

AN ABSTRACT OF THE THESIS OF

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Title: ZINC-65 UPTAKE BY A BACTERIUM ISOLATED FROM
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Bacteria were isolated from water at Alder Slough, Oregon. Of 15 isolates grown successfully in the medium employed, 100% showed measurable uptake of Zn-65. A growth curve was established for one isolate, a gram-negative rod designated AS-1. Increasing Zn-65 uptake was found generally to correspond with increasing growth of AS-1. The optimum growth temperature for this isolate was 31.0°C, with very little growth at 37.5°C and 6.7°C. Growth and Zn-65 uptake occurred in a 35 ppt medium from a temperature of 31.0°C to 13.9°C but little growth and no Zn-65 uptake was observed at 8.6°C and 4.4°C. Growth and Zn-65 uptake in a 0 ppt medium occurred from 31.0°C to 5.0°C, with little growth but measurable Zn-65 uptake at 8.9°C and 5.0°C.

It was found that 200 ppm Mg added to a culture which had already taken up Zn-65 failed to displace the zinc from the cells. A

interaction of the energy source, Casamino Acids, with the Zn-65 spike influenced the results. When cells were lysed, spiked with Zn-65, and the debris removed by centrifugation, 84.8% of the Zn-65 remained in the supernatant. But when cells grown first in Zn-65 spiked medium were lysed and centrifuged, 74.4% of the Zn-65 was found in the debris.

It was concluded that bacterial uptake of Zn-65 and other metal cations must be considered in determining the fates of these materials released into the environment. Chemical adsorption phenomena were determined to be a major factor controlling this uptake, with other chemical and biological factors, such as competitive binding by the medium and active uptake or exclusion by the cells, exerting a significant influence that requires further investigation to characterize.

Zinc-65 Uptake by a Bacterium Isolated
from Alder Slough, Columbia River Estuary

by

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ZINC-65 UPTAKE BY A BACTERIUM ISOLATED FROM ALDER SLOUGH, OREGON

INTRODUCTION

The tendency for certain metals, such as zinc, to become concentrated in the biosphere (Krumholz, Goldberg, and Boroughs, 1957) has important consequences both for removal of these metals from the rest of the environment and for the biosphere itself. Substances concentrated by an organism are unavailable to its neighbors, unless one of its neighbors eats it. In that case, the substances have proceeded a step in the food chain. The entry of substances into the food chain and their paths through it are of interest because a knowledge of these processes is fundamental to an understanding of the ecosystem in which the food chain operates. Also, man is a part of the food chain. He may receive these substances, some of them further concentrated along the way, in harmful amounts. Since it is frequently man's own activities that have put these materials into the environment in the first place, a knowledge of the ways in which they might return to harm him could help him modify his output to prevent their return.

The purpose of this research was to investigate one way that a heavy metal, zinc, might enter the food chain: by its incorporation into bacteria in the water of a slough. It was hoped to demonstrate

whether or not bacteria, under a variety of conditions, could be expected to remove zinc from the environment and to investigate some of the mechanisms governing this removal.

MATERIALS AND METHODS

Experimental Method

One basic method, with appropriate variations, was employed in all the experiments. Radioactive zinc (Zn-65) was added to a standard medium in which the isolates were grown. After incubation under the various experimental conditions the bacteria were removed from the medium by filtration. Radioanalysis of the filters then indicated the amount of Zn-65 the bacteria had taken up. The filtration and radioanalysis procedures are described in the appendix.

Obtaining Cultures

Cultures used in these experiments were isolated by the spread plate method from a water sample obtained at Alder Slough, Oregon, from a depth of approximately 0.5 m during low tide. Except for the Survey experiment, a single isolate, designated AS-1, was used in all the experimental work. AS-1 is a motile gram-negative rod that swims with a vigorous spiral motion. It occurs singly and sometimes in chains. No further taxonomic identification was attempted.

Medium

All experimental cultures were grown in medium made in one or two-liter batches according to the following procedure:

1. Two parts Millipore (0.45 micron) filtered aged seawater mixed with 33 parts distilled water for a salinity of approximately 2 ppt (parts per thousand).
2. 0.1% Casamino Acids (Difco).
3. 0.05M tris (hydroxymethyl) aminomethane (Sigma 7-9, Sigma Chemical Company).

The pH of the solution was adjusted to 7.0 with 6N HCl. The medium was then dispensed into the culture flasks or tubes. Sterilization was effected by autoclaving 12 minutes at 121°C and 2 atm. The final pH was 7.0.

Experiments

Uptake of Zn-65 by AS-1

The first experiment was conducted to determine if AS-1 would take up Zn-65 and to establish a radioactivity balance for the filtration procedure.

One set of triplicate 1-liter Erlenmeyer flasks was filled with 290 ml growth medium, a second set with 300 ml. All six flasks were spiked with Zn-65 and sterilized. When cool, each of the triplicates

containing 290 ml was inoculated with 10 ml from a culture of AS-1. The flasks containing 300 ml sterile medium served as filter blanks for the experiment (see the appendix).

All flasks were incubated at 10°C in the dark. Turbidity developed within two days. Approximately 36 hours after inoculation, a 10 ml sample was removed from each flask. Five ml from each sample were set aside untreated and an equal volume was filtered through 0.45 micron Millipore filters. The amounts of Zn-65 on the filters, in the filtrates, and in the untreated portions of each sample were determined by radioanalysis as described in the appendix. The Zn-65 in the untreated portion was compared with that in the filters plus filtrates to determine if any activity had been lost in the filtration procedure.

To avoid contaminating pH electrodes with radioactive zinc, pH was monitored in two separate flasks. These were treated the same as the experimental flasks except that an extra 100 ml of medium were added and the flasks were not spiked. The extra 100 ml were removed immediately after autoclaving, allowed to cool, and then used to obtain a pH reading. This was taken as the initial pH of the medium. When turbidity developed and the experimental flasks were sampled, another 100 ml were removed from each pH flask, allowed to warm to room temperature, and again used for a pH reading. This was taken as the pH of the medium at the time of sampling.

Since maximum pH change was 0.2 unit in the Uptake experiment, no further pH checks were run. In this procedure, it was assumed that the high specific activity Zn-65 solution used as the spike did not significantly affect the pH in the experimental flasks.

Survey: Uptake of Zn-65 by Isolates from Alder Slough

The purpose of this experiment was to determine how many of those bacterial species that would grow in the medium used in this research took up Zn-65 and to what extent.

Isolates were again obtained at Alder Slough from a depth of approximately 0.5 m during low tide. Medium was prepared, spiked with Zn-65, and dispensed in 6 ml portions into 27 polypropylene counting tubes. After sterilization, 20 of the tubes were radioanalyzed. Zn-65 activity was found to be the same for all the tubes within one standard deviation so the mean value was taken as the initial activity present in the medium.

From a plate with 89 colonies, 27 were picked at random and each placed in one of the tubes with a sterile inoculating loop. Because the filtration process for removing the bacteria required at least 15 minutes, the tubes were inoculated at half-hour intervals. They were then filtered for radioanalysis in the same order, every half-hour, each after 30 hours incubation in the dark at 10°C.

Growth Rate and Zn-65 Uptake by AS-1

The purpose of this experiment was to establish a growth curve for AS-1 and to determine the rate of Zn-65 uptake at various stages of its growth.

Optical Density (OD) of the culture was taken as a measure of relative population density and was monitored at 600 millimicrons by a spectrophotometer (Bausch and Lomb, Spectronic-20) using a distilled-water blank. Duplicate 500 ml flasks, A and B, equipped with nephelometer tubes, were filled with 100 ml each of Zn-65 spiked medium. They were each inoculated with a 10% inoculum of a 24-hour culture of AS-1 and kept in the dark at 10°C. The nephelometer portions of the flasks fit into the spectrophotometer for measurements of optical density, which were plotted against time. At the intervals shown in Figure 1, samples were removed from A, the next from B, then from A, alternating flasks until the end of the experiment. Ten 10 ml and six 5 ml samples were taken. Duplicate samples were not possible for the following reasons: (1) To use the nephelometer tubes, the flasks could be filled with no more than 100 ml, thus restricting the number of samples that could be taken; (2) At least 15 minutes were required to filter each sample.

The 10 ml samples were treated as in the Uptake experiment; the 5 ml samples were filtered, and no untreated subsamples were

saved. For the initial radioactivity values of these samples, the mean value from the 10 ml samples was used (they were within 3 standard deviations of one another).

Since recovery of activity was never less than 85% for the two preceding experiments, filtrates were not saved in this and in the remaining experiments and no more radioactivity balances were determined.

Optimum Growth Temperature Determination

The optimum temperature for growth of AS-1 was determined with a polythermostat similar to that described by Oppenheimer and Drost-Hansen (1960). Culture tubes (18 X 120 mm) containing 10 ml sterile medium were equilibrated overnight in the apparatus, which provides 15 different temperatures in quadruplicate, simultaneously. A 10% inoculum of a 24-hour culture of AS-1 grown at 10°C was introduced into each tube. Growth was estimated by the change in optical density at 600 millimicrons on the spectrophotometer.

Influence of Temperature and Salinity on Growth and Zn-65 Uptake

The polythermostat and the spectrophotometer were again used as in the optimum growth temperature determination. But instead of quadruplicates with normal medium, two different sets of duplicates were placed in the apparatus. One set contained medium in which the

2 ppt salinity solution had been replaced by pure distilled water; in the other set, the 2 ppt solution was replaced by pure aged seawater. Both sets were spiked with Zn-65. After the last readings on the spectrophotometer, the spiked duplicates were filtered and untreated portions saved from each for radioanalysis.

Displacement of Zn-65 by Mg

To determine if magnesium ions would displace Zn-65 from AS-1, 1.1 gm $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per liter were added to two of the flasks used in the uptake experiment immediately after they were sampled for Zn-65. This concentration of Mg (200 ppm) was chosen after considering the work of Abelson and Aldous (1950), who found that 200 ppm Mg inhibited bacterial uptake of cobalt, nickel, and manganese.

Twenty-four hours later, all three flasks were sampled again for Zn-65 radioactivity. The third flask, to which no Mg had been added, served as a control.

Zn-65 Uptake as a Function of Protein Content

The purpose of this experiment was to determine if Zn-65 content per unit of protein varied with time and growth phase of the organism.

Triplicate 500-ml flasks were each filled with 100 ml of Zn-65 spiked medium. A 10% inoculum of a 24-hour culture of AS-1 kept at 10°C was introduced into each flask. The flasks were incubated at the

optimum growth temperature, 31°C, in a Gyrorotary shaker-water bath (New Brunswick Scientific Company) at 106 strokes/min. with a 1.2 cm stroke. After 8 hours, 14 hours, and 24 hours, 11 ml samples were removed from each flask. Two 0.5 ml quantities were dispensed from the sample into duplicate 12 X 75 mm spectrophotometer tubes for protein analysis by the Lowry method as modified by Straw and Ingraham (1967). The remaining 10 ml were saved for filtration and radioanalysis. As in the growth rate determination, the flasks were inoculated and then sampled half an hour apart to allow for filtration time.

Zn-65 Uptake With and Without an Energy Source

The purpose of this experiment was to determine whether or not AS-1 requires an energy source to take up Zn-65, i. e., whether uptake of Zn-65 is a passive attachment or an active, energy-requiring process.

A 10% inoculum of a 24-hour culture of AS-1 was placed into 450 ml of sterile medium in a 2800 ml Fernbach flask. After 12 hours incubation at room temperature, 45 ml portions were removed from the flask into each of 10 polypropylene 50 ml centrifuge tubes. The cells were washed by centrifugation followed by suspension in 45 ml per tube of medium from which the Casamino Acids had been omitted. Centrifugation was done at 53,000 g for 12 minutes in a

Sorvall RC2-B refrigerated centrifuge at 3°C. The cells were centrifuged and suspended again in 5 ml per tube of the same solution as before, then pooled into a single centrifuge tube.

The washed cells were introduced into two sets of duplicate 18 X 120 mm culture tubes, one set containing normal Zn-65 spiked medium and the other containing spiked medium without nutrients.

The tubes were prepared prior to washing the cells in the following manner: Forty ml of medium without the Casamino Acids were prepared and spiked with 0.1 microcurie Zn-65. Eight ml from this solution were dispensed into each of the duplicates containing no nutrients. Twenty ml were dispensed into another tube, to which 0.05 gm Casamino Acids were then added. From this tube, eight ml were dispensed into each of the duplicates containing nutrients. Both sets of duplicates were then sterilized.

Twelve ml of the washed cell suspension were dispensed into each of the culture tubes prepared as described above and allowed to stand in the spiked media for half an hour before the initial filtration. Introduction was again carried out serially at half-hour intervals to allow for filtration time. Before filtering, duplicate 0.5 ml samples were removed from each of the tubes for protein analysis. In the first filtration, 10 ml were removed from the tube so the initial Zn-65 concentration in the medium could be determined. A second and a third filtration were performed after 2.25 and 3.17 hours. Only 5 ml were

removed in each of these filtrations and all of it filtered. The Zn-65 concentration in the medium was assumed to be the same as it had been in the first filtration.

Zn-65 Distribution Within the Bacterial Cell

This experiment was conducted to determine whether the Zn-65 spike became associated primarily with the cell wall or with the cellular material remaining in the supernatant when the walls were separated from a suspension of lysed cells by centrifugation. The experiment consisted of two parts. In the first, the Zn-65 spike was added to cells already lysed. A 12-hour culture was grown and the cells washed and concentrated as in the preceding experiment, except that only 8 centrifuge tubes were used and the second suspension was done in 6 ml portions, to give 48 ml of pooled cell suspension. The suspension was then slowly (over approximately 36 hours) frozen and thawed twice to lyse the cells. To the lysed cell suspension was added 0.05 microcurie of Zn-65. After half an hour, 6 ml portions of the suspension were removed into three 17 X 100 mm polypropylene counting tubes, each containing 5 ml of sterile medium without Cas-amino Acids. From one of the tubes 1 ml was removed for protein analysis and the remaining 10 ml used for the determination of the initial radioactivity in the medium. The other two tubes were centrifuged at 53,000 g for 20 minutes and the supernatants separated from

the centrifugates. One ml was removed from each of the supernatants for protein analysis and then the supernatants and the centrifugates were radioanalyzed.

In the second part of the experiment, a 12-hour culture was grown in medium spiked with Zn-65. The cells were washed and centrifuged as before, but 20 ml portions were removed from the culture flask into the 8 centrifuge tubes. Twenty ml medium without Casamino Acids were used in each tube for the first suspension, 5 ml for the second, for 40 ml of pooled cell suspension. The suspension was slowly frozen and thawed twice, then distributed in 11 ml portions among three polypropylene counting tubes, which were sampled for protein, centrifuged, and radioanalyzed as in the first experiment.

To determine what percentage of the cells had been lysed by the freezing and thawing process, direct microscopic counts were made of intact bacteria in the suspension before and after the procedure in a Petroff-Hausser Bacteria Counter (C.A. Hausser and Son, Philadelphia).

RESULTS AND DISCUSSION

Uptake

Table 1 shows that AS-1 did incorporate the Zn-65 after 36 hours, removing up to 24.1% of the activity present in the medium at the time of sampling. The lowest recovery of total activity was 94.1%, indicating that little was lost in the filtration procedure used in this research.

This recovery figure does not represent a complete radioactivity balance for the experiment. It is not known how much of the Zn-65 spike was lost to adsorption onto the glassware, but it was assumed that zinc bound to the sides of the container was not available to the bacteria. Loss by adsorption to the container probably was minimal at the pH of the medium used (Tomlinson, 1970) and an attempt was made to prevent adsorption by coating the glassware with a silicone preparation (Siliclad, Clay-Adams, Div. of Becton, Dickinson and Co.). The coating, however, did not appear to last.

Table 1. Uptake of Zn-65 by an Alder Slough bacterium, AS-1 after 36 hours incubation in Zn-65 spiked medium. Cells were removed by filtration and radioanalyzed.

Replicate	Percent Uptake	Percent in the Filtrate	Percent Recovery
A	16.6	77.6	94.2
B	11.0	83.1	94.1
C	24.1	72.5	96.6

Survey

A survey of Alder Slough water bacteria for their uptake of Zn-65 in the medium used in the experiments is represented in Table 2.

Fifty-six percent of the bacterial colonies caused perceptible turbidity in the liquid medium. All of those isolates that produced turbidity showed measurable uptake of Zn-65. Visual inspection (before the results of radioanalysis were known) indicated that the two isolates with the lowest values, numbers 9 and 11 in Table 2, were less turbid than the other isolates at the time of sampling, i. e., the two lowest uptake values were associated with the lowest cell density.

Table 2. Uptake of Zn-65 by bacterial isolates from Alder Slough. Cultures were incubated 30 hours in Zn-65 spiked medium. Cells were removed by filtration and radioanalyzed.

Isolate	Percent Uptake
1	2.09
2	1.84
3	8.97
4	9.32
5	1.48
6	2.37
7	1.80
8	3.73
9	1.44
10	2.47
11	0.77
12	2.62
13	2.34
14	2.83
15	2.58

Growth Rate and Zn-65 Uptake

Figure 1 shows the growth rate at 10°C of AS-1 and the uptake of Zn-65 in the same time period. The open and closed circles on the uptake curve distinguish the duplicate flasks, which, as explained in the materials and methods section, could not be sampled simultaneously.

The graph shows that growth was rapid for 12-14 hours, then began to slow. Density continued to increase more slowly until 40-44 hours, after which it remained constant. Uptake of Zn-65 was not closely correlated with the growth rate, but generally increased with increasing density of the population and did not increase further when growth stopped.

Optimum Growth Temperature

The effect of temperature on the growth of AS-1 is represented in Figure 2. Optimum growth occurred at 30-31°C. Very little growth occurred at the maximum and minimum temperatures tested, 37.5°C and 6.7°C, respectively.

The optical density at 31°C was plotted against time for a growth rate curve at the optimum growth temperature (Figure 3). Growth appears to be logarithmic until 12-14 hours.

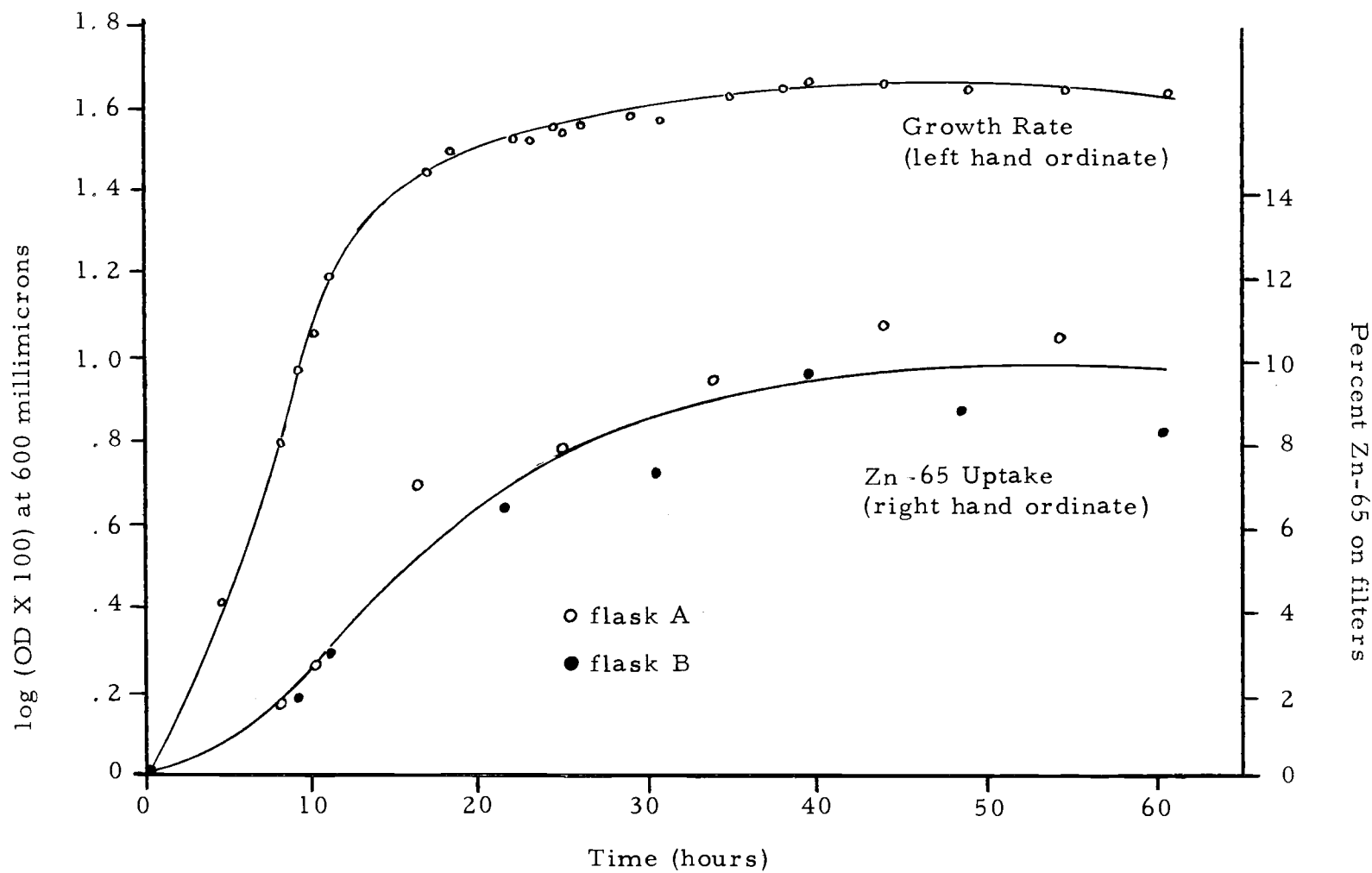


Figure 1. Growth rate of an Alder Slough bacterium, AS-1 and rate of Zn-65 uptake in 500 ml flasks at 10°C.

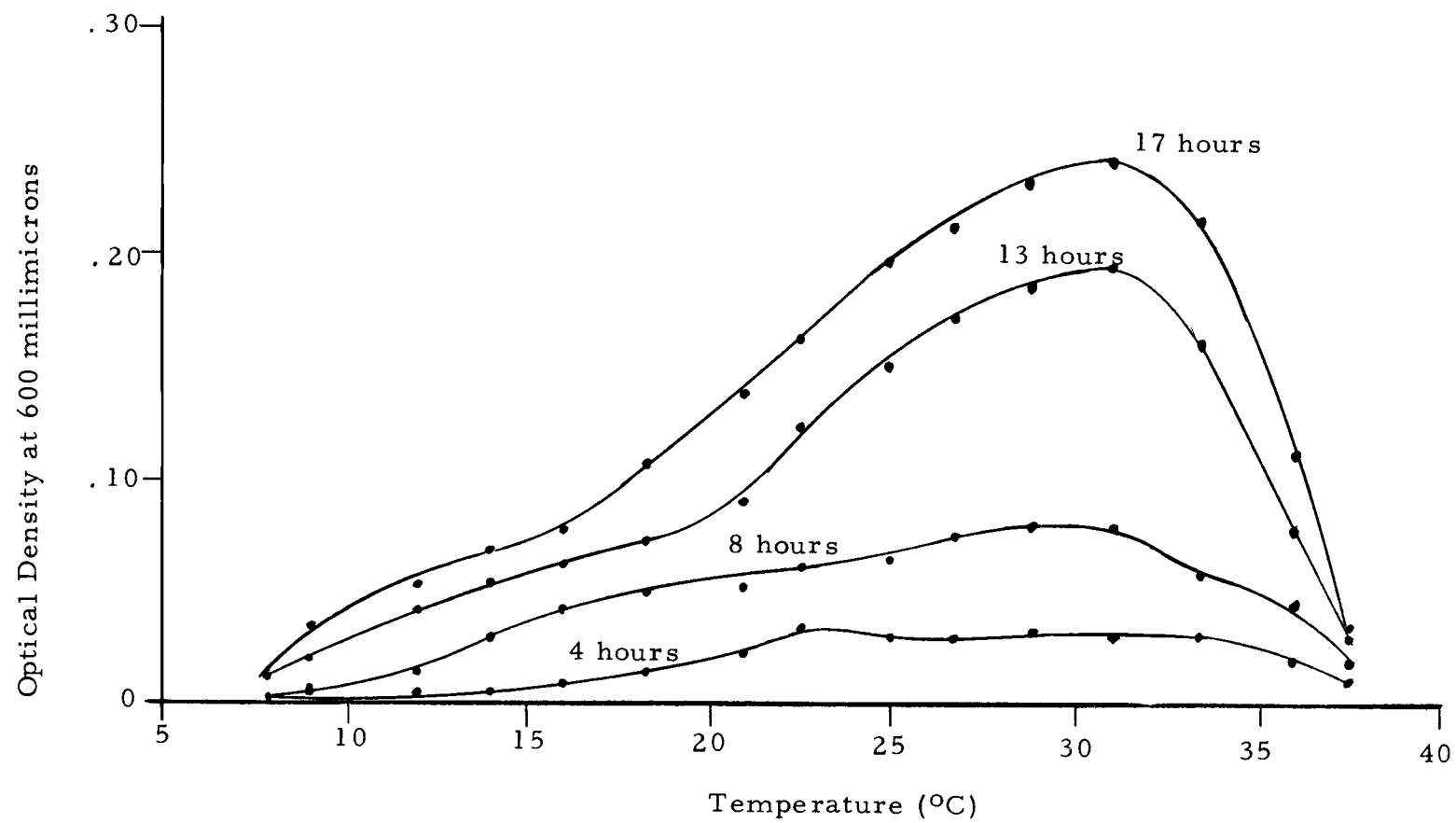


Figure 2. The influence of temperature on the growth of an Alder Slough bacterium, AS-1 at a salinity of 2 ppt.

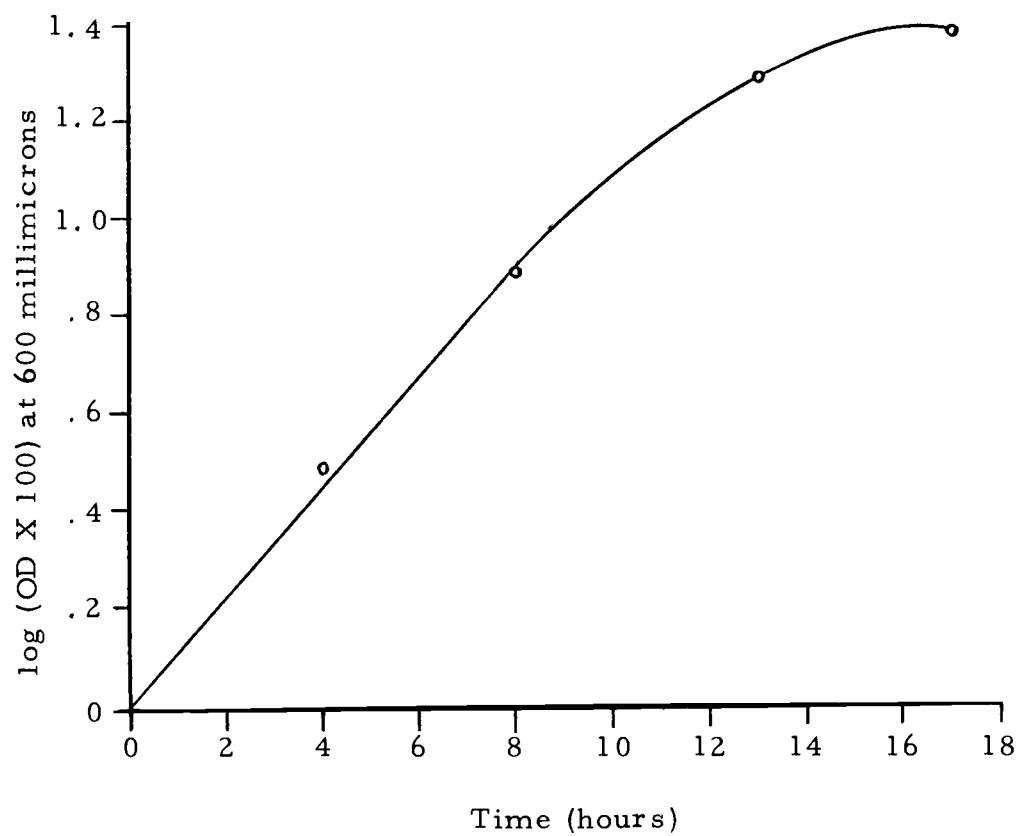


Figure 3. Growth rate of an Alder Slough bacterium, AS-1 in 18 X 120 mm culture tubes at its optimum temperature, 31°C.

Influence of Temperature and Salinity

The effect of two different salinities on the growth and Zn-65 uptake of AS-1 over a range of temperatures is represented in Tables 3 and 4. The uptake values showed considerable variability, which resulted in variable uptake/unit OD values.

In general, measurable uptake was shown to occur in both extremes of salinity over most of the temperature range (over the entire range in freshwater). Uptake/unit OD was lower in seawater than in fresh at temperatures 25°C and lower. The possible significance of this difference will be considered in the discussion on mechanisms of zinc uptake.

Mechanisms of Zinc Uptake

The remaining experiments deal with the mechanisms of zinc's association with the bacterial cell. Since most previous work has been in this area, and the scope of the present project is limited, the following discussion will depend heavily upon the literature.

Table 3. The influence of temperature on growth and Zn-65 uptake by an Alder Slough bacterium, AS-1 at a salinity of 35 ppt.

Temperature (°C)	Optical Density	Percent Uptake	Percent Uptake/unit OD	Average Percent Uptake/unit OD
31.0	.140	4.17	29.8	44.4
	.140	8.27	59.1	
28.2	.230	4.53	19.7	31.8
	.211	8.86	42.0	
27.3	.257	6.17	24.0	31.6
	.273	10.68	39.1	
25.0	.376	12.10	32.2	23.3
	.337	4.87	14.4	
22.0	.335	7.97	23.8	18.8
	.355	4.90	13.8	
19.8	.239	3.62	15.1	19.5
	.305	7.30	23.9	
17.9	.211	2.03	9.62	17.1
	.191	4.71	24.6	
13.9	.114	0.48	4.21	12.1
	.119	2.38	20.00	
8.6	.068	0		
	.067	0		
4.4	.056	0		
	.008	0		

Table 4. The influence of temperature on growth and Zn-65 uptake by an Alder Slough bacterium, AS-1 at a salinity of 0 ppt

Temperature (°C)	Optical Density	Percent Uptake	Percent Uptake/unit OD	Average percent Uptake/unit OD
31.0	.267	9.94	37.2	43.4
	.273	13.54	49.6	
29.3	.287	11.57	40.3	40.6
	.293	11.96	40.8	
26.9	.292	9.37	32.1	50.6
	.297	20.51	69.0	
24.6	.292	-	-	31.7
	.297	9.42	31.7	
22.0	.295	16.59	56.2	51.4
	.272	12.66	46.5	
19.6	.291	11.38	39.1	36.6
	.295	10.10	34.2	
18.0	.302	14.73	48.8	41.4
	.292	9.95	34.1	
13.9	.159	4.93	31.0	39.5
	.168	8.07	48.0	
8.9	.059	2.57	43.6	44.8
	.059	2.71	45.9	
5.0	.009	-	-	79.4
	.017	1.35	79.4	

- sample lost

The Role of Zinc in the Cell

Zinc apparently is used by the cell in two kinds of enzymes. In their investigations into the metabolic role of zinc, Vallee, Kägi and Hoch (1958) grew baker's yeast in Zn-65 labelled media and extracted from the cells a yeast alcohol dehydrogenase (YADH) coupled with the radioactive metal ($[YADH]Zn_4^{65}$). The radioactive zinc was non-exchangeable with contaminating stable zinc. Hoch and Vallee (1958) distinguished this type of metalloenzyme, in which the metal was firmly bound and non-exchangeable, from metal-enzyme complexes, in which the metal cation could be exchanged for another similar cation. The "metalloenzymes" discussed by MacLeod and Snell (1950), in which zinc appeared to be a non-active (enzymatically) substitute for several other cations, seem to have been the metal-enzyme complexes described by Hoch and Vallee (1958).

The Bacterial Cell as an Agent in Cation Exchange

A study of the bacterial cell as an agent in cation exchange began with McCalla (1940a). "Since the bacterial cell contains proteins, approaches inorganic colloids in size, and is negatively charged, it seems logical that it might function as an agent in the adsorption of positively charged ions." (p. 23). McCalla was able to demonstrate that bacteria did adsorb cations, that the adsorbed cations were

exchangeable with certain other cations, and that results were the same with living or dead bacteria. Measuring displacement of adsorbed methylene blue by various cations added to the medium, he went on to establish an order of adsorption strength for the cations: $H > Fe > Al > Mn > Ba > Ca > Mg > K > NH_4 > Na$ (1940b). He found that basic stains acted as cations and combined with the cell in an adsorption exchange process (1941). Bartholomew, Roberts, and Evans (1950) also established an exchange order with salts of cations:

$Cu > Ca > Fe > Mg > Ba > St(\text{sic}) > Na$, which they noted was consistent with McCalla's (1940b). They, too, found that basic dyes were exchangeable with other basic dyes and with salts of cations, but that the cations replaced the dyes more slowly than other basic dyes. The dye exchanges proceeded according to mass action laws for reversible reactions. They pointed out that basic dyes are known to react at carboxyl group sites chemically. Serény (1967) provided further evidence that the adsorption of dyes takes place according to the mechanism of ion exchange and that basic dyes act as cations.

The "ion exchange" described above, in which cations replace each other according to a definite order, should be distinguished from true ion exchange, in which addition of any cation would cause the displacement of the ion already adsorbed. Johnson (1966) discusses the two kinds of exchange in connection with sorption of Zn-65 to river sediments and terms exchange that is not true ion exchange "specific

sorption". He cites the work of several authors to show that transition metal cations display specific orders of displacement from silicate minerals ($\text{Cu}^{++} > \text{Co}^{++} > \text{Zn}^{++} > \text{Fe}^{++} > \text{Ni}^{++} = \text{Mn}^{++}$), and from organic soil ($\text{Cu}^{++} > \text{Co}^{++} > \text{Zn}^{++} > \text{Ni}^{++}$) and that the latter order is consistent with the cations' order of stability in coordination complexes with organic ligands ($\text{Pd}^{++} > \text{Cu}^{++} > \text{Ni}^{++} > \text{Pb}^{++} > \text{Zn}^{++} > \text{Fe}^{++} > \text{Cd}^{++} > \text{Mn}^{++} > \text{Mg}^{++}$). He shows that the ionization potentials for transition metals of similar ionic radii follow an order, $\text{Cu} > \text{Zn} > \text{Ni} > \text{Co} > \text{Fe} > \text{Mn}$, that is consistent, except for Ni, with all the above orders, "suggesting that specific sorption is related to the electronic configuration of the metal ions." (p. 19)

Since the displacement orders found by McCalla (1940b) and Bartholomew, et al. (1950) are consistent with those cited by Johnson (1966), it is possible that cation exchange in bacteria is governed by some of the same mechanisms governing specific sorption to minerals and to organic soils and might, in fact, account for at least part of the exchange observed in the latter.

Johnson (ibid.) found that seawater, with its rich complement of Mg and Na ions, failed to displace Zn-65 sorbed onto river sediments. This observation indicated that the zinc was held by a stronger process than ordinary ion exchange. Table 5 provides similar evidence that zinc is held by bacteria by some mechanism stronger than ordinary ion exchange; the data show that 200 ppm Mg failed to displace

measurably the zinc taken up by AS-1.

Table 5. Displacement by 200 ppm magnesium of Zn-65 incorporated by an Alder Slough bacterium, AS-1. Initial uptake data is from radioanalysis of cells grown in Zn-65 spiked medium for 36 hours. Magnesium (200 ppm) was added and the cells radioanalyzed again 24 hours later.

Flask	Percent uptake initial	Percent uptake 24 hours after Mg added	Percent increase
A	24.1	29.2	21.1
B ¹	11.0	16.8	52.7
Control no Mg added	16.6	20.7	24.7

Tables 3 and 4 indicate that growth in seawater may result in less uptake of zinc, but it would be difficult to sort out displacement effects from the effects of growth in seawater by a bacterium taken from as low a salinity environment as Alder Slough (salinity usually = less than 10 ppt). Abelson and Aldous (1950) found that results from displacement of a metal already sorbed may not be comparable to results from interference with uptake of the metal. They found that 200 ppm Mg in their medium decreased bacterial uptake of Co^{++} by

¹ Flask B was noticeably less turbid than the others at the time of the initial sampling. At the second sampling, cell density in B seemed to be closer to that in the other flasks. This "catching up" by flask B might account for its larger percent increase in Zn-65.

8.8 but that growth in both Mg^{++} and stable Co^{++} failed to change the concentration of Co-56 already taken up by the cells.

Other experiments by Abelson and Aldous (ibid.) indicated that cation uptake might be controlled by biological as well as chemical processes. They found an initial uptake of cobalt with or without an energy source, glucose, but further accumulation occurred only when glucose was present and the organisms were increasing in density. The results in Table 6 indicate that AS-1 also continued to accumulate zinc only during the period that cultures were increasing in density (refer also to Figure 3).

Table 6. The ratio of Zn-65 uptake to protein content in three stages of growth in a culture of an Alder Slough bacterium, AS-1 at its optimum temperature, 31°C.

Time	Flask	Percent Uptake	Micrograms Protein/ml	Percent uptake
				Micrograms protein/ml
8 hr	A	14.1	472	.0299
	B	16.3	368	.0443
	C	14.7	354	.0415
	\bar{X}	15.0	398	.0386
14 hr	A	22.2	450	.0493
	B	22.6	390	.0579
	C	22.8	396	.0576
	\bar{X}	22.5	412	.0546
24 hr	A	23.0	472	.0487
	B	-	-	-
	C	22.1	400	.0552
	\bar{X}	22.6	436	.0518

A comparison of zinc uptake by a culture of AS-1 with an energy source to one without also was made. The results in Figure 4 show that zinc uptake was higher in the culture without an energy source. It first appears from this result that the cells need energy to exclude zinc, whereas Abelson and Aldous (ibid.) found that cobalt uptake was enhanced by an energy source. Further evidence for exclusion of zinc was provided by the experiment in the present research in which the cells were lysed. Only 30% of the cells were lysed by the procedure used (freezing and thawing); nevertheless, 84.8% of the zinc was found in the supernatant when the spike was added to the lysed cells and the debris removed by centrifugation, indicating that Zn-65 has a high affinity for the material inside the cell. But when cells were grown in Zn-65 spiked medium, then lysed and centrifuged, 74.4% of the Zn-65 was found in the centrifugate. These results indicate that whole living cells were able to exclude zinc from inside their walls, despite the high affinity of zinc for the material inside.

However, because the energy source for this experiment was Casamino Acids, another explanation for the results in Figure 4 must be considered in addition to active exclusion of zinc by the cells. There is evidence that the reduced uptake in the presence of Casamino Acids could be due to reduced availability of zinc in the medium. Duursma (1968) reported significant chelation of Zn-65 by the amino acid leucine. Serény (1967) cited previous evidence that amino acids

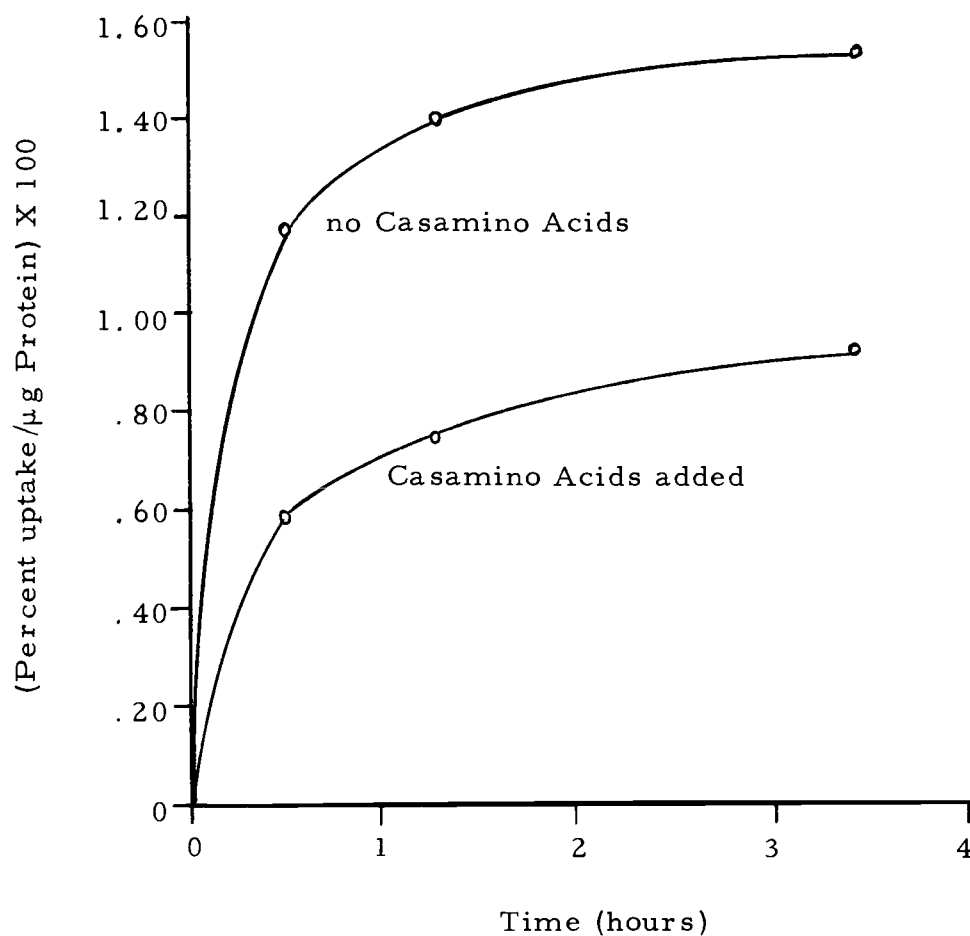


Figure 4. Uptake of Zn-65 by an Alder Slough bacterium, AS-1 in normal medium compared to Zn-65 uptake in a medium deprived of an energy source, Casamino Acids. A 12-hour culture of AS-1 was washed and equal portions were dispensed into normal and into deficient medium, both labelled with Zn-65.

decreased the anti-bacterial effects of various cations and noted his own results in which L-cysteine and L-histidine reduced the growth-inhibiting effect of cobaltous nitrate. MacLeod and Snell (1950), testing two different growth media, found that Zn^{++} and Mn^{++} precipitated far more in one medium (I) than the other (II). They attributed the greater solubility of the cations in medium II to the presence of partial hydrolysis products of casein, which bound the cations. They found that zinc toxicity was reduced in medium II.

MacLeod and Snell (ibid.) also found, however, that one of their test species, Lactobacillus arabinosis, showed a growth response to considerably smaller amounts of Mn^{++} in medium II, where it presumably was bound, than in medium I. They pointed out that biological availability and availability for precipitation were not necessarily related.

This last result indicates that zinc may still be biologically available when bound to amino acids. However, it also indicates that it may not be valid to compare results with Zn^{++} to results with Mn^{++} . MacLeod and Snell (ibid.) did observe that toxicity of zinc was reduced in medium II. This discussion has shown that different cations have different exchange capacities, presumably with organic molecules in the medium as well as with bacterial cells. They may also undergo different biological reactions with the cells. In the previously cited experiments, Abelson and Aldous (1950) showed that once Co-56 was

taken up by the cells neither Mg nor stable Co changed the cells' concentration of Co-56 significantly. But in a similar experiment with Mn-52, both Mg and stable Mn displaced large amounts of Mn-52 from the cells.

The evidence of Serény (1967) for the binding of zinc by amino acids depends upon the binding of other cations, but that of Duursma (1968) and of MacLeod and Snell (1950) does not. Therefore, decreased availability of zinc for adsorption to the cells in the Casamino Acid medium still must be considered along with active exclusion of zinc as an explanation for Figure 4.

CONCLUSIONS

Environmental Implications

In every one of the experiments performed, uptake of Zn-65 by bacteria occurred. At least one strain, AS-1, was shown to take up significant amounts over a wide range of temperatures and salinities. The conclusion follows that bacteria, as a link in the food chain and as a significant component of water and sediments, cannot be overlooked as an important factor in determining the fates of stable and radioactive metals released into estuaries.

Mechanisms of Zinc Uptake

The evidence reported in the literature and the experiments in the present research indicate that bacteria take up Zn-65 primarily by a special kind of cation exchange process similar to the "specific sorption" discussed by Johnson (1966). There is also evidence, however, that this uptake may be modified by other chemical variables, such as competitive binding by the medium, and by biological processes, such as active uptake or exclusion by the cells. Further investigation is needed to determine the nature and the extent of the influence of these other factors on the final disposition of zinc and other cations in bacterial cultures.

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APPENDIX

APPENDIX

Sampling for Zn-65Removal of the Sample

All samples from all the experiments were withdrawn into 12 ml polypropylene syringe barrels (Monoject), from which they were dispensed either into the counting tubes or onto the filters. The barrels alone were too short to reach the liquid in the flasks and so had to be fitted with either a length of 4mm (outer diameter) Tygon tubing or a 2-inch needle.

The Tygon tubing was used in the uptake experiment, in which the liquid had to be withdrawn from the bottom of the 1-liter Erlenmeyer flasks. When the sample had been withdrawn and freed of bubbles, the tubing was detached and the sample dispensed directly from the barrel. The risk of spilling radioactive medium was reduced by permanently affixing a short piece of more rigid polypropylene tubing to one end of the Tygon tubing. This rigid length was more easily attached to and loosened from the syringe barrel than the flexible Tygon tubing.

The 2-inch needle was used for all the other experiments, in which the culture vessels were smaller. Since the needle caused no inconvenience in handling the syringe, it was left on the barrel in dispensing the samples.

Filtration

The filtration assembly consisted of four Swinnex-13 units (Millipore), each draining into a counting tube inside an aspirator flask. Each flask was connected to a vacuum line. All four units were used for each sample, since filtering time was inordinately long with only one filter.

When the units were first used, medium remained trapped in the tops, so the tops were removed. The filters were placed on the bases of the units, the vacuum turned on, and the samples distributed carefully among the units. By proper use of the plunger in the syringe, a drop at a time could be introduced onto a filter until a fair-sized bead, held by surface tension, covered the filter. More drops could be added to the beads as they were depleted by the filtration until the syringe was emptied.

When no more water could be seen standing on the filters, the vacuum was turned off and the filters removed to a counting tube. The vacuum was turned on again and one ml of distilled water was passed through each unit. The vacuum was again turned off and the filtrates removed and pooled into one counting tube. For the Uptake and the Survey experiments, in which the filtrates were saved, one ml of distilled water was distributed among the three collecting tubes as a rinse and poured into the pooled filtrate. The collecting tubes

were then rinsed with distilled water three times, wiped, and replaced in three of the units. For the other experiments, the filtrates were discarded.

To determine if the collecting tubes retained any activity, the distilled water rinse was omitted after an experiment and the collecting tubes "counted". No activity was detectable within the same interval of time used in "counting" the filtrates.

Radioanalysis

Spiking the Cultures

Zinc-65 was obtained from New England Nuclear Corporation as $^{65}\text{ZnCl}_2$ dissolved in 0.5M HCl. Activity was 1.0 microcurie/ml. Five ml of this solution were removed into 45 ml distilled water to make a working solution with 0.1 microcurie Zn-65/ml. All experimental cultures were spiked with one ml working solution per 100 ml medium, or 1000 picocuries Zn-65 per ml medium.

Processing the Samples

Zn-65 emits a 1.114 MeV photon, which can be detected by means of a NaI(Tl) crystal well detector with appropriate instrumentation. Counts per minute in each of 256 energy channels were obtained as a typed readout. The counts in the photopeak and in the

four channels on either side were summed and then divided by the counting time in minutes. Background was automatically subtracted.

Polypropylene counting tubes (17 X 100 mm) were used to contain all samples in the well crystal. To insure constant geometry in the well, the liquid samples were all brought up to the same volume with distilled water. The filters were digested in acetone and allowed to stand overnight. As the acetone evaporated, the filter and its contents were precipitated uniformly over the bottom of the tube. To compare the values obtained from the filters with those from the filtrates and untreated samples, a volume correction was made from a curve of observed counts plotted against volume for a sample containing a constant amount of Zn-65.

The filters were further corrected (before the volume correction) by subtraction of a filter blank. The same volume of sterile medium with the same Zn-65 concentration as that in the sample was filtered. The value obtained from radioanalysis of these filters was subtracted from the values for the sample filters. All uptake values in this project were expressed as percent initial radioactivity on the filters = (corrected filter counts per minute/corrected initial counts per minute from the untreated samples) X 100.