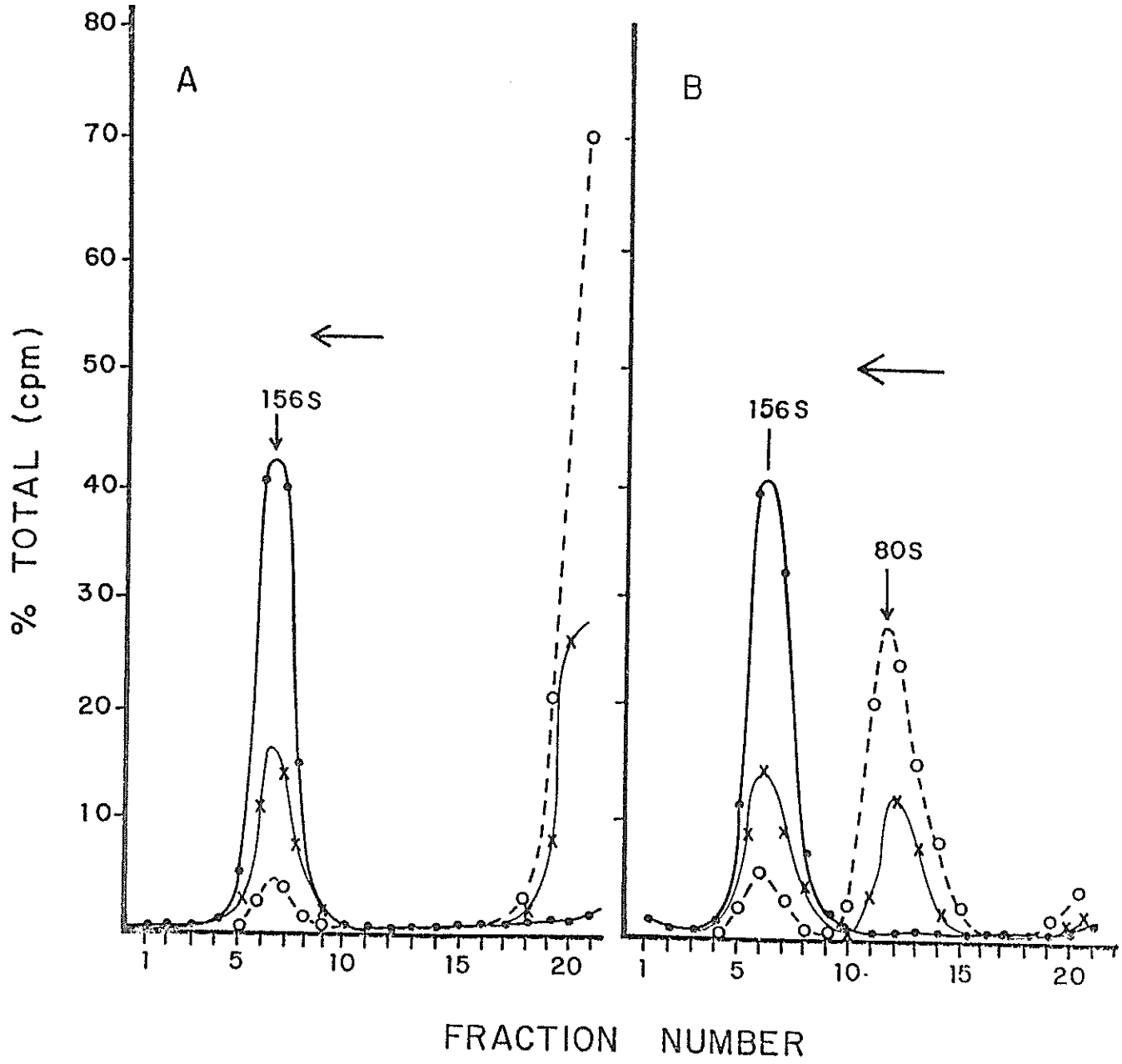

Figure 4. Glycerol gradient profiles of poliovirus particles after inactivation in saturated soil. Purified suspensions of RNA-labeled (A) and capsid-labeled poliovirus (B) were adsorbed to soil and incubated at 37°C. Initial samples were eluted (●), and samples were subsequently eluted on day 7 (×) and day 15 (○). The eluates were assayed for recoverable infectivity and analyzed by density-gradient centrifugation (15 to 30% glycerol, 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, SW41 rotor, 30,000 rpm, 3 h, 4°C). The arrow indicates the direction of sedimentation.

To determine the extent of degradation of the RNA released from the virions during incubation in moist soil, the total RNA associated label recovered from the soil after 15 d was further analyzed on 5 to 30% glycerol gradients. RNA extracted from infectious [^{3}H] uridine labeled polioviruses was used as a marker in a separate but identical gradient. These gradient profiles (Fig. 5) show that the RNA released from the virions in saturated soil was extensively degraded because its sedimentation coefficient decreased from 35S to less than 5S.

Because intact poliovirus RNA is known to be infectious, it was of interest to determine if the viral genomes were released intact into the moist soil during virus inactivation and then degraded by nuclease activity in the soil. To examine this possiblity, an identical experiment was done in which [^{3}H] uridine labeled polioviruses were suspended in sterile groundwater and adsorbed to autoclaved, washed soil. The viruses were eluted with sterile T.E. buffer adjusted to pH 8 rather than pH 11.5 to ensure that the RNA was not hydrolyzed during elution.

As shown in Fig. 6, most of the viral RNA was released in a degraded form which was found by subsequent analysis in 5 to 30% gradients to sediment at less than 5S. These results suggest that biological activity in the soil was not required for the degradation of viral RNA noted in the moist soil.

In summary, these results show that polioviruses lose their RNA genomes during incubation in moist soil. Furthermore, the extensive degradation of the released RNA indicates that irreversible damage

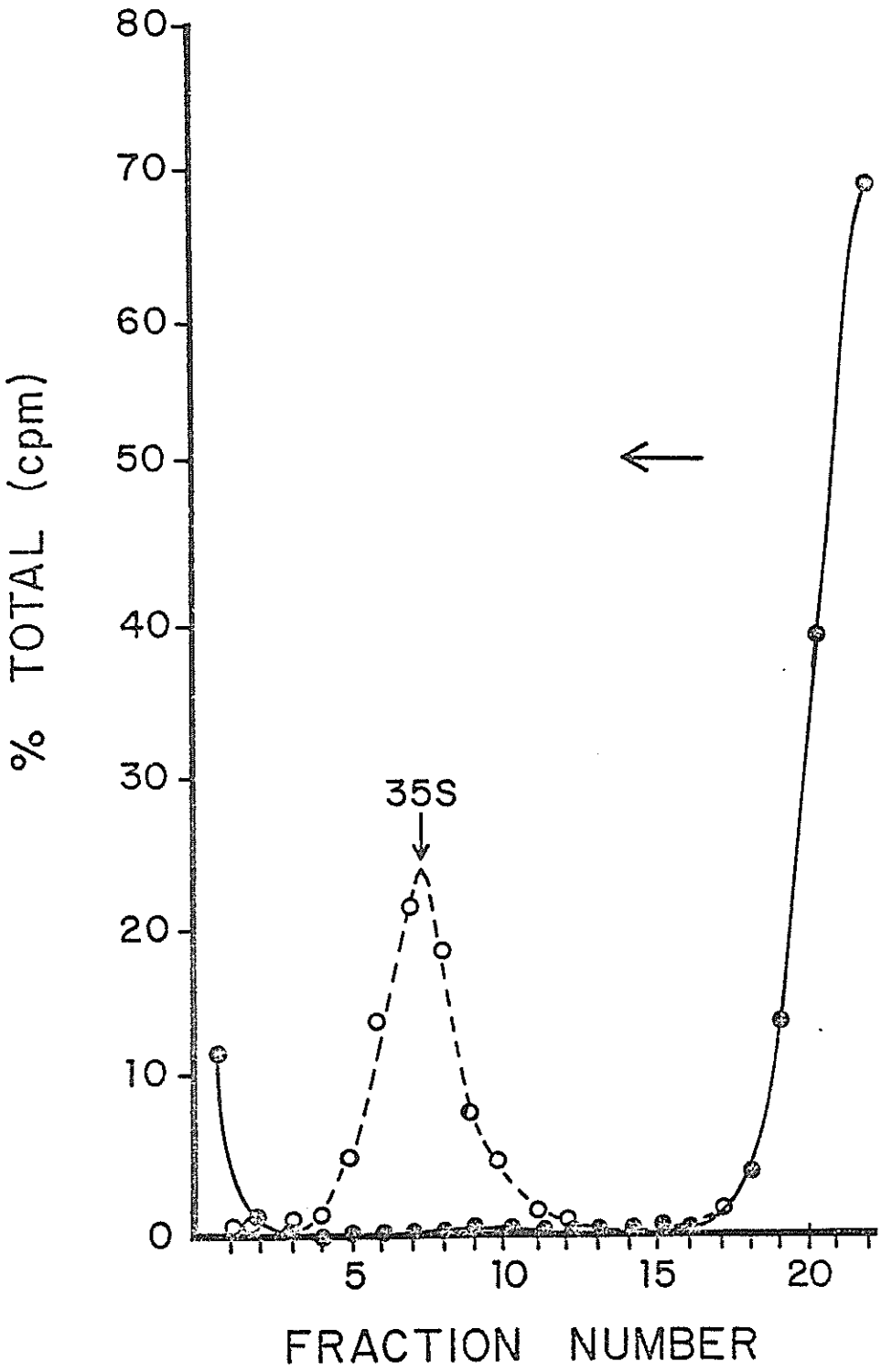

Figure 5. Glycerol gradient profiles of [^{3}H] uridine labeled RNA extracted from infectious polioviruses (o) and recovered from moist soil after 15 days of incubation at 37°C (●). The total RNA labeled material recovered as described in the legend for Figure 3 was analyzed by sedimentation in 5 to 30% glycerol gradients (SW65 rotor, 65,000 rpm, 90 min, 4°C). The arrow indicates the direction of sedimentation.

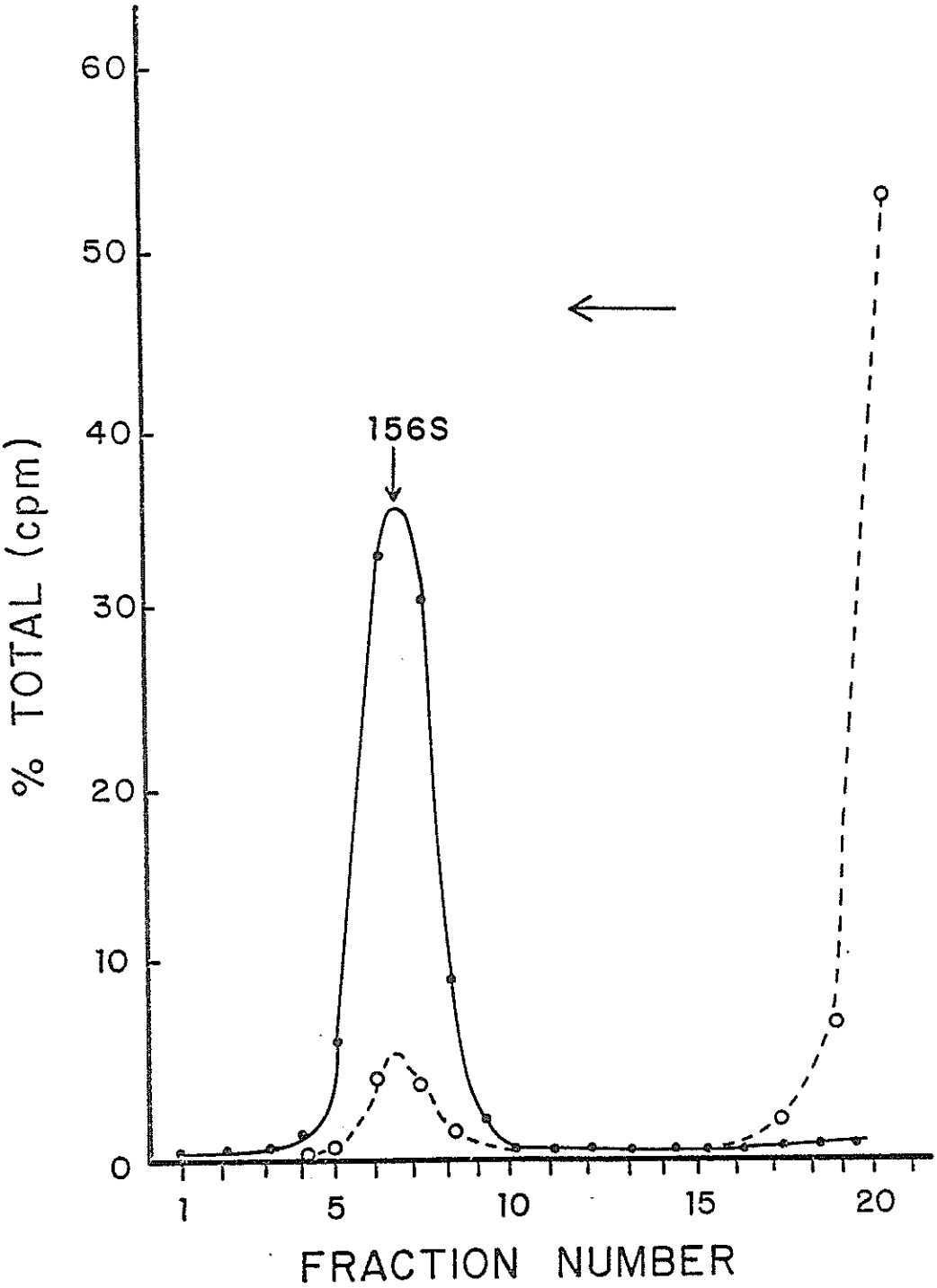

Figure 6. Density gradient profiles of [^{3}H] uridine labeled poliovirus eluted from sterile, moist soil. Viruses were adsorbed to sterile soil and eluted with T.E. buffer initially (●) and after 15 days of incubation at 37°C (○). Samples were analyzed in 15 to 30% glycerol gradients as described in the legend for Figure 4. The arrow indicates the direction of sedimentation.

to the virion, rather than masking of infectivity, was responsible for the decreased virus infectivity seen in moist soils.

Poliovirus inactivation in soils dried by evaporation. The results presented in Table 9 strongly suggested that poliovirus RNA and capsid proteins were dissociated as the viruses were incubated in the drying soil. Further studies were carried out to more closely examine the inactivated virions and to confirm this suggestion.

For these studies, labeled polioviruses were adsorbed to soil samples as previously described. An initial sample was taken and refrigerated without drying to serve as a control. The other samples were incubated in dessicators at 37°C. Viruses were resuspended in pH 11.5 buffer when the sample was gravimetrically dry (4 d), and eluates were assayed for infectivity and radioactivity.

As in the previous experiments with capsid labeled viruses, no infectivity was recovered from the dried soil samples. Additionally, so little capsid-associated radioactivity was recovered from the dried soil that further analysis was impossible. Attempts were made to recover the capsid-associated label from the soil by a variety of techniques, but only 1% of the radioactivity was ever recovered.

The gradient profiles of the viruses eluted before and after soil drying were compared in Fig. 7. As was suggested by the results shown in Table 9, these profiles clearly demonstrated that RNA was released from the virions as a result of inactivation in drying soil. Furthermore, all of the RNA was recovered.

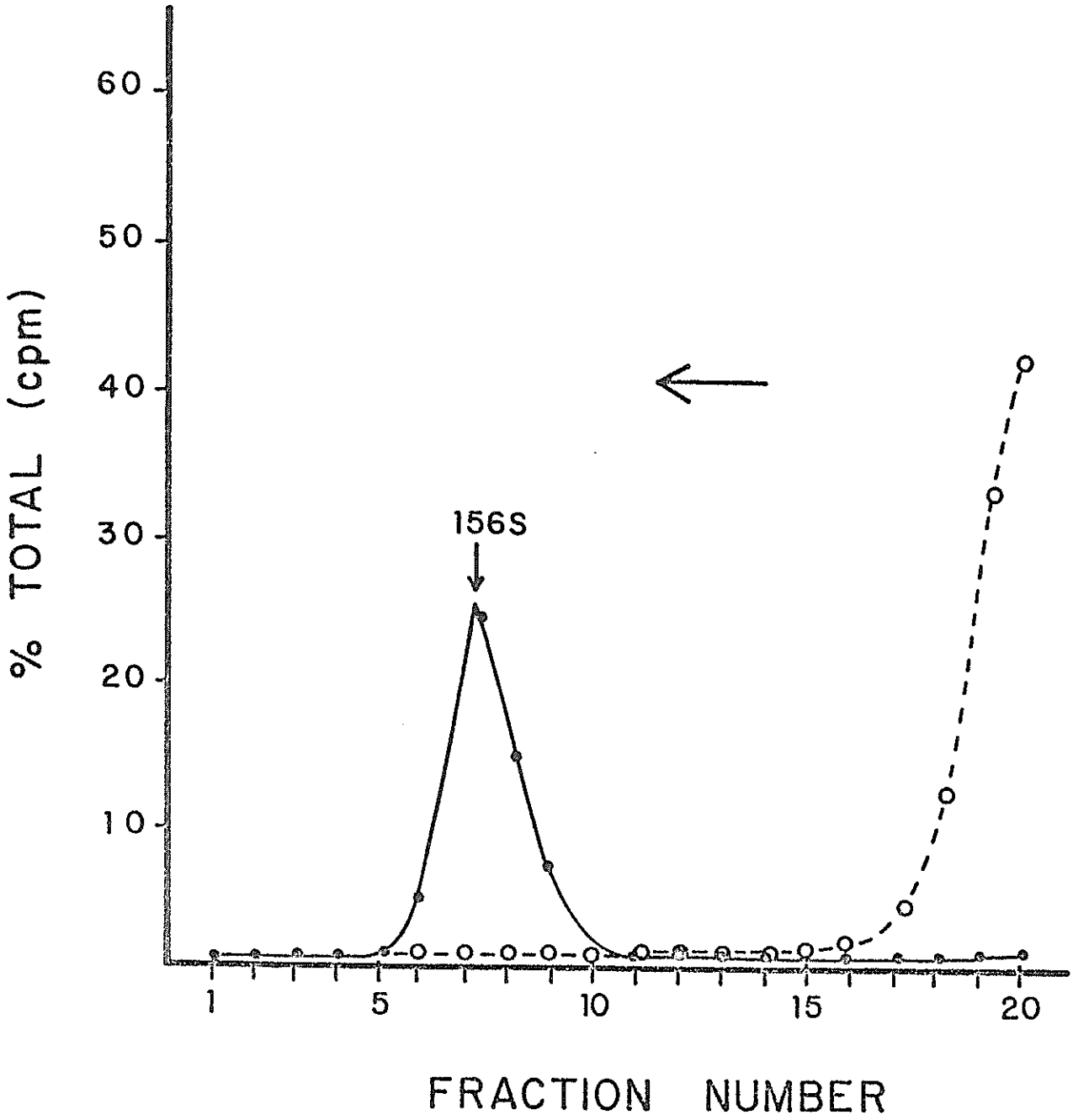

Figure 7. Density gradient analysis of [^{3}H] uridine labeled poliovirus recovered from dried soil. Viruses were adsorbed to soil and eluted initially (●) and after 4 days when an open sample had dried (o). Samples were analyzed by centrifugation in 15 to 30% glycerol gradients as described in the legend for Figure 4. The arrow indicates the direction of sedimentation.

The recovered RNA was further analyzed to determine the extent of degradation in the dried soil. The 5 to 30% gradient profiles (Fig. 8) showed that, similar to moist soils, the RNA was extensively degraded to fragments sedimenting at less than 5S. Thus, irreversible inactivation of polioviruses occurs in dried soils.

In an attempt to determine if virus inactivation in dried soil was accompanied by the release of intact or degraded RNA molecules from the poliovirus capsids, the preceding experiment was repeated under the sterile conditions previously described. As found after drying in non-sterile soil, no radioactivity remained in the 156S peak (Fig. 9). However, the released RNA did not remain at the top of the gradient as was the case in the non-sterile soil (see Fig. 7). Analysis of the RNA eluted from sterile soil on 5 to 30% glycerol gradients showed that most of the RNA sedimented as a homogenous peak corresponding to the position of marker 35S RNA extracted from infectious polioviruses (Fig. 10).

The results obtained from these experiments in dried soil clearly showed that irreversible inactivation of polioviruses resulted from the drying of the viruses in soil. As in moist soil, degraded RNA was recovered from dried, non-sterile soil. However, in contrast to the results found after inactivation in moist soils, most of the RNA recovered from sterile, dried soil sedimented near 35S, suggesting that the mechanisms of inactivation may be dissimilar under the two conditions.

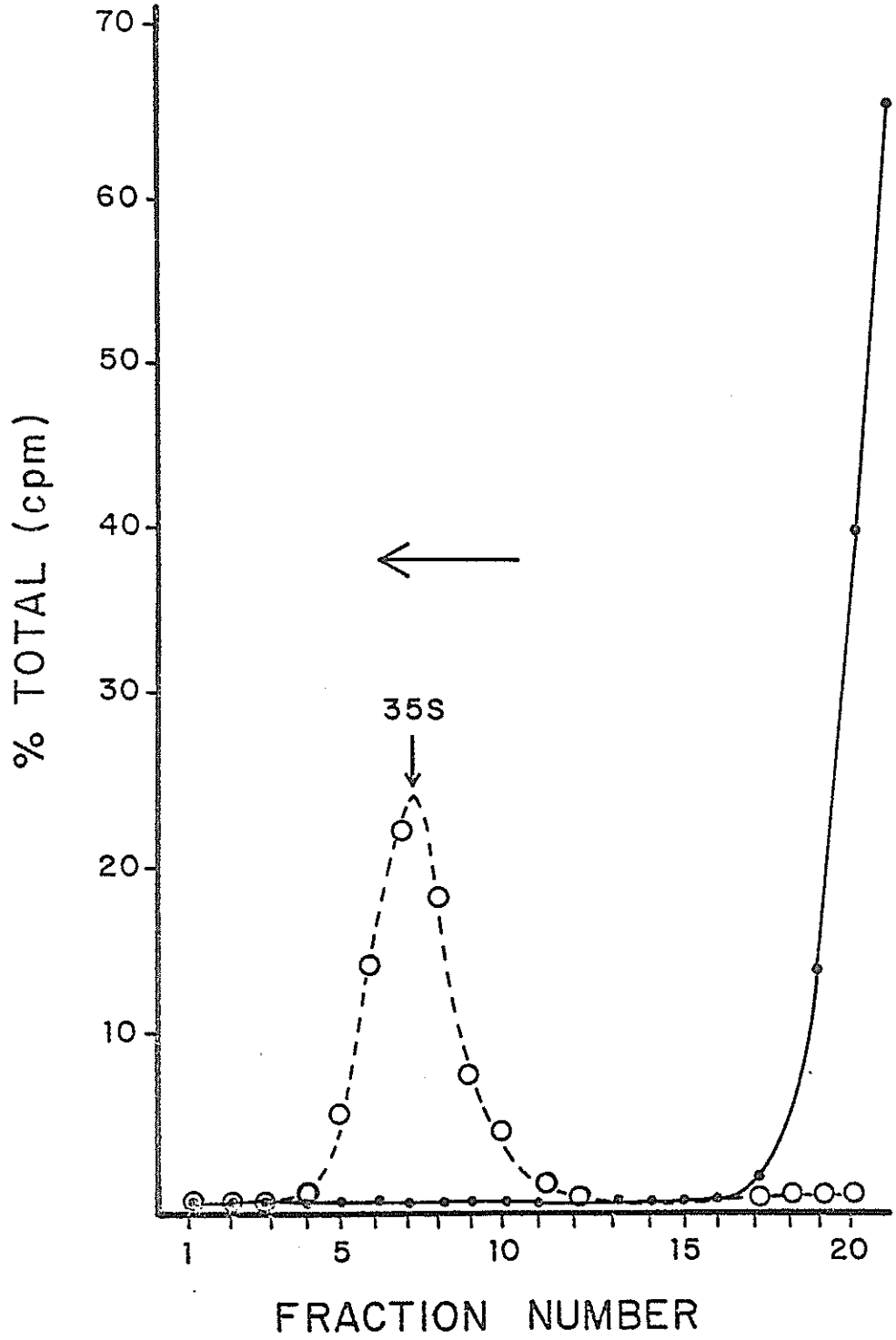

Figure 8. Glycerol gradient analysis of [^{3}H] uridine labeled virus components recovered from dried soil after 4 days of incubation. The labeled RNA material described in Figure 7 (●) was further analyzed by centrifugation in 5 to 30% glycerol gradients as outlined in the legend for Figure 5. Labeled RNA extracted from infectious viruses was used as a 35S marker (O). The arrow indicates the direction of sedimentation.

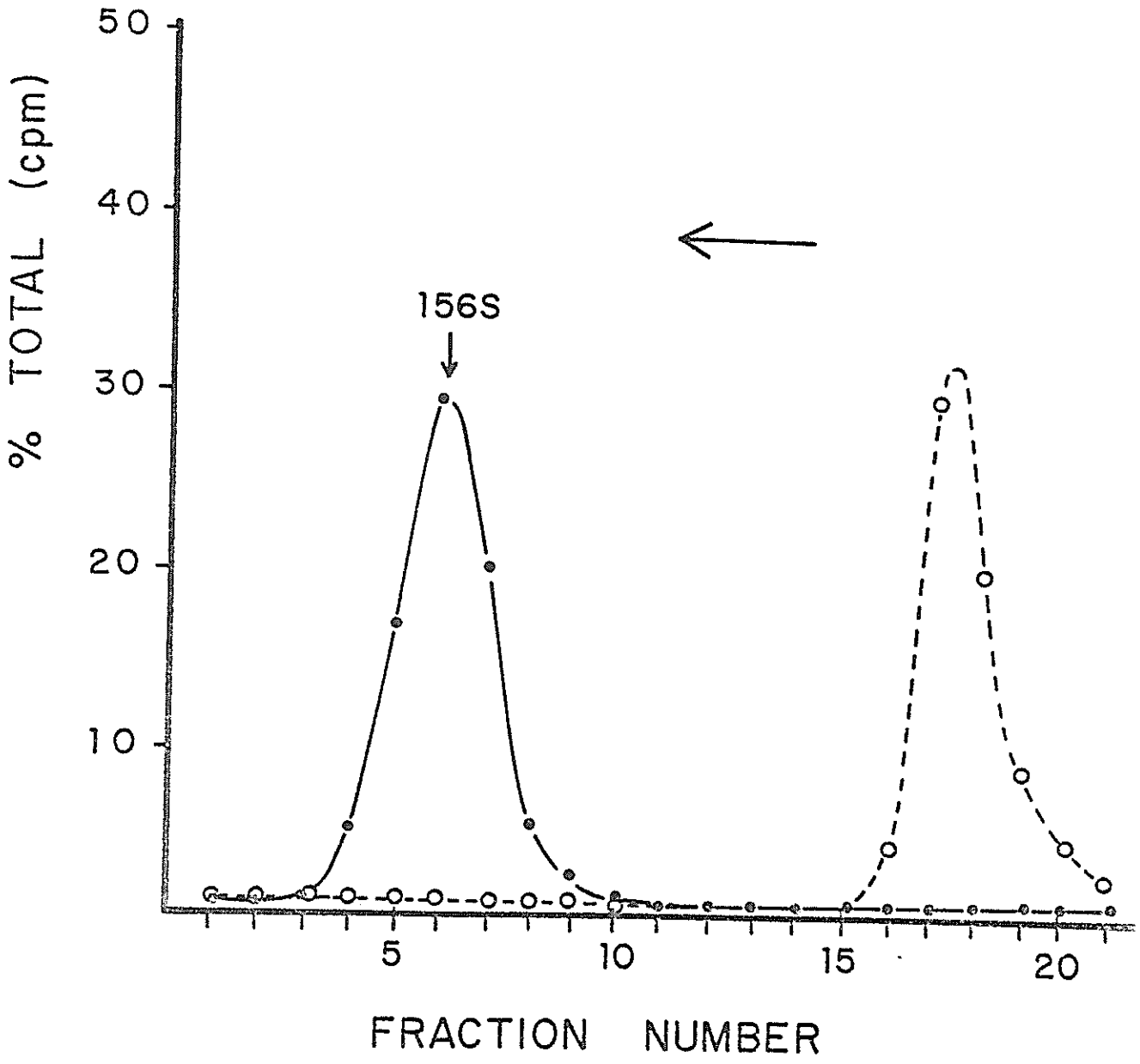

Figure 9. Effect of dessication on the sedimentation of [^{3}H] uridine labeled poliovirus. Viruses were adsorbed to sterile soil and eluted initially (●) and after drying (○) with T.E. buffer. The samples were centrifuged in 15 to 30% glycerol gradients as previously described. The direction of sedimentation is indicated by the arrow.

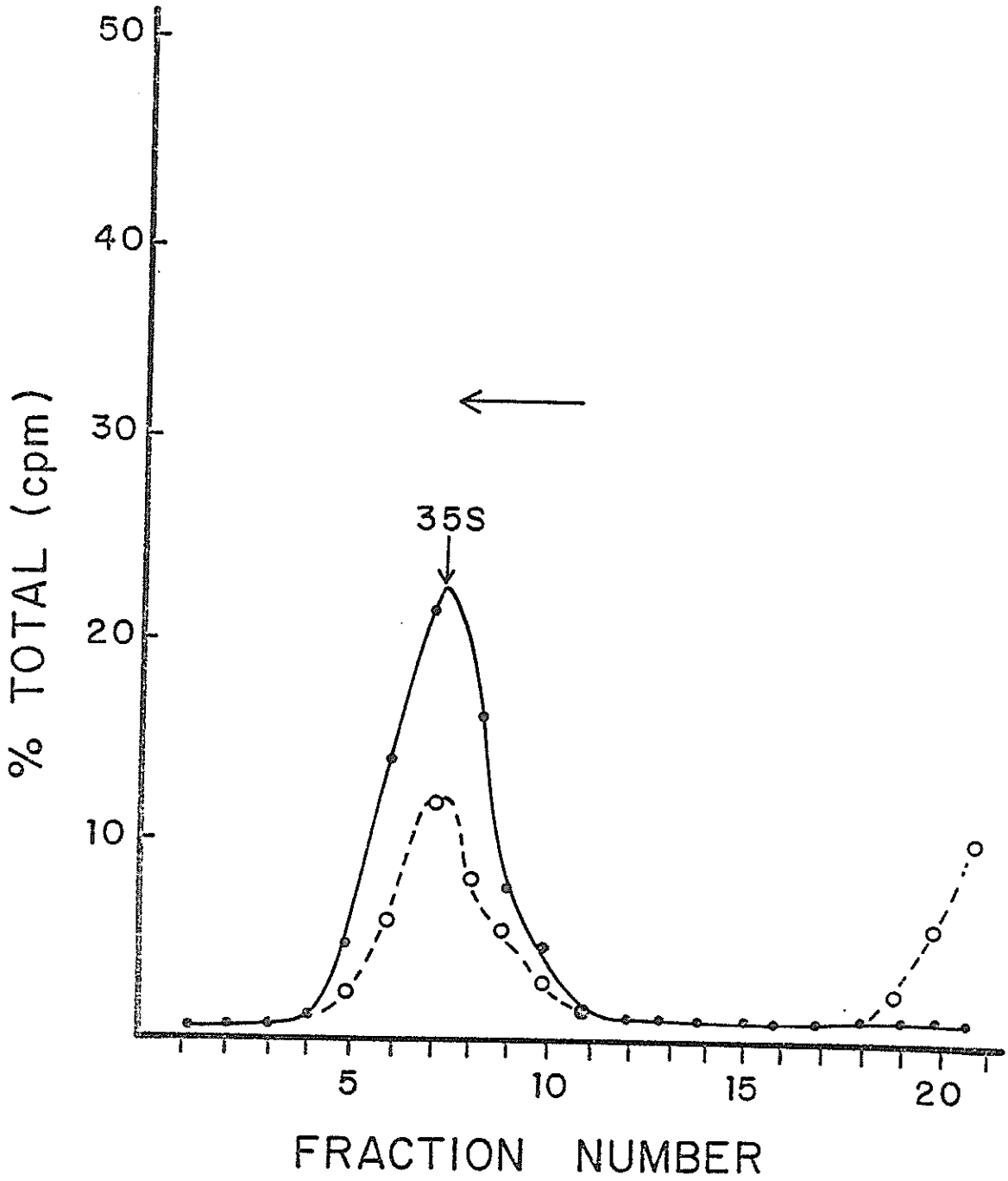

Figure 10. Gradient analysis of [^{3}H] uridine labeled RNA recovered from sterile dried soil. The RNA labeled material eluted as described in the legend to Figure 9 (o) was further analyzed by sedimentation in 5 to 30% glycerol gradients. Phenol extracted [^{3}H] uridine labeled RNA was used as a marker (●). The arrow indicates the direction of sedimentation.

Examination of capsid changes associated with drying of poliovirus. Because previous experiments showed that irreversible binding of poliovirus capsids to soil occurred during the drying process, it was impossible to study the capsid changes in the soil environment. In order to mimic the soil environment as closely as possible and to determine if some soluble soil component was responsible for conversion of poliovirus capsids to a conformation that led to irreversible binding, studies were carried out in soil extracts. These experiments were done to answer three questions: 1) can viruses be recovered following drying, 2) what capsid changes have occurred as a result of the drying process, and 3) are the adsorption properties of the capsids altered in such a way that they irreversibly bind to soil?

A soil extract was prepared by mixing 100 g of soil with 100 ml of distilled water for 1 h. The soil suspension was centrifuged to remove soil particles and filtered through 0.45 µm cellulose acetate filters. Capsid labeled polioviruses were then suspended in the soil-filtrate and rapidly evaporated to dryness with a stream of sterile dry air in order to avoid most of the time-dependent effects that could influence the course of virus inactivation. The residue was resuspended in glass-distilled water and aliquots were examined for recovered radioactivity and infectivity.

No infectivity was recovered and approximately 50% of the added radioactivity was recovered from the soil filtrate residue. The resuspended material was further examined to characterize changes in sedimentation characteristics and capsid conformation resulting from the drying process.

The resuspended material was layered onto 15 to 30% glycerol gradients to measure any alterations in sedimentation characterstics in the dried viruses. A preparation of [^{3}H] uridine labeled poliovirus was used to mark the 156S sedimentation coefficient of native polioviruses, and the 80S position of empty poliovirus capsids was marked with a heated (56°C for 5 min) ^{14}C-capsid labeled poliovirus preparation (4). The gradient profiles (Fig. 11) demonstrate that the viruses dried in the soil filtrate sedimented in the same 80S location as the heated virus marker. Thus, the drying of the viruses in the soil filtrate apparently led to the loss of viral RNA as noted in the experiments in drying soil. Additionally, it appeared that no extensive capsid degradation occurred as a result of the drying procedure.

To further examine the 80S particles produced during the drying of the soil extract, the resuspended viruses were analyzed by isoelectric-focusing using the technique of Korant and Lonberg-Holm (30). A reference preparation of labeled poliovirus was also focused (Fig. 12A) to demonstrate the bimodal isoelectric pH distribution described for polioviruses by Mandel (35). The dried viruses stabilized at an isoelectric pH near 4.5 (Fig. 12C) along with the poliovirus suspension heated to 56°C for 5 min (Fig. 12B). These results indicated that major conformational changes in poliovirus capsids accompanied the conversion of the particles to 80S in the dried soil extract.

In order to determine whether the conformational alterations noted in the previous experiments could result in altered binding of

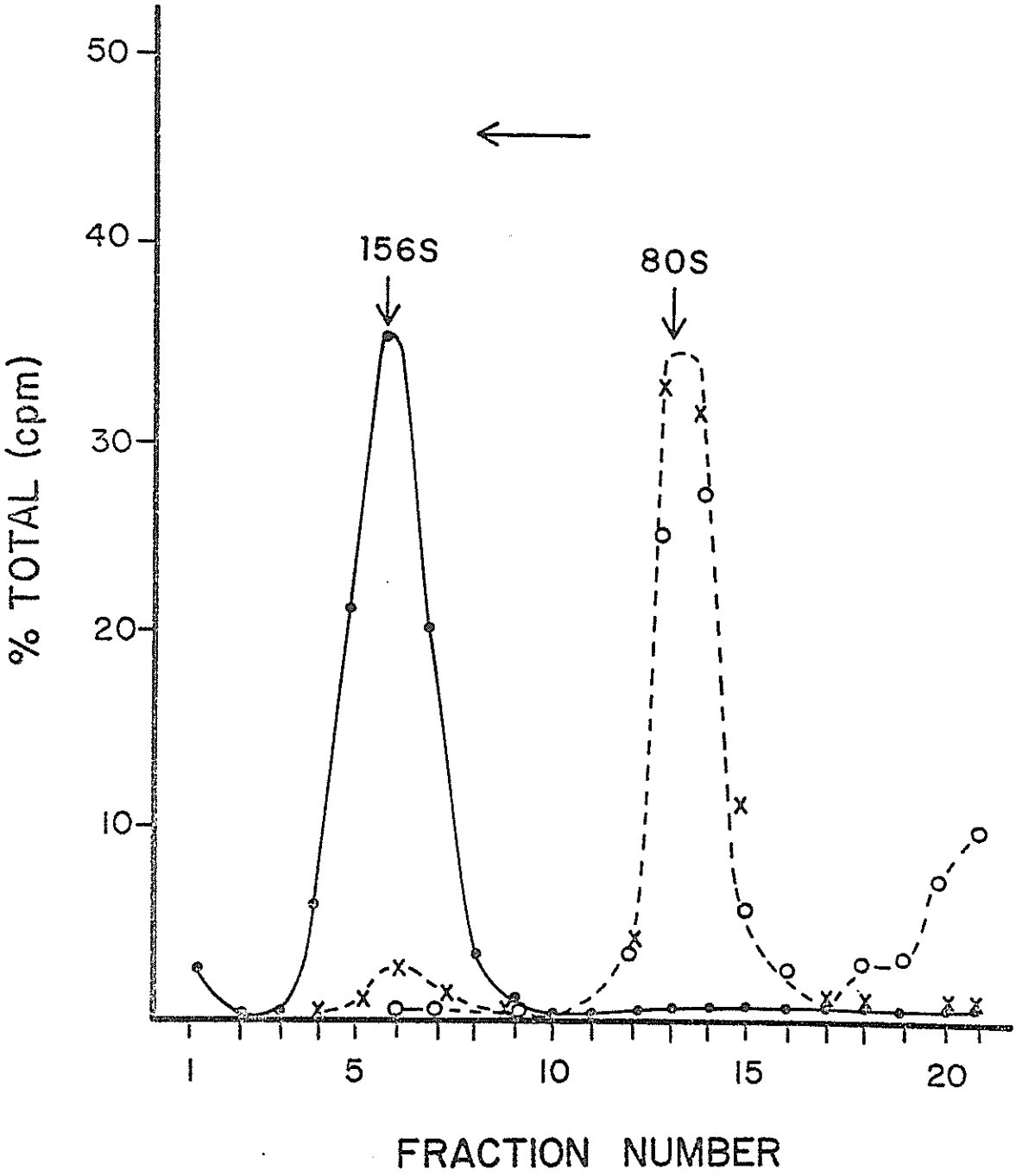

Figure 11. Effect of dessication on the sedimentation coefficient of poliovirus type-1. Purified ^{14}C capsid-labeled poliovirus was suspended in soil extract and either heated to 56°C for 5 min (×) or evaporated to dryness and resuspended in distilled water (o). These samples and an untreated control preparation (●) were analyzed by density-gradient centrifugation as described in the legend to Figure 4. The arrow shows the direction of sedimentation.

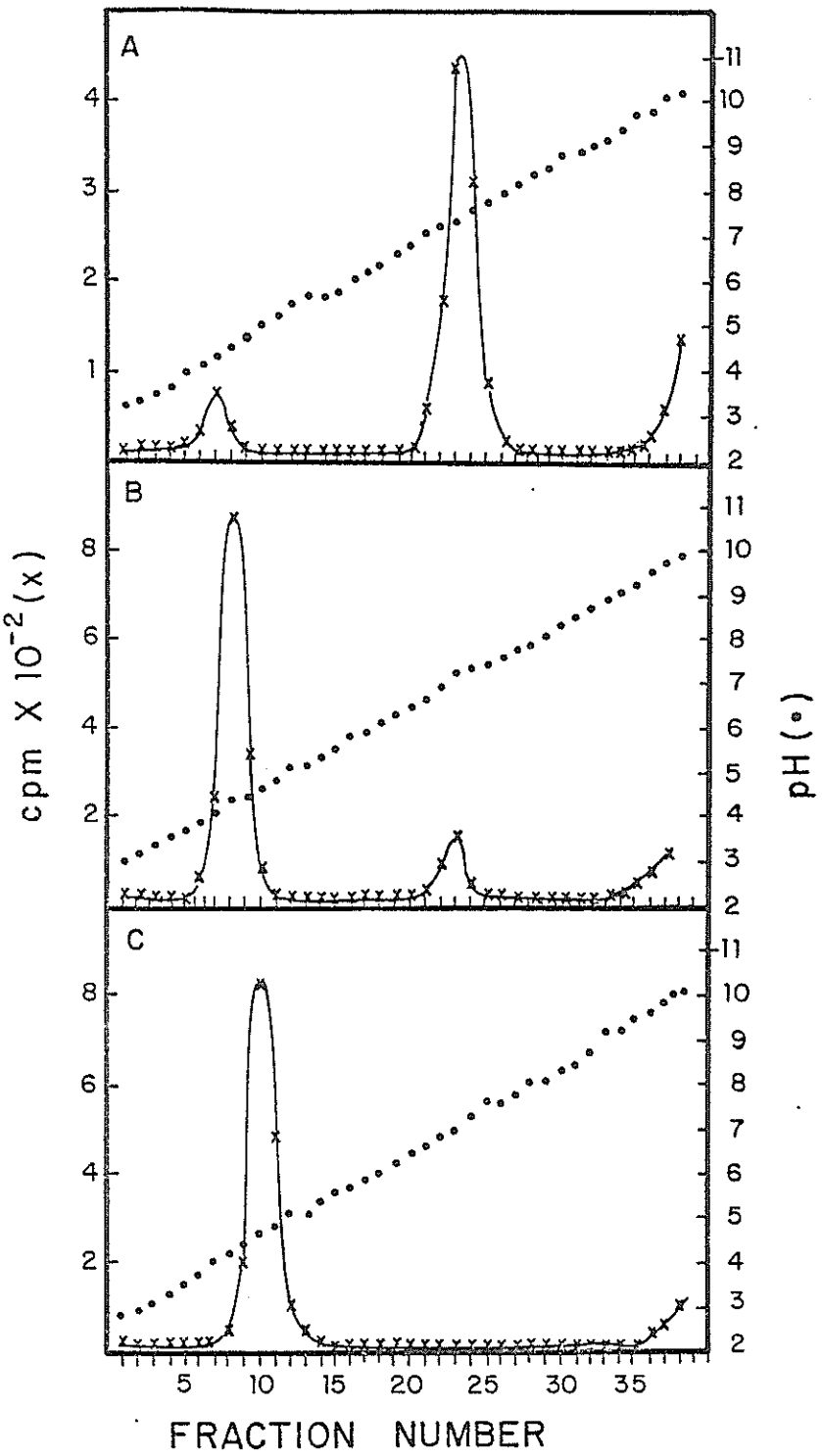

Figure 12. Effect of dessication on the isoelectric-point of poliovirus-1. Purified ^{14}C capsid labeled poliovirus diluted in soil-extract was either heated at 56°C for 5 min (B) or evaporated to dryness and resuspended (C). The samples, along with an untreated control (A) were analyzed by isoelectric-focusing in sucrose gradients.

the poliovirus capsids to soil, aliquots of untreated, heated and dried-resuspended viruses were adsorbed to soil in 5% soil suspensions. After mixing for 1 h, the soil particles were removed by sedimentation at 8,000 x g, and the supernates were assayed for radioactivity. The results presented in Table 10 show that over 99% of the radioactivity was adsorbed to soil from the untreated and heated virus preparations while approximately 94% of the label was absorbed from the dried, resuspended preparation. The soil pellets were then eluted with pH 11.5 glycine, EDTA, calf serum buffer as previously described. This elution resulted in the recovery of 82.3% of the adsorbed radioactive label from the soil treated with the dried preparation compared to greater than 90% elution of label from the soils treated with the other virus suspensions. Thus, drying of viruses in soil extract did not lead to a significant alteration in the adosrption properties of the dried virions to moist soils.

These experiments, simulating the drying of viruses in soil, have demonstrated clearly that major structural and conformational changes were associated with drying in soil filtrates. While slightly decreased recovery of the dried preparations from soil pellets was noted in the last experiment, the magnitude of this difference was insufficient to explain the lack of recovery of viruses noted in earlier experiments in which viruses were dried in the presence of soil. Also, this series of experiments indicated that no soluble component of soils was sufficient to alter the adsorptive properties of the virions during drying. Even though the

Table 10. Effects of heating and dessication on the adsorption of poliovirus to soil[a].

Virus Treatment	cpm added	% cpm adsorbed	cpm eluted	% adsorbed cpm recovered
Untreated	26,586	99.6	25,559	96.5
Heated	19,125	99.7	17,370	91.0
Dried	32,669	94.1	25,301	82.3

[a] Capsid-labeled poliovirus type-1 was suspended in groundwater and either heated to 56°C for 5 min or dried with sterile, dry air. The dried sample was resuspended in distilled water, and it, along with the heated and control virus preparations, was adsorbed to soil in 5% suspensions. After 1 h, the suspensions were centrifuged to sediment soil particles. The supernatant was assayed for radioactivity and the adsorbed cpm were eluted with pH 11.5 buffer.

nature of the capsid changes leading to irreversible binding of viruses to soil is not clear, it appears that drying in the presence of soil may be a necessary condition.

Discussion

The results presented in Chapter II showed decreases in virus infectivity in soils under both moist and dry conditions. The experiments discussed in this Chapter addressed the molecular events that accompanied this apprent inactivation in soils. These results clearly showed that under natural, unsterile conditions, polioviruses were inacitvated in moist and dry soils by virtue of irreversible d-mage to virions. This damage included the dissociation of viral components and the degredation of viral RNA.

Further analysis of the events accompanying virus inactivation in moist soils showed that, with both natural and sterile conditions, the viral RNA lost from viral capsids was recovered from the soils in a degraded form. This result suggested that the poliovirus RNA was damaged prior to its release from the capsid. The exact cause of this RNA degradation is not clear, but the results obtained in sterile, moist soil suggest that no biological nuclease activity is responsible. These observations are consistent with Dimmock's (14) hypothesis that, at "low" temperatures, viruses are inactivated by damage to viral RNA rather than by damage to viral protein that occurs at elevated temperatures. Even though the soil used in these experiments was sterilized by autoclaving, it is possible that the degradation of viral RNA was due to nuclease activity present in the virus preparation after purification. It has been suggested that nuclease activity may be an integral part of intact picornaviruses (13, 18), and it is possible

that this putative nuclease activity was stimulated by conditions in the moist soil. While it was not possible to differentiate between these two hypotheses in these experiments, the results obtained in sterile, dry soil suggested that if nuclease activity was associated with the virus preparation, it was poorly expressed under the drying conditions.

In dried, non-sterile soils the results were very similar to those noted in moist soil. Complete loss of viral infectivity resulted from soil drying, and the RNA recovered from these dried soils was degraded in a manner similar to that in moist soils. However, when viruses were dried in sterile soil, most of the RNA genomes were released and recovered as intact molecules. This result suggests that different mechanisms of virus inactivation are operating in moist and drying soils. The release of intact RNA from virions described here is similar to the results obtained by deJong and coworkers (10, 11, 12) when several picornaviruses, including poliovirus, were inactivated in aerosols held at different relative humidities. Similarly, Ward and Ashley (58) noted that polioviruses released their genomes during evaporation in distilled water. Examination of their data suggests that the released RNA may be sedimentable in a manner similar to that observed in these studies. The irreversible binding of poliovirus capsids that occurred as a result of drying in the soil environment is still unexplained, but the results reported here indicate that the binding only occurs when the virions are dried in the presence of soil.

In view of the proceeding discussion suggesting different virus inactivation mechanisms in moist and dry soils, it is tempting to speculate that two general mechanisms of picornavirus inactivation may occur in the environment. In the first mehcanism, virus inactivation occurs under saturated, temperate conditions as a result of RNA damage as suggested by Dimmock (14). The dissociation of virions into intact RNA and isoelectrically altered capsids noted in dried soil also appears to occur in aerosols (10, 11, 12) and under a variety of other physical stresses such as heat, ultraviolet irradiation, and dessication (35). These observations suggest that this may be a second general type of environmental virus inactivation mechanism.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The results presented in Chapter II of this paper show that enteric viruses can persist for extended periods in Mesilla Valley soils under laboratory and simulated field conditions. Thus, any viruses entering these soils from septic tanks or other wastewater discharge have the potential for surviving long enough to be translocated through the soil to the groundwater aquifer and to wells or irrigation drains. The actual hazard posed by these viruses would depend on site-specific conditions such as distance to groundwater, groundwater flow-rate, and the lateral distance to wells or receiving drains. However, reports showing lateral movement of viruses for distances up to 600 feet from the point of application, indicate that, under proper conditions in the groundwater aquifer, viruses may be transported for considerable distances (46).

The experiments described in Chapter II also support the prior observations of other researchers who have shown that virus persistence in soil depends largely upon soil temperature and moisture, and to a lesser extent on such factors as virus type, soil type, and the medium in which viruses are applied to soil (3, 22). Further, these studies have demonstrated the existence of a critical soil-moisture threshold. When soil moisture content dropped below this threshold, the rate of soil associated virus inactivation was markedly accelerated. The demonstration of this moisture threshold supports the results of others who have noted that virus recovery from soil is decreased with

a lowering of the soil moisture content by evaporation (1, 16, 27, 37). These studies differ from those just cited because radioactively labeled viruses were used to show that the decreased recovery was due to virus inactivation rather than to the inability to recover viruses from the soil.

The research presented in Chapter III demonstrates conclusively that the loss of virus infectivity in soils noted in Chapter II was due to inactivation of the virions in the soil environment rather than to irreversible soil binding. In moist soils, virus inactivation appeared to result from irreversible damage to viral RNA while the genome was still associated with the virus capsid. Results in sterile, moist soil reinforced the notion that the genomes were damaged while still capsid-associated. Additionally, the results obtained in the steril, moist environment suggested that no biological activity was required for the RNA degradation. The causative agent(s) of this activity is not known, but these results are consistent with Dimmock's (14) hypothesis that picornaviruses are inactivated at low temperatures by damage to their RNA genomes. He further hypothesized that virus inactivation at high temperatures in due to irreversible damage to viral structural proteins.

The experiments on virus inactivation in drying soil showed that the viruses were inactivated due to irreversible damage in the drying soil. As in moist soils, the damage in drying soil included the loss of viral RNA. However, the experiments in sterile, drying soil indicated that intact and presumably infectious RNA genomes were released from the virus capsids. In non-sterile drying soil this

this released RNA was rapidly degraded by nuclease activity in the soil. Thus, under natural conditions the risk from this infectious RNA that is released into the soil upon drying would probably be small. Poliovirus capsids became tightly bound to the soil particles as a result of the drying process, and characterization of the molecular events associated with this pehnomenon was impossible. However, studies on virus drying in soil extracts showed that capsid alterations resulting from the drying process slightly altered the soil-binding characteristics of the viruses. The magnitude of these changes was insufficient to explain the nearly total lack of recovery seen when viruses were dried in the soil. However, the data clearly indicate that drying in close proximity to soil particles may be a necessary condition for this irreversible binding pehnomenon.

The results presented here along with the results of other investigations, predict extended survival of enteric viruses in moist soils. The demonstration of a soil-moisture critical threshold associated with enhanced virus inactivation in drying soils suggests that any virus hazard associated with the application of wastewater or sludge to agricultural land can be greatly reduced or eliminated by allowing the soil to dry for a specified period of time after waste application.

More field studies are needed to more clearly define the relationship between soil moisture content and virus inactivation under natural conditions. The persistence of viruses in moist soils poses a potential health hazard in areas where septic tanks or other wastewater sources discharge to soil overlying shallow groundwater

tables. The potential for virus movement from septic tanks or other waste sources through to soil to irrigation or domestic wells deserves continued investigation.

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ENTEROVIRUS INACTIVATION IN SURFACE WATER, GROUNDWATER, AND SOIL

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TECHNICAL COMPLETION REPORT

Project No. A-052 NMEX

New Mexico Water Resources Research Institute
in cooperation with
Department of Biology
New Mexico State University
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Contents of this publication do not necessarily reflect the views and policies of the United States Department of the Interior, Office of Water Research and Technology, nor does mention of trade names or commercial products constitute their endorsement or recommendation for use by the United States Government.

INTRODUCTION

The research accomplished on this project has been described in the technical reports and open literature publications listed below and the reader is referred to these reports for detailed discussion of methods and results.

"A Comparison of Inactivation of Bacteriophage and Enterovirus During Activated Sludge Treatment." 1976: Report No. 079.*

"Enterovirus and Bacteriophage Inactivation in Subsurface Water and Translocation in Soil." 1977: Report No. 083.*

"Survival of Enteroviruses in Septic Tanks and Septic Tank Drain Fields." 1979: Report No. 108.*

"Enterovirus Inactivation in Soil and Structural Changes Associated with the Inactivation of Soil-Bound Viruses." 1979: Report No. 111.*

"Inactivation of Polioviruses and Coxsackieviruses in Surface Water." Appl. Environ. Microbiol. 33: 334-340. 1977.

"Enterovirus Inactivation in Soil." Appl. Environ. Microbiol. 38: 694-701. 1979.

"Structural Changes Associated with Poliovirus Inactivation in Soil." Appl. Environ. Microbiol. 38:702-709. 1979.

"Structural and Compositional Changes Associated with Chlorine Inactivation of Polioviruses." Appl. Environ. Microbiol. 38: 1034-1039. 1979.

"Inactivation of Bacteriophage and Enterovirus During Activated Sludge Treatment." Water Research. In Press 1980.

It is evident from the titles listed above that the investigation was broadly based. This report will briefly summarize the findings of each segment of the research and where appropriate problems on viruses in the environment which warrant future study will be indicated.

* Available from New Mexico Water Resources Research Institute, P.O. Box 3167, NMSU, Las Cruces, New Mexico 88003.

VIRAL INACTIVATION IN WATER

More than 100 viruses are excreted by man. Treatment of wastewater containing human waste will remove up to 90 percent of the viruses; however, in the absence of a disinfection stage such as chlorination or ozonation, substantial numbers of infective viruses are released into water and soil receiving wastewater. Thus, information on the fate of viruses in water and soil is needed in order to properly assess potential public health hazards.

Studies supported by this project included extensive in situ and laboratory experiments on virus inactivation in surface and groundwater. The results of these studies are described in detail elsewhere (4,5). However, briefly summarized, the results showed that viral persistence in the water environment was highly variable. Among the decisive environmental factors which affected virus inactivation were water type, water temperature, and dissolved solids. With respect to water type, viruses generally were inactivated more rapidly in surface water than in groundwater and seawater was generally more viricidal than freshwater. The rates of inactivation were more rapid as the water temperature was increased regardless of the nature of the water in which the viruses were suspended. The effects of dissolved solids on virus inactivation are less clear cut. The data shown in Table I illustrate the effects of dissolved solids on virus inactivation in water samples taken from various locations in the southern United States. Further evidence that dissolved solids enhance virus inactivation is shown in Table 2 where it can be seen that artificial seawater was progressively less viricidal when diluted with distilled water.

TABLE 1

Comparison of virus inactivation rates in water samples taken from various locations in the U.S.[1]

Location	pH	spc[2]	conduct. mmhos	log/redn time[3] days
Sterile glass dist. H_2O	5.2	0	0	38.9
Phos. buffered saline	7.2	0	18	2.1
San Carlos, MX	8.0	410	70	1.1
Galveston Bay, TX (marine)	8.4	143	34	1.2
Gulf of Mexico MS (marine)	7.1	80	16	1.3
Sabine Lake, TX (marine)	8.1	1720	30	1.5
Laguna Bay, CA (marine)	7.8	-	-	1.5
Monterrey Bay, CA (marine)	7.9	738	52	1.6
San Francisco Bay CA (mar)	7.5	960	35	1.6
Bodega Bay, CA (marine)	7.8	50	55	1.7
San Juan Capistrano CA (mar)	7.9	71	75	1.8
Rio Grande, NM	8.1	56	0.8	2.5
Potomac River, Wash DC	7.6	110	0.35	2.0
Trinity River, TX	9.1	40	0.35	2.2
Red River, AR	7.8	61	0.75	2.4
Mississippi River, South LA	8.1	180	0.3	2.5
Lake Hubbard, TX	7.9	124	0.25	2.7
Sabine River, TX	7.5	3580	2.0	3.2
Maui, HI (marine)	-	-	-	3.3
Colorado River, TX	8.6	430	0.45	3.4
Display Hot Springs, AR	7.6	0	0.2	4.9
Mississippi River, North LA	8.3	210	0.3	5.0
Mississippi River, TN	7.6	740	0.3	5.6
Mobile Bay, MS (estuarine)	7.5	265	0.2	6.0
Arkansas River, AR	8.0	50	0.3	6.4
Hondo River, NM	-	-	-	6.8
Lake Ouchita, AR	8.4	19	0.1	6.8
Kentucky Lake, TN	8.1	600	0.1	8.9
Chattahoochee River, GA	8.0	390	0.1	18.1
Clinch River, TN	7.9	90	0.15	25.4
Tugaloo River, TN	8.1	10	0.1	28.2

[1]Water samples were collected in sterile 500 ml bottles and frozen until returned to the lab. Five (5) mls of each sample were seeded with approximately 1 x 10^5 pfu/ml of poliovirus-1. These samples were incubated at 27°C in screw-cap tubes with stirring, and aliquots were taken over a 5 day period. The aliquots were assayed on HeLa cells and the log reduction time determined.

[2]Aerobic standard plate count

[3]Time for 90% less of infectivity

TABLE 2

Effect of dilution on poliovirus inactivating capacity of artificial seawater

Dilution of RilaSalts	% infectivity remaining 24h	72h	Conductivity millimhos
1:10	30	2.9	5.5
1:50	44	10	1.2
1:100	84	67	0.6

In none of our studies, was there any relationship between the bacterial count in water and the rate of virus inactivation.

Experiments with radioactively labeled viruses indicated that the primary mechanism of virus inactivation in water was damage to genome. Capsid damage and breakdown occurred subsequent to genome damage. Some preliminary experiments to determine the presence of specific antiviral factors in water were inconclusive. It was found that heating the water consistently reduced the virus inactivating capacity of the water whereas filtration of the water give varied results. At this time, no specific viricidal factors have been identified in water; however, the possibility that such factors are present in some water merits additional study.

VIRUS INACTIVATION DURING ACTIVATED SLUDGE TREATMENT

The activated sludge process is extensively used as a wastewater treatment process. With respect to viruses the process has been shown to be fairly effective in virus removal from wastewater. However, the studies done on virus removal did not distinguish between removal by adsorption of viruses to mixed liquor suspended solids and virus inactivation.

In this project, virus inactivation was done in situ in a large scale four chambered reactor using membrane dialysis chambers (1,2). The inactivation rates of poliovirus and indigenous coliphages were compared and the results indicated that poliovirus and phages were inactivated at similar rates. Furthermore, it was found that during the initial 5 h of treatment most of the virus removal was due to adsorption to suspended solids.

If treatment was continued beyond 10 h, the adsorbed viruses were inactivated. Most activated sludge processes do not have detention times longer than 6 h, thus the sludge will contain infective viruses and disinfection of the sludge must be considered. A convenient and promising process for disinfection would be to dewater the sludge by evaporation.

VIRUS INACTIVATION IN DOMESTIC SEPTIC TANK SYSTEMS

Septic tank systems provide wastewater disposal for an estimated 50,000,000 Americans. Septic tanks in sparsely populated areas probably do not represent a major polution source for groundwater. However, with increasing population densities in suburban areas not served by municipal sewage systems, domestic septic tanks are of greater concern, as a pollution source.

In this study, poliovirus inactivation was studied in a domestic septic tank and in observation wells placed in the drain field (3). It was found that 10 percent of the viruses inoculated into septic tank liquor were still infective after six days. Similar results were obtained in groundwater in the observation wells. Thus, these results indicated that viruses remained infective for sufficiently long period of time to contaminate groundwater and, depending on the nature of the aquifer and the soil, could migrate for considerable distances.

Further studies were done in which Mesilla Valley soil cores were infiltrated with poliovirus inoculated septic tank liquor. Migration of the viruses was followed as the liquor percolated through the soil. It was found that the viruses were distributed throughout the fractions of the

core which were moist. However, the highest number of viruses recovered from the cores were within 1 cm of the top of the core and at the moisture front as it percolated through the core. Thus in unconfined aquifer such as occur in the Mesilla Valley, virus migration in soil is possible in both the the lateral and vertical directions.

VIRUS INACTIVATION IN SOIL

The final repository of viruses released into the environment in wastewater is frequently soil. While the public health significance of viruses in soil has not been fully evaluated, data on virus inactivation in the soil environment are needed in view of the increasing interest in land disposal of sludge. This component of the project was designed to explore the nature of virus-soil interactions and to elucidate the molecular events associated with virus inactivation in soils of the Mesilla Valley (5,6,7,8).

Results obtained in the study showed that viruses could persist for extended periods in the soil. The dominant environmental factors which influenced virus inactivation rates were soil temperature and soil moisture. With respect to soil moisture, there was a critical soil-moisture threshold at approximately 3 percent (w/w) below which the rate of virus inactivation was accelerated. Using radioactively labeled virus preportions, it was found that the decreased virus recovery in soils dewatered by evaporation was due to inactivation and not to binding of infective viruses to soil particles.

The molecular events associated with virus inactivation in moist and dry soils were different. In moist soils virus inactivation was due to irreversible damage to the viral RNA genome while the genome was still associated with the virus capsid. In drying soil, inactivation was due to separation of the RNA and the capsid. The RNA was released intact; however, indigenous soil nuclease activity rapidly degraded the RNA. It was also found that the viral capsids became irreversibly bound to soil particles during drying indicating that the capsid surface characteristics were altered.

CONCLUSIONS

From the results of this study, the following conclusions can be made:

1. Enteric viruses can persist for extended times in surface water. Viral persistence is directly related to the water temperature and to some extent the dissolved solids content.
2. Enteric viruses can persist for extended times in groundwater, and depending on soil type, virus type and the nature of the groundwater can migrate laterally and vertically in the soil environment.
3. Enteric viruses remain infective sufficiently long in domestic septic tank systems to migrate out of the drain fields. As indicated above, these viruses represent a potential source of pollution to groundwater.
4. Viral persistence in the soil environment is dependent primarily on soil temperature and the moisture content.
5. Evaporative dewatering of soil containing infective viruses would be an effective method of eliminating viruses in soil.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. More extensive studies need to be done on virus inactivation under field conditions. More specifically, field studies on the effectiveness of dewatering soil by evaporation on virus inactivation should be done, especially where land disposal of sludge is practiced or contemplated.
2. The interactions between viruses and soils need future detailed study.
3. Further studies on removing viruses during wastewater treatment are needed. In particular, effective, safe, terminal disinfection processes need to be developed.

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