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UREA/AMMONIUM ION REMOVAL SYSTEM FOR THE ORBITING FROG OTOLITH EXPERIMENT

By Jon R. Schulz and Robert T. Anselmi, PhD

January 1976

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AMES RESEARCH CENTER
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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Final Report

January 1976

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SUMMARY

The objective of this program was to determine the feasibility of using free urease enzyme and ANGC-101 ion exchange resin to remove urea and ammonium ion for space system waste water applications, specifically, the prevention of urea and ammonia toxicity in a 30-day Orbiting Frog Otolith (OFO) flight experiment.

The study has shown that free urease enzyme used in conjunction with ANGC-101 ion-exchange resin and pH control can control urea and ammonium ion concentration in unbuffered recirculating water. In addition, the resin does not adversely effect the bullfrogs by lowering the concentration of cations below critical minimum levels. Further investigations on bioburden control, frog waste excretion on an OFO diet, a trade-off analysis of methods of automating the urea/ammonium ion removal system and fabrication and test of a semiautomated breadborad were recommended as continuing efforts.

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SYMBOLS

Å	angstrom
^{o}C	degrees centrigrade
ca	approximately
Ca^{++}	calcium ion
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
CO_2	carbon dioxide
db	decibel
^{o}F	degrees Fahrenheit
enz.	enzyme
g	gram
HCl	hydrochloric acid
in.	inch
K^{+}	potassium ion
KCl	potassium chloride
M	molar or gram molecular weight/liter of solution
mg	milligram
Mg^{++}	magnesium ion
$MgSO_4$	magnesium sulfate
min.	minute
ml	milliliter
mm	millimeters
mmole	millimole

N	nitrogen
N_2	molecular nitrogen
Na^+	sodium ion
NaCl	sodium chloride
NaOH	sodium hydroxide
NAS	National Academy of Sciences
NH_3	ammonia
NH_4^+	ammonium ion
NH_4Cl	ammonium chloride
NO_3^-	nitrate ion
O_2	oxygen gas
O_3	ozone
OFO	Orbiting Frog Otolith
pH	negative log of the hydrogen-ion molarity
ppm	parts per million
psi	pounds per square inch
rpm	revolutions per minute
sec.	second
P	differential pressure
l or ℓ	liter
μg	microgram
μ mole	micromole
	micron

INTRODUCTION

This report presents Martin Marietta Corporation's continued technical effort to develop a method of removing urea and ammonium ion from recirculated waste water in a 30 day Orbiting Frog Otolith experiment. The post-Skylab flight experiment programs will require greater quantities of potable water which may necessitate water recovery from waste water (urine) for reuse. An attractive method of water reclamation from waste water (urine) in space systems is reverse osmosis. Because reverse osmosis membranes are permeable to urea, prior removal of urea would be required. Furthermore, experiments such as the Orbiting Frog Otolith (OFO) flight experiment, require urea removal from the proximity of the experimental organisms to avoid the urea toxicity problem.

Urease is an enzyme which catalyzes the hydrolysis of urea to ammonia and carbon dioxide. These hydrolysis products may also reach toxic concentrations; therefore, control of their concentrations within nontoxic limits was investigated. Because the OFO A experiment had methods for controlling the CO_2 levels, only the problem of controlling ammonia levels was addressed in this technical effort.

The first phase of the technical effort was accomplished in 1974 by Dr. Richard Husted by performance of three tasks. Task 1 - Immobilized Urease Technology Analysis, and Task 2 - Analysis of Ammonia Removal Techniques were conducted simultaneously and led into the performance of Task 3 - Combined Urea/Ammonia Removal Systems Test. Early Task 3 results led to modifications of the combined urea/ammonia removal system which required extension of the technical effort from the proposed six months to six and one-half months. The results of the first phase are reported in a report, NASA CR-137596.

The second phase of the technical effort was accomplished by performance of two tasks. Task 4 - System Component Testing and Experiment Simulation, and Task 5 - 30 Day System Performance Test.

Early Task 4 results led to modification of Task 4 to include urease activity testing which required extension of the technical effort from the proposed eight months to eight and one-half months.

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TASK 4

SYSTEM COMPONENT TESTING AND EXPERIMENT SIMULATION

Ion Exchange Resin Affinity for Cations

In this portion of Task 4 the question of whether or not the ion exchange resin, ANGC-101, can be "preloaded" with certain cations was addressed. The total alkalinity (total ionic strength) recommended for amphibians by the National Academy of Sciences is 150-250 ppm as $CaCO_3$(4). The cation concentration range should be roughly 60-100 ppm. Dr. George Nace of the University of Michigan believes an adult bullfrog could be kept in distilled water with no adverse effects, if the frog is eating normally. A frog can usually obtain all of the ions it needs from the food it eats. Concentrations of cations in naturally occurring water vary tremendously, making definition of a "normal" water virtually impossible. However, as an approximation the ratios of cations in natural water were used to define an ion solution in the 60-100 mg cations/liter range. Na^+ and K^+ occur in approximately equal amounts, ranging from 1-10 ppm. Calcium to magnesium ratios commonly vary from 5 to 1. Ion Solution I was prepared with a ratio of 2.5 Ca^{++} to 1 Mg^{++}. Ion Solution I contained: 8 ppm Na^+, 8 ppm K^+, 48.3 ppm Ca^{++} and 11.8 ppm Mg^{++}.

Dr. Richard Husted's investigations on immobilization of urease and his early investigations with free urease enzyme were all done in 0.05M sodium phosphate buffer since urease is most active in buffered solution and is inactivated by radical pH change (1). This concentration of buffer, in addition to radically changing the 1:1 Na^+-K^+ ratio, adds 1846 ppm of Na^+ to the solution. This concentration of sodium and phosphate is certainly much higher than found in natural waters and is probably undesirable from the frog's point of view. For this reason the ion solution passed through the last set of resin columns in the affinity tests contained no buffer. Also, Ion Solution I in 0.05M sodium phosphate buffer gives precipitates. Even when the buffer concentration was cut to 0.025M, the fine precipitates formed partially clogged the first pair of resin columns (1 and 2).

No assays were performed on columns 1 and 2 and a new ion solution was prepared for columns 3 and 4 which produced no precipitates in 0.05M sodium phosphate buffer. Resin columns were prepared by: (1) washing 10 gram quantities of ANGC-101 ion exchange resin three or four times with glass distilled water to get rid of air and extra fine particles of resin, (2) transferring each 10 g sample of resin into a buret that had a layer of glass wool and ground pyrex glass (47-48 mesh) in the bottom, (3) leaving distilled water just covering the resin to avoid trapping air bubbles.

Each set of columns which were assayed are discussed sequentially below. Specific information on preparation of ion solutions, materials and methods, and equipment are found in the appendices. Tabulated data on most tests are placed for reference in an appendix.

Columns 3 and 4: The solution for this test contained Ion Solution II (8 ppm K^+, 5.1 ppm Ca^{++}, 1.2 ppm Mg^{++}) and 1846 ppm Na^+ from 0.05M sodium phosphate buffer. 100 ml aliquots were prepared and passed through the resin columns. The 1st 100 ml aliquot passed through a column experiences a slight dilution by the distilled water covering the resin after washing. Therefore, the data on the 2nd, 3rd, and 4th aliquots are probably more meaningful. All cation assays performed during this contract have been accomplished using atomic absorption and flame emission spectrometry. Materials and methods are included in the appendices. The ppm values for the aliquots are a mean of the values obtained for each separate column in a pair. Data from this test is tabulated in the tables section of the appendices and is graphically shown in Figures 1 and 2.

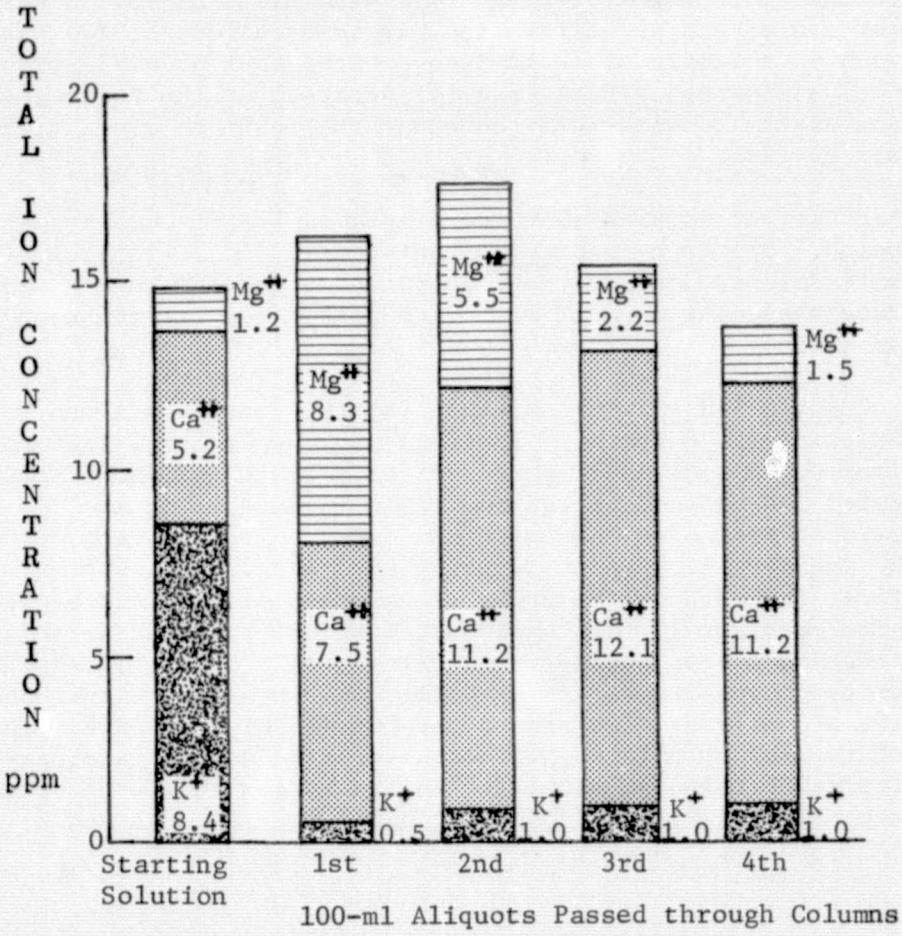

Figure 1 Assayed Cation Concentrations in Solutions from Columns 3 and 4.

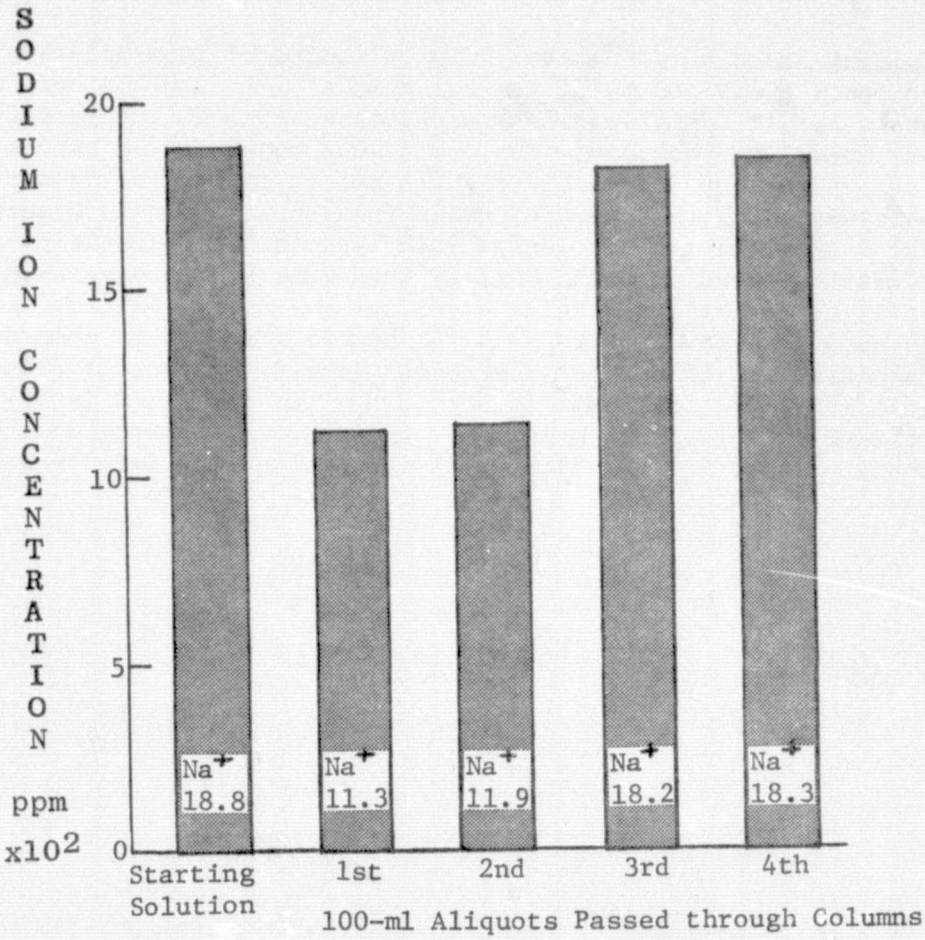

Figure 2 Sodium Ion Concentration in Solutions from Columns 3and 4

The data shown in Figures 1 and 2 for columns 3 and 4 show that given Ion Solution II in 0.05M sodium phosphate buffer the ANGC-101 resin has a high affinity for potassium and a lower affinity for sodium, and the resin actually gives up calcium and magnesium to the solution. It appears that an end point, a cation-loaded condition could be extrapolated for magnesium and sodium, but the resin absorbed the same amount of K^+ from each of the last three aliquots and gave up approximately the same amount of Ca^{++} to the solution each time (not allowing extrapolation to an end point).

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Columns 5 and 6: Separate 100/ml aliquots of Iron Solution II (8 ppm K^{+}, 1.2 ppm Mg^{++}, and 5.1 ppm Ca^{++}) in 0.0125M sodium phosphate buffer (461.5 ppm Na^{+}) were passed through two freshly prepared resin columns. The ppm values shown in the tabulated data in the appendices and figures are a mean of the values obtained for each separate column in a pair. Data in Figures 3 and 4 show that in a solution with less buffering capacity (i.e. 0.0125M instead of 0.05M buffer) the resin has an even higher affinity for Na^{+}, and still gives up some Mg^{++}. Calcium, however, is no longer given up to solution but is absorbed to some degree. Examining the values for the second, third, and fourth aliquots does show that the resin is not loaded yet and there is insufficient data for extrapolation. Information gathered on columns 3 and 4 and 5 and 6 indicates that the situation is more complex than anticipated.

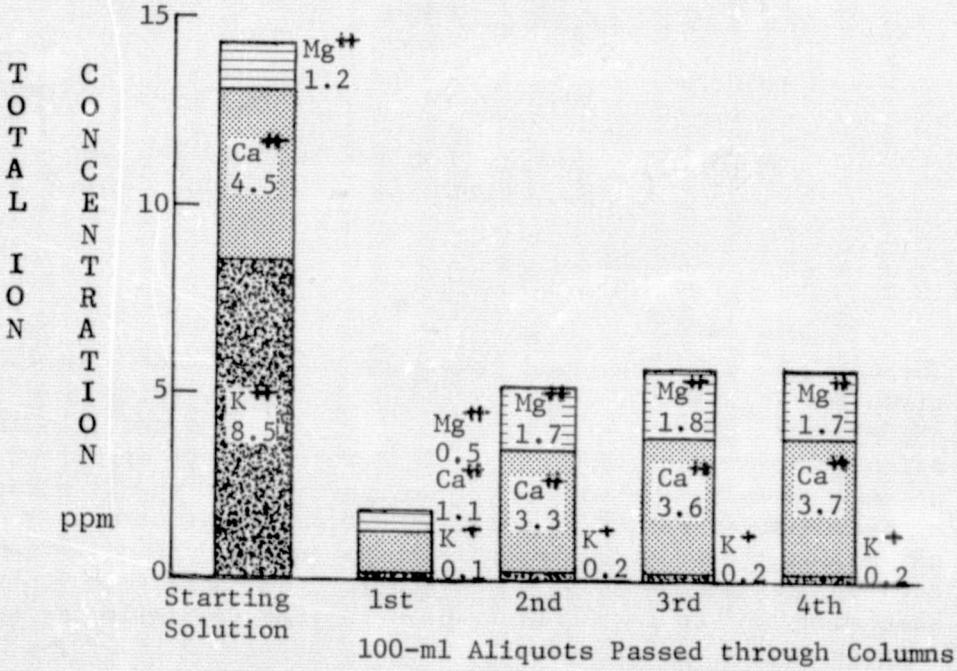

Figure 3 Assayed Cation Concentrations in Solutions from Columns 5 and 6.

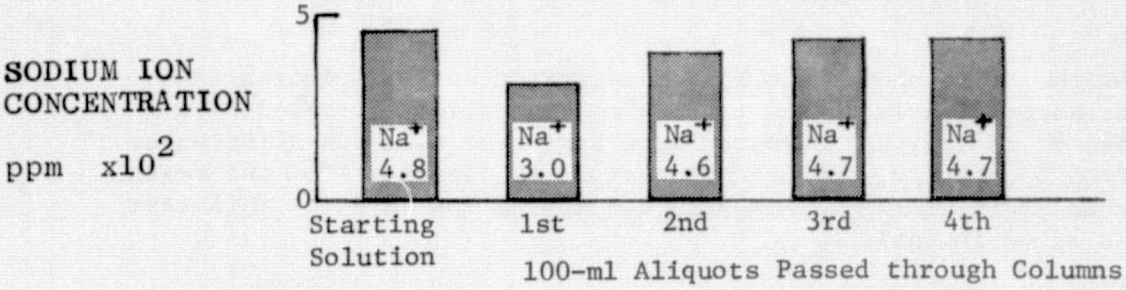

Figure 4 Assayed Sodium Ion Concentration in Solutions from Columns 5 and 6

Columns 5.1 and 6.1: Since the major concern is whether or not the resin will adversely alter the cation balance in the presence of NH_4^+ and other substances, tests on columns 5 and 6 were continued in the presence of ammonium ion. An ion solution containing no K^+, 0.5 mg Mg^{++}, 1.1 mg Ca^{++} and 160 ppm Na^+ was split into three samples, a start sample, and two -450 ml samples which were passed through columns 5 and 6 ten times. The results are shown in Table 1.

Table 1. Assayed Cation Concentrations in Solutions Containing Ammonium Ion From Columns 5.1 and 6.1. All Concentrations in ppm.

Element	Starting Solution (100 ml)	Solutions Passed Through Columns 10X		
		Column 5.1 (450 ml)	Column 6.1 (450 ml)	Mean Value
Mg^{++}	0.52	0.81	0.70	0.76
Ca^{++}	1.1	1.8	3.0	2.40
K^+	$\ll 0.01$	4.90	5.00	5.0
Na^+	160	655	780	717.5

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As the table shows, the Mg^{++} concentration in the solution increased slightly again. The Ca^{++} concentration increased by a factor of 2.2, Na^{+} increased by a factor of 4.5, and K^{+} increased from 0 to 5 ppm. Ammonium ion seems to displace K^{+} on the resin when it is present. Ammonium ion absorption data for this test is shown in Table 2.

Table 2. Ammonium Absorption Data From Columns 5.1 and 6.1

Ammonia	Starting Solution (100 ml)	Solutions Passed Through Columns 10X	
		Column 5.1 (450 ml)	Column 6.1 (450 ml)
Calculated mg NH_3 (from NH_4Cl) before contact with resin	23.93	107.57	107.51
Calculated ppm NH_3	239.3	239.0	238.9
Adjusted ppm NH_3 [1]	---	224.7	224.6
Adjusted mg NH_3 [1]	---	101.12	101.07
Assayed ppm NH_3	225.0	66.0	70.0
Assayed mg NH_3	22.5	29.70	31.50
mg NH_3 Trapped by 1.0g of ANGC-101 resin [2]	---	7.14	6.96
Mean Value mg NH_3 Trapped by 1.0g ANGC-101 resin	---	7.05	
mg Nitrogen Trapped by 1.0g ANGC-101 resin		5.81	

[1] $\frac{\text{Assayed value of starting solution}}{\text{Calculated value of starting solution}} \times$ Calculated Value of Sample

[2] Adjusted $mgNH_3$ - assayed $mgNH_3$ ÷ 10 g resin

The columns were deliberately overloaded with ammonium ion to determine trapping ability. It should be pointed out that all ammonium ion in the samples was converted to ammonia gas by basification with 10N NaOH for assay with an ammonia-gas electrode. Starting pH of the solution before passage through the columns was 7.2 and after passage was 7.1.

Columns 7 and 8: A fourth pair of columns were prepared and exposed to Ion Solution II plus 10 ppm of Na^+. Since there is a good possibility that the use of buffered water will not be possible, this pair of columns were never exposed to buffer and were exposed to an ion solution containing ammonium ion. Table 3 shows the composition of the starting solution and the concentration of ions in the 450 ml aliquots after passage through each of the columns six times.

Table 3. Assayed Cation Concentration in Solutions Containing Ammonium Ion From Columns 7 and 8. All Concentrations in ppm.

Element	Starting Solution (100 ml)	Solutions Passed Through Columns 6X		
		Column 7 (450 ml)	Column 8 (450 ml)	Mean
Mg^{++}	1.26	0.27	0.86	0.57
Ca^{++}	4.4	1.7	1.5	1.6
K^+	8.6	4.7	4.8	4.8
Na^+	10.5	296	308	302

Compared to solutions containing phosphate buffer, neither Mg or Ca increased in the solutions from Columns 7 and 8. When NH_4^+ is present Na^+ concentration actually increases significantly in the solutions, some Na^+ actually being dumped by the resin. Ammonium data for this test is shown in Table 4.

Table 4. Ammonium Data from Columns 7 and 8

Ammonia	Starting Solution (100 ml)	Solution Passed Through Columns 6X	
		Column 7 (450 ml)	Column 8 (450 ml)
Calculated mg NH_3	23.81	107.29	107.36
Calculated ppm NH_3	238.1	238.4	238.6
Adjusted ppm NH_3[1]	---	245.3	245.5
Adjusted mg NH_3[1]	---	110.38	110.48
Assayed ppm NH_3	245	51.2	46.8
Assayed mg NH_3	24.5	23.04	21.06
mg NH_3 Trapped by 1.0g of ANGC-101 resin	---	8.73	8.94
Mean Value mg NH_3 trapped by 1.0g ANGC-101 resin	---	8.84	
mg N trapped by 1.0g resin	---	7.28	

[1] $\frac{\text{Assayed value of starting solution}}{\text{Calculated value of starting solution}}$ x Calculated ppm of sample

[2] Adjusted mg NH_3 - Assayed mg NH_3 ÷ 10 g resin

The 6.4 pH of the solution did not change after passage through the columns. All solutions were basified for analysis with the ammonia-gas electrode.

Columns 7.1 and 8.1: A second batch of Ion Solution II plus 10 ppm Na^+ and 84.6 ppm ammonium ion was passed through the columns 5 times. This test was conducted 27 days after the first test on columns 7 and 8.

Table 5. Assayed Cation Concentrations in Solutions Containing Ammonium Ion From Columns 7.1 and 8.1

Element	Starting Solution (100 ml)	Solutions passed Through Columns 6X		
		Column 7.1 (450 ml)	Column 8.1 (450 ml)	Mean
Mg^{++}	1.16	0.50	0.69	0.6
Ca^{++}	5.0	1.0	1.5	1.3
K^{+}	7.7	2.1	2.3	2.2
Na^{+}	9.7	69.0	44.0	57.0

Compared to the first solutions passed through these columns, Mg^{++} and Ca^{++} were absorbed to about the same degree, K^{+} was more strongly absorbed and much less Na^{+} was dumped by the resin.

Table 6. Ammonium Ion Absorption Data for Columns 7.1 and 8.1

Ammonia	Starting Solution (100 ml)	Solutions Passed Through Columns 5X	
		7.1-450 ml	8.1-450 ml
Calculated mg NH_3 in Sample	26.62	119.79	119.79
Calculated ppm NH_3 in Sample	84.60	84.60	84.60
Assayed ppm NH_3 (mg/e)	90.08	36.4	48.0
Assayed mg NH_3 in Sample	9.1	16.38	21.60
mg NH_3 trapped by 1.0g ANCG-101 resin	---	11.22	10.69
Mean Value mg NH_3 trapped by 1.0g resin	---	10.95	
mg N trapped by 1.0g resin	---	9.02	

The first ion solution containing ammonium ion that was passed through these columns was reduced from 110 to 22 ppm NH_3. The second was reduced from 85 to about 19 ppm NH_3. Why the resin absorbed that much ammonium ion when it was supposedly saturated may be related to the time lag between the first and second tests. Microbial conversion could have occurred since neither solution or resin were sterile.

Evaluation of a Potential Adverse Influence of ANGC-101 on Perfusion Fluid Cation Balance: Late during this phase of the testing, values for minimum allowable cation concentrations in the OFO Experiment became available and are specified below (8).

MINIMUM ALLOWABLE CATION CONCENTRATIONS	
Sodium	0.05 ppm
Calcium	0.006 ppm
Potassium	0.003 ppm (critical)
Magnesium	(not specified)

If the criteria for determining whether or not the resin adversely alters the cation balance in the perfusion fluid is based on the minimum values, no adverse effects are anticipated from use of the resin. The ANGC-101 resin does alter the ratio of cations in the perfusion fluid depending on a variety of factors, but does not lower the concentration of any cation more than about one order of magnitude. The minimum concentrations of cations present in the ion solutions after contact with the ANGC-101 resin are still two or three orders of magnitude above the minimum levels. Since earlier studies had shown that the presence of ammonium or phosphate buffer alters the relative affinity of the resin for different cations, it was expected that the addition of pure urea, urease enzyme, or frogs and their complex waste products would also alter the resin's cation affinity. Cation studies did form a part of the test plan on the accelerated 30-day test (10 days) and the final system performance test (30 days).

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Discussion of Resin Ammonium Ion Trapping Ability: Dr. Richard Husted found that one of the resin columns he worked with was able to absorb 5.7 mg N/gram of ANGC-101 in 0.05M sodium phosphate buffer (1). The first set of columns exposed to ammonium ion in these tests (5.1 and 6.1) were also exposed to 0.05M sodium phosphate buffer. A trapping ability of 5.8 mg N/gram of resin was found, showing close correlation to Dr. Husted's figure. Columns 7 and 8 which were never exposed to buffer and were always exposed to ammonium ion demonstrated a trapping ability between 7.3 and 9.0 mg N/gram of resin, indicating large amounts of sodium and/or phosphate interfere with ammonium ion absorption.

Urease Activity Testing

When the potential problems associated with use of a buffered perfusion fluid became evident, the function of urease enzyme in non-buffered environments had to be examined. A series of tests were conducted to determine the influence of temperature, ion solutions, buffer, ion exchange resin and antibiotics on urease function. The three test systems employed are shown in Figures 5 , 6 and 7. Each test is described separately in the following pages.

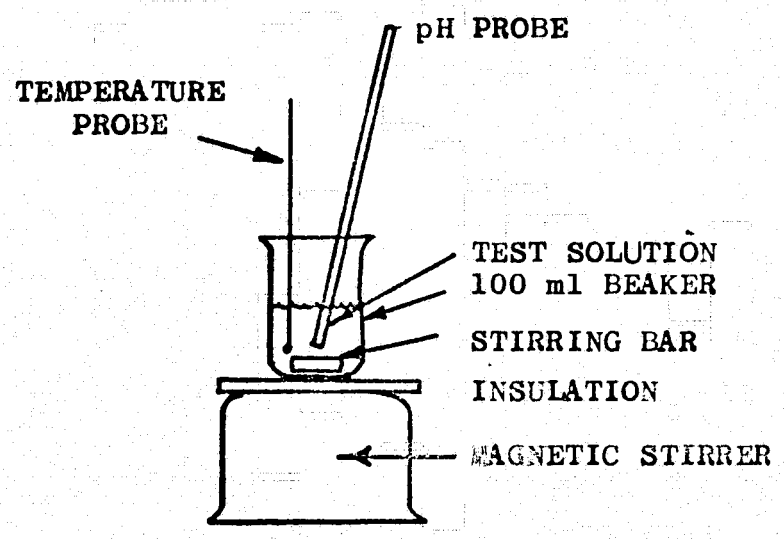

Figure 5 System for a Low Volume Test at Ambient Temperature.

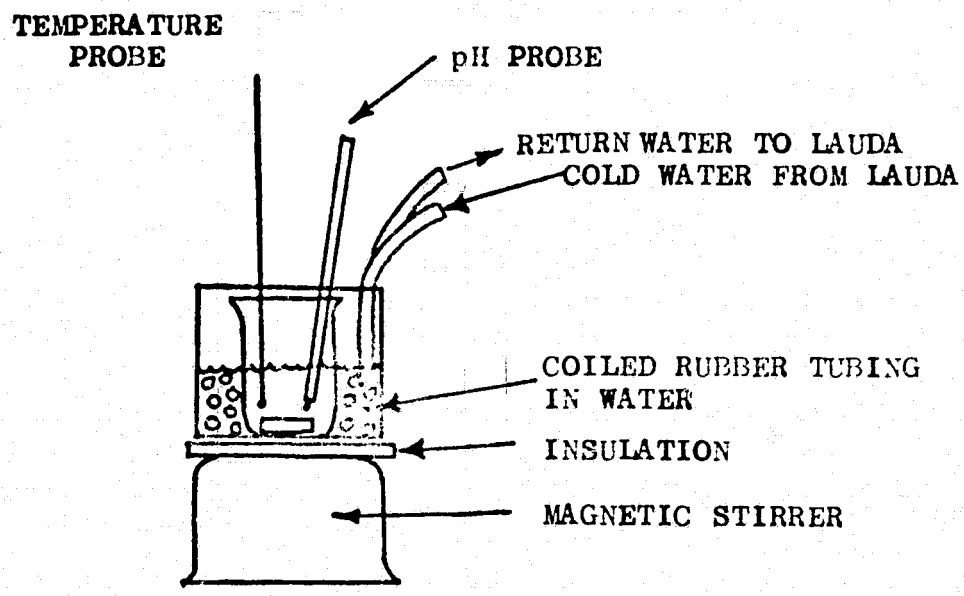

Figure 6 System for a Low Volume Test at 16.5 C.

Test a - OBJECTIVE: Determine free urease activity under OFO similar conditions.

DESCRIPTION: 3.8 liters of Ion Solution II plus 8 ppm Na^+ was placed in the Lauda bath (Figure 7) and allowed to stabilize at 16.5°C. The temperature was monitored with a digital thermometer. The pH was monitored and recorded using a Beckman, Zerometic pH meter and Varian recorder. Oxygen gas was sparged into the solution. 152.0 mg urea (Baker analyzed) was added and allowed to mix for 15 in the bath giving a final concentration of 40 mg urea/liter. At that time 51.8 mg jackbean urease from Baker Chemical Co. was combined (it did not dissolve well) with ion solution and added

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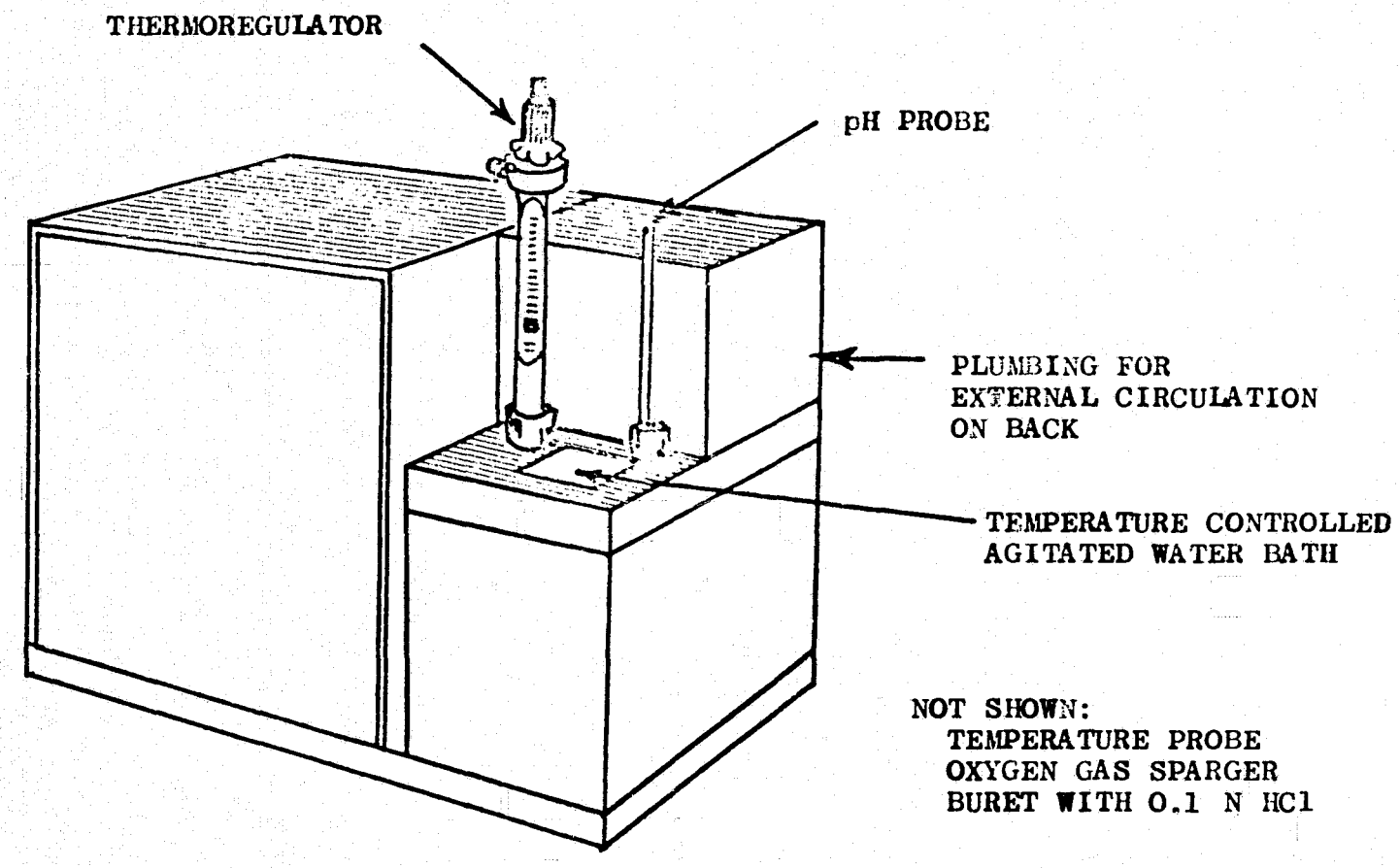

Figure 7 System for a Large Volume Test at 16.5 C. Lauda Constant Temperature Circulator

to the bath. Samples for urea and ammonium assay were periodically drawn and flash frozen in liquid nitrogen.

RESULTS: The initial pH was 6.9, rising to 8.2 about 1 hour after urease addition. It then slowly declined to pH 7.6 over the next 5 hours. Urea and ammonium samples were assayed by methods described in the appendices and the results are shown in Table 7.

Table 7 Urea and Ammonium Assay Data: 16.5°C, no buffer, no ion exchange resin, in Ion Solution II plus 8 ppm Na^+; Baker, jackbean urease

Time in minutes	mg NH_4^+/liter	mg urea/liter (ppm)
0	-	36.0
40	1.7	30.7
70	2.0	33.2
130	4.0	33.5
201	4.8	34.8
243	4.8	33.2
300	7.1	33.5
360	5.0	27.2

DISCUSSION: The data presented in Table 7 indicates there was very little or no enzyme activity in the system. There were several possible explanations postulated: (1) something was wrong with the enzyme, (2) the enzyme may require phosphate buffer to function, or (3) the enzyme may not function in Ion Solution II plus 8 ppm Na^+.

The question was also raised as to how much NH_3 would be scrubbed from the system by the sparged oxygen at pH 8.2 at 16.5°C.

Test b - OBJECTIVE: Determine extent of NH_3 stripping at pH 8.2 at 16.5°C in Ion Solution II plus 8 ppm Na^+ containing 20 ppm NH_4^+.

DESCRIPTION: 3.8 of Ion Solution II plus 8 ppm Na^+ containing 24.0 ppm NH_4^+ was adjusted to pH 8.2 with NaOH in the Lauda bath. The solution was circulated and oxygenated. Samples were withdrawn for ammonium assay.

CONCLUSION: No stripping of NH_3 takes place. Ammonium concentration was the same (within analytical error) at the start and at the finish 5 hours later.

Test c - OBJECTIVE: Ascertain the real activity of Baker Chemical Co.'s jackbean urease enzyme.

DESCRIPTION: A urease activity assay was carried out as described in the appendices.

CONCLUSION: Our assay indicates Baker's enzyme had only 2.8 units/mg[1] of activity. It was very insoluble in aqueous solution. Inactive enzyme was apparently the major cause of failure in Test a.

Test d - OBJECTIVE: Ascertain the activity of Worthington Biochemical Corporation's urease Lot URC 44E040 (we had the enzyme in cold storage for about one year).

DESCRIPTION: A standard activity assay was carried out.

CONCLUSION: Worthington's urease showed an activity of 59.8 units/mg. It dissolved easily in aqueous solution.

Test e - OBJECTIVE: Same as Test a: Determine free urease activity under OFO similar conditions.

DESCRIPTION: Same as Test a except 180.0 mg urea (Baker analyzed) was added and allowed to mix for 1 hour 24 minutes (a final concentration of about 47 mg/l) before 50.0 mg of Worthington's urease lot URC 44E040 was added. Samples were drawn as before for ammonium and urea assays. A microbial assay for total count was also performed at the final assay time.

RESULTS: The pH immediately increased from 7.0 to about 8.7-8.8 in the first minute and remained there for about 30 minutes. It then slowly declined to 7.6 over the next two hours and again increased to 7.7-7.8 in the next 2.5 hours after that. The pH stabilized at that level and

1. 1 unit consists of a conversion ability of 1 micromole/min.

did not change in the next 21 hours.

Urea and ammonium assay data are shown in Table 8.

Table 8 Urea and Ammonium Assay Data: 16.5°C, no buffer, no ion exchange resin, in Ion Solution II plus 8 ppm Na^+, Worthington Urease - URC 44E040

Time in minutes	ppm NH_4^+	ppm urea
0	-	36.0
27	1.0	35.9
42	1.4	43.5
72	1.7	41.5
130	2.0	40.0
192	1.5	32.5
334	1.4	40.0
1555	1.6	42.0

An assay of the test solution at 1555 minutes (about 26 hours) revealed a total microbial count of 2.0×10^4 microorganisms/ml. A dilution series was prepared from the solution, duplicate spread plates on Trypticase Soy Agar (TSA) were made from the dilution series and the plates were inverted, incubated for 48 hours at 32°C prior to counting. All of these organisms produced basic materials and an odor of NH_3 growing on TSA. There were four bacterial colony types, one fungus, and possibly one actinomycete on the plates.

DISCUSSION: Assay data for this test appears quite erratic. Examining ammonium assay data alone, it would appear that there was some low level of enzyme activity, however, when compared to the erratic urea data it is clear that, again, the enzyme did not work to eliminate urea from the system. Postulated explanations for the failure included the following: (1) rapid change in pH causes inactivation of the enzyme, (2) enzyme is inactivated by the preparation, dissolution technique used prior to adding it to the system, and/or (3) enzyme may be inactivated by Ion Solution II plus 8 ppm Na^+.

No explanation is advanced as to why the urea concentration seemed to increase during the test. The validity of our urea assay technique was reverified. Because of these tests, different facets of the problem were examined on a smaller scale (50 ml reaction volume).

Test f - OBJECTIVE: Ascertain the activity of a new activity of a new shipment of Worthington urease - URC 35A616.

DESCRIPTION: A standard activity assay was carried out on the new enzyme which had an activity of 78 units/mg marked on the bottle.

CONCLUSION: The assay showed an activity of 31 units/mg, probably sufficient for the system.

Test g - OBJECTIVE: Determine if the enzyme is denatured by Ion Solution II plus 8 ppm Na^+.

DESCRIPTION: 10 mg of urease (URC 35A 616) was dissolved in 10 ml of 0.02M potassium phosphate buffer to give a clear solution. A portion of the solution (1 ml) was diluted to 50 ml with Ion Solution II plus 8 ppm Na^+ and assayed by a standard enzyme activity assay. Reaction was carried out at 25°C in a Figure 5 system.

CONCLUSION: The enzyme assayed at 33.5 units/mg indicating the enzyme was fully active in Ion Solution II plus 8 ppm Na^+.

Test h - OBJECTIVE: Determine if urease is active in Ion Solution II plus 8 ppm Na^+ with no buffer or pH control at 25°C.

DESCRIPTION: A second portion of the enzyme - Ion Solution II plus solution (25 ml) (from g) was added to 25 ml of urea solution (100 ppm, made up in Ion Solution II plus 8 ppm Na^+ to give a final solution of 50 ml containing 1 mg urease and 50 ppm urea. A test system like Figure 5 was used. Samples of 1.0 ml were removed periodically and flash frozen in liquid nitrogen for urea assay later.

RESULTS AND CONCLUSIONS: Urea assay data shown in Table 9 demonstrate urea was not removed under conditions of the test.

Table 9 Urea Assay and pH data: 25°C in Ion Solution II plus 8 ppm Na^+, no buffer or pH control, no ion exchange resin

Time in Minutes	pH	ppm urea
2	-	50
5	6.80	45
15	6.90	46
30	6.95	45
60	6.95	40

Test i - OBJECTIVE: Determine if urease is active in potassium phosphate buffer at 25°C.

DESCRIPTION: Using a test system like Figure 5 , 25 ml of 100 ppm urea solution was added to 25 ml of solution containing 1.0 mg of urease enzyme. Both solutions were prepared in 0.05M potassium phosphate buffer at pH 7.0. Samples were withdrawn and flash frozen for later assay.

RESULTS AND CONCLUSION: The urea assay data shown in Table 10 indicate the enzyme is very effective in hydrolyzing urea in phosphate buffer at 25°C.

Table 10 Urea Assay Data: 25°C in potassium phosphate buffer, no Ion Solution II plus 8 ppm Na^+, no ion-exchange resin

Time in minutes	ppm urea
2	50
5	46
15	39
30	30
60	22
72 hours	Not detectable

Test j - OBJECTIVE: Determine if urease's active in Ion Solution II plus 8 ppm Na^+ in the presence of ANGC-101 ion exchange resin at ambient temperature (about 25°C) with pH control.

DESCRIPTION: 25 ml of solution containing 100 ppm urea and 200 mg ANGC-101 resin (pre-washed 3 times with glass distilled water to remove fines) prepared in Ion Solution II plus 8 ppm Na^+ was added to 25 ml of ion solution II plus 8 ppm Na^+ containing 1 mg urease. The pH was maintained by the addition of 0.1 N HCl. A test system like Figure 5 was used.

RESULTS AND CONCLUSION: The results shown in Table 11 clearly show that the enzyme is effective in removing urea at 25°C if the resin is present and the pH is controlled.

Table 11 Urea assay and pH data: 25°C in Ion-Solution II plus 8 ppm Na^+ with ANGC-101, no buffer

Time in minutes	pH	Addition of 0.1 N HCl	ppm urea
2	-	-	21
5	-	-	9
7	9.00	-	-
9	7.25	2 drops	-
19	8.25	-	-
20	-	-	0
21	-	1 drop	0
30	7.70	-	0
45	7.70	-	0
60	7.72	-	0

A control experiment was performed concurrently with Test j. It was identical in every respect except no enzyme was present. Results shown in Table 12 demonstrate that there is no significant interaction between the resin and urea in solution.

Table 12 Urea Assay and pH Data: 25°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101, no buffer, no urease

Time in minutes	pH	ppm urea
1	6.7	50
30	6.7	46
60	6.7	44
20 hours	6.7	48

Test k - OBJECTIVE: Determine the effectiveness of urease urea removal in ion solution II plus 8 ppm Na^+ with ANGC-101 ion exchange resin at 16.5°C.

DESCRIPTION: A test system similar to Figure 6 but with an open rather than a closed external loop on the Lauda circulator was used for the test. In all other respects it was set up just like Test j conducted at 25°C.

RESULTS AND CONCLUSION: An open external loop on the Lauda is a problem because the pumped flow must be adjusted to the rate of gravity flow back into the bath. Because of difficulties in adjusting the flow the test started at 15.9°C instead of 16.5°C and did not reach 16.5°C until 25 minutes into the test. After that time temperature was stable at 16.5°C. Urea assay data is shown in Table 13 . Figure 9 shows the data from this test and Test m, both conducted at 16.5°C. Solution pH during the test is shown in Figure 8 .

Table 13 Urea Assay Data: 16.5°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 ion exchange resin

Time in minutes	ppm urea
0.0	50.0
1.5	32.2
6.0	37.2
10.0	31.2
40.0	29.6
50.0	28.7
60.0	26.0

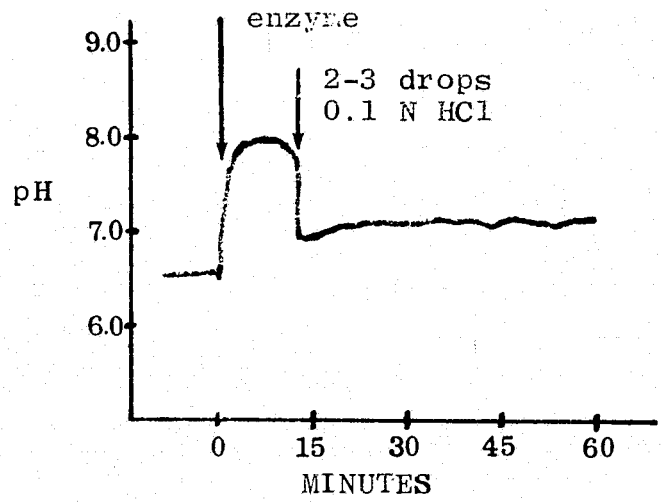

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Figure 8 Solution pH During Test k

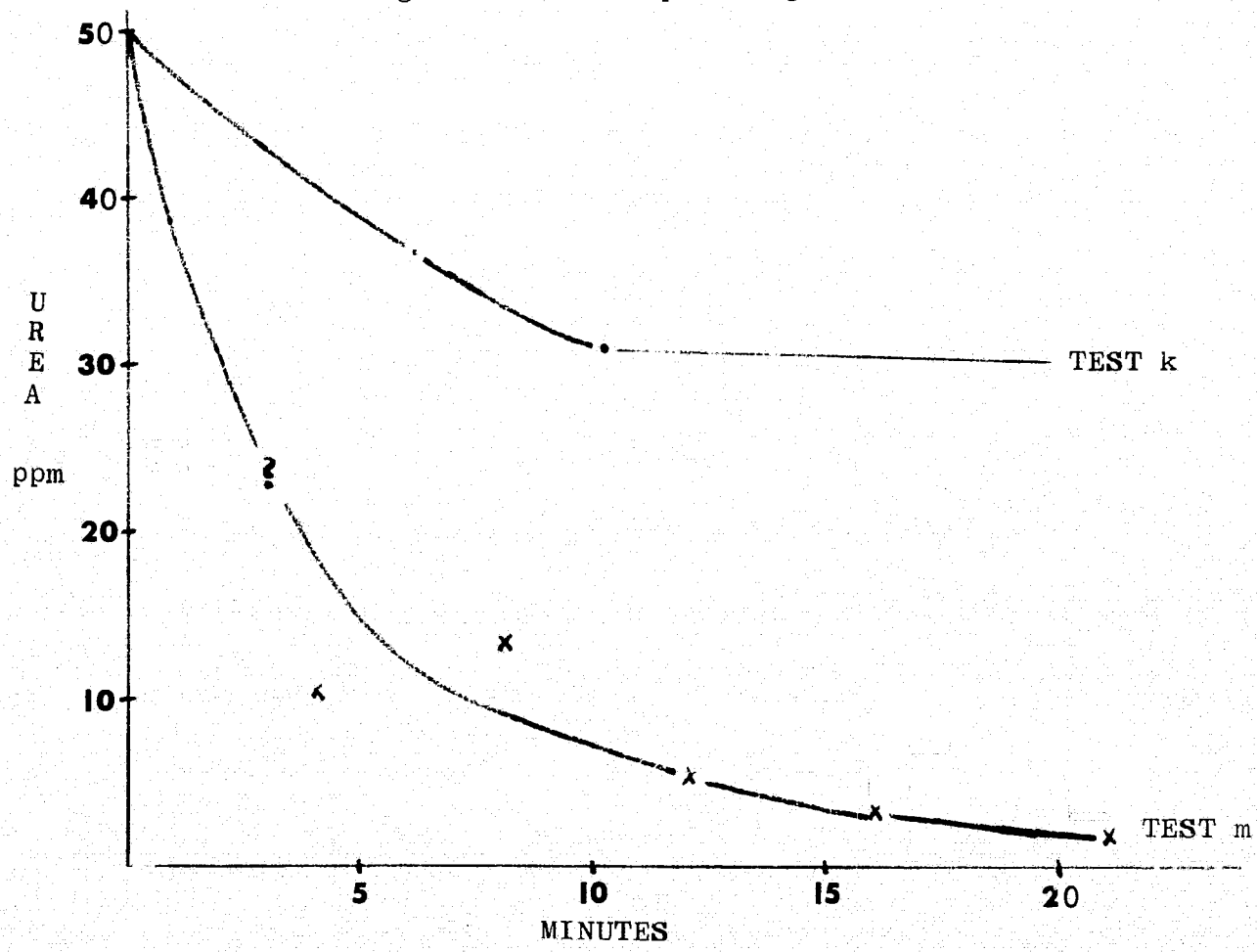

Figure 9 Urea Hydrolysis by Urease at 16.5°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 and pH control

Examining Figure 9 it would appear there was some hydrolysis occurring during the first ten minutes of the test. On addition of the enzyme, the pH of the solution rose to pH 8.0 and remained there until 2 to 3 drops of 0.1 N HCl was added 13 minutes into the test which reduced the pH to about 7.0. Negligible hydrolysis occurred after the first 10 minutes of test. The inactivation of the enzyme is attributed to the uncontrolled pH change which occurred during the first three to four minutes of the test.

Test 1 - OBJECTIVE: Verification of enzyme activity at ambient temperature in Ion Solution II plus 8 ppm Na^+ with ANGC-101 ion exchange resin and pH control.

DESCRIPTION: A test system like Figure 5 was used. This test was identical in every respect to Test j, conducted at ambient temperature (about 25°C). No active temperature control was employed, so the solution in this test did gradually increase in temperature from 25.4°C to 27.7°C.

RESULTS: Urea assay data is shown in Table 14. Figure shows the data from this test plus Test j for comparison. Figure 11 shows solution pH during the test including urease and 0.1 N HCl addition.

Table 14 Urea Assay Data: Same as Test j (about 25°C, Ion Solution II plus 8 ppm Na^+ with ANGC-101)

Time in Minutes	0	5	10	15	20	30	40	50	60
ppm urea	50	6.2	3.7	1.5	1.0	0.6	0.4	0.4	0.4

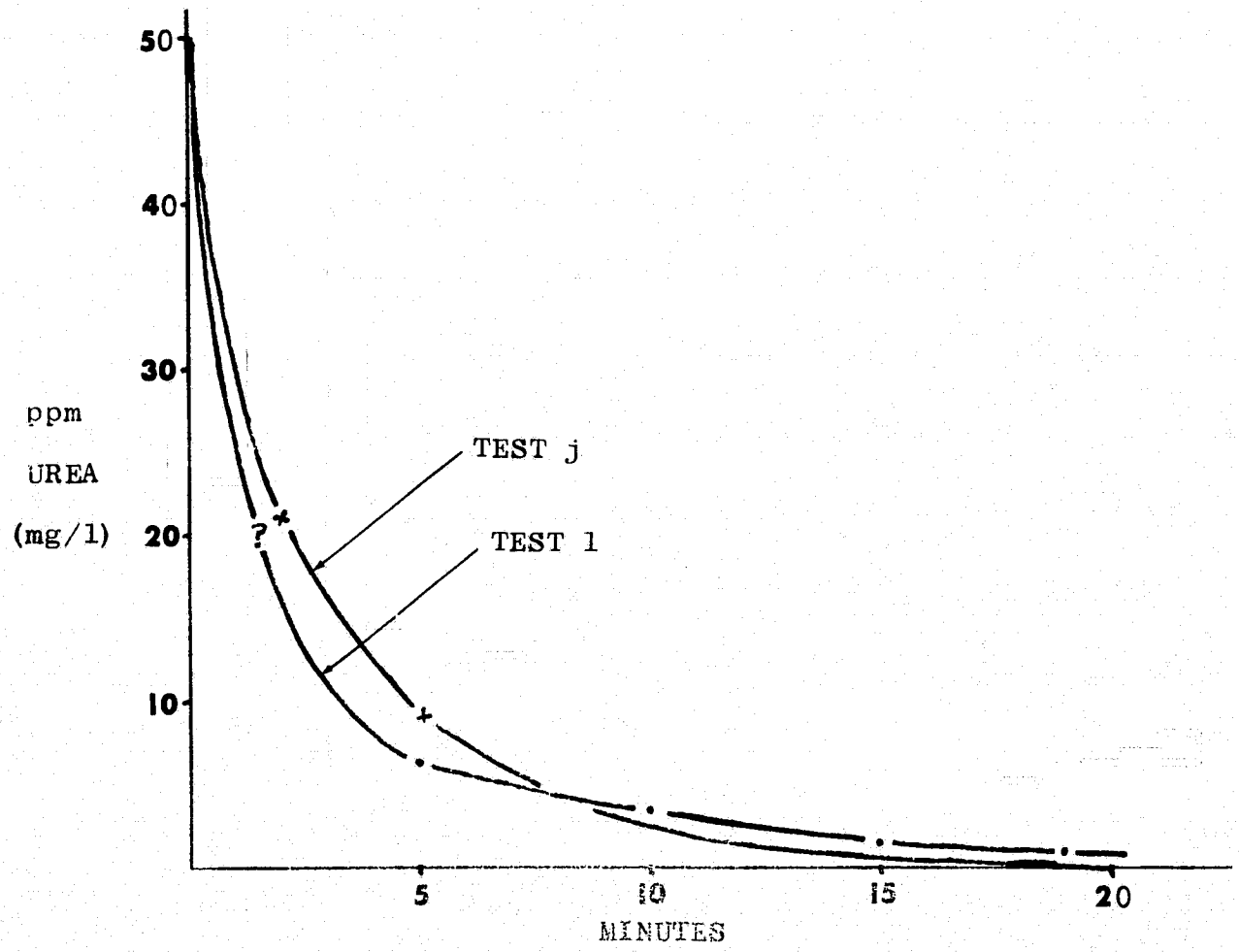

Figure 10 Urea Hydrolysis by Urease at 25°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 Ion Exchange Resin and pH Control

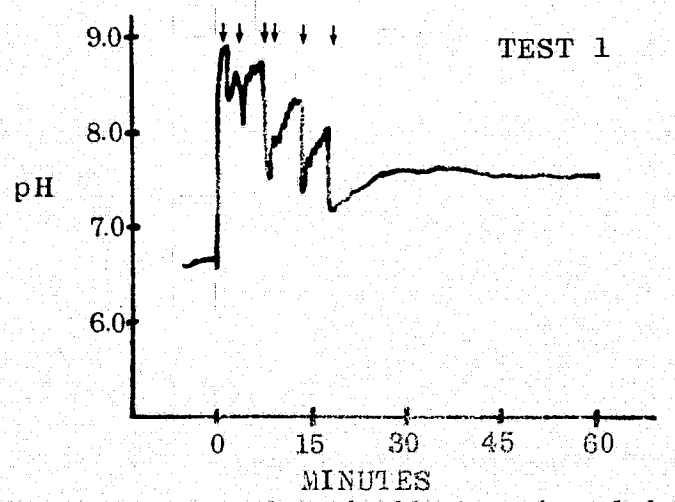

Figure 11 Solution pH and Acid Additions (↓ = 1 drop 0.1 N HCl)

CONCLUSION: Data on the two tests is comparable. In both tests the concentration of urea was reduced to 2% or less of the starting value in 20 minutes in Ion Solution II plus 8 ppm Na^+ with ANGC-101 and pH control at about 25°C.

Test m - OBJECTIVE: Verification of enzyme activity at 16.5°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 ion exchange resin.

DESCRIPTION: Same as Test k in all respects except a closed loop was used on the Lauda for temperature control, i.e., a Figure 6 test system.

RESULTS: Urea assay data is in Table 15. Figure shows data from both tests conducted at 16.5°C. Figure shows the solution pH and urease and 0.1 N HCl additions during the test.

Table 15 Urea Assay Data: Same as Test k

Time in minutes	0	4	8	12	16	21	26	36	46	56
ppm urea	50	10.2	13.5	5.4	3.2	2.0	2.0	1.8	4.9	3.5

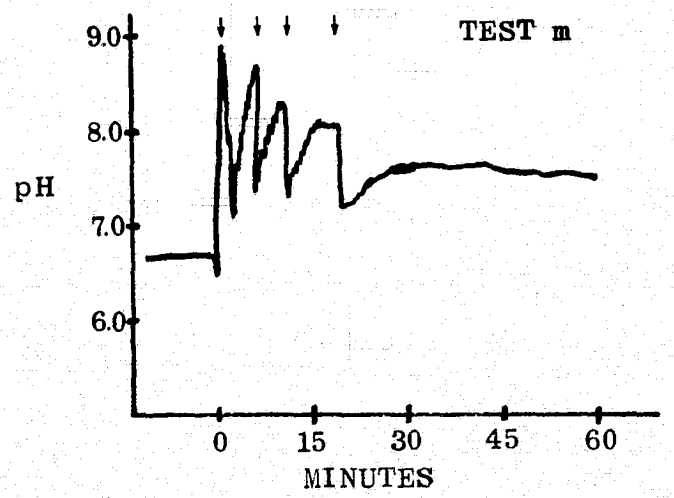

Figure 12 Solution pH and Acid Additions (↓ = 1 drop 0.1 N HCl)

CONCLUSION: As the graph in Figure 9 shows for Test m the concentration of urea was reduced to about 4% of the starting value in twenty minutes. This means a temperature of 16.5°C will not impact system performance.

Test n - OBJECTIVE: Determine the influence of kanamycin antibiotic on enzymatic hydroysis of urea at 16.5°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 ion exchange resin and pH control.

DESCRIPTION: Identical to Test m except 1.0 ml of diluted Kantrex (Kantrex is 1g kanamycin/3 ml) diluted 0.1 ml to 66.6 ml H_2O) was added to the solution containing the urea giving a final concentration of kanamycin of 0.5 mg/50 ml of solution or 10 mg/l. Also, the amount of resin was increased to 300 mg.

RESULTS: Urea assay data is shown in Table 16. Comparison of this data and data from Test o on tetracycline HCl is shown on Figure 13. Solution pH, and additions of urease and 0.1 N HCl during the test are shown on Figure 14.

Table 16 Urea Assay Data: Same as m, but with Kanamycin

Time in Minutes	0	2	4	7	10	15	20	30	40	50	60
ppm urea	50.0	21.2	15.0	13.0	8.7	7.4	6.4	4.2	3.8	4.3	6.0

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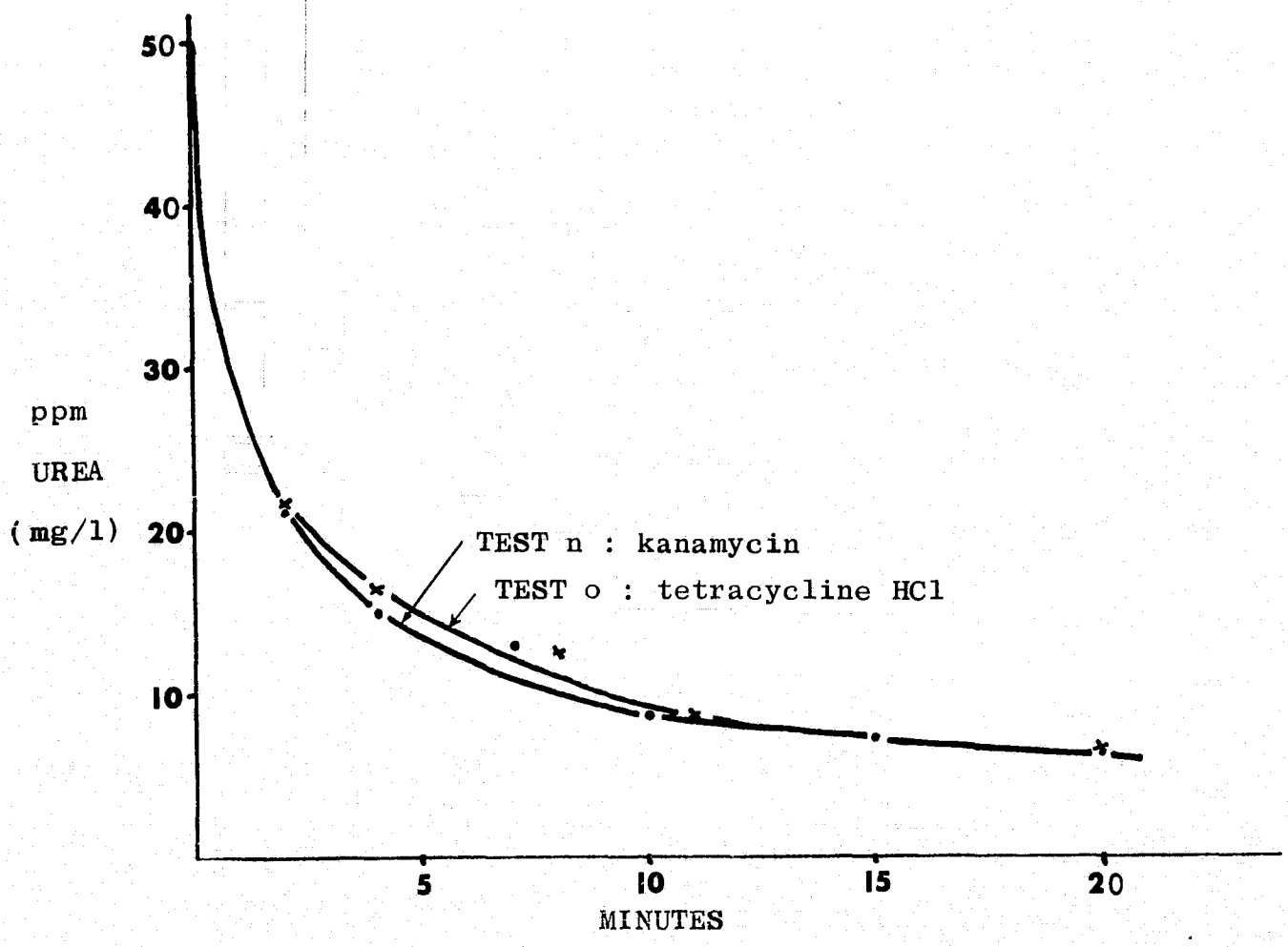

Figure 13 Urea Hydrolysis by Urease at 16.5°C in Ion Solution II plus 8 ppm Na^+ with ANGC-101 and pH Control in the Presence of Antibiotics

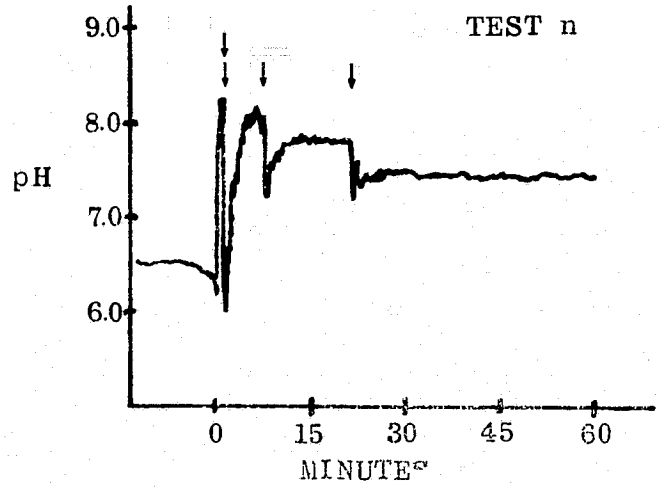

Figure 14 Solution pH and Acid Additions (↓ = 1 drop 0.1 N HCl)

Test o - OBJECTIVE: Determine the influence of tetracycline HCl on enzyme activity at 16.5°C in ion solution II plus 8 ppm Na^+ with ANGC-101 resin and pH control.

DESCRIPTION: Like Test m or n, except tetracycline HCl was added to the urea solution instead of kanamycin. Final concentration of the antibiotic was 0.55 mg/50 ml. 300 mg ANGC-101 resin was used.

RESULTS: Urea assay data is shown in Table 17 . Examine Figure 13 for a comparison of the influence of tetracycline and kanamycin on urea hydrolysis. Solution pH and acid additions are shown in Figure 15 .

Table 17 Urea Assay Data: Same as m but with Tetracycline

Time in Min.	0	2	4	8	11	15	20	30	40	50	60
ppm urea	50.0	21.8	16.5	12.5	8.9	7.5	6.5	7.5	5.0	8.5	6.0

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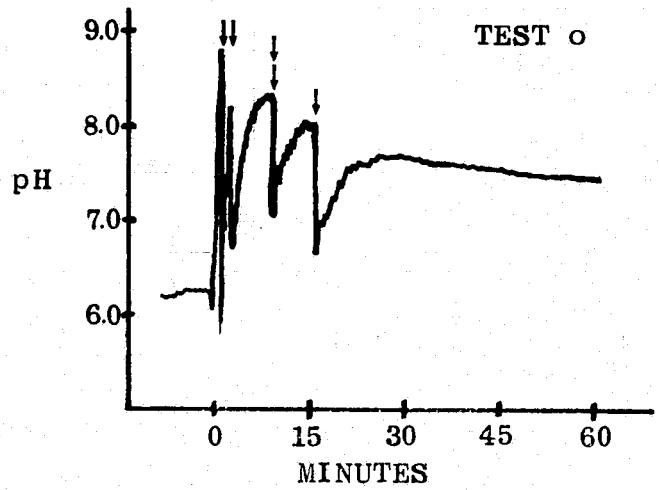

Figure 15 Solution pH and Acid Additions (↓ = 1 drop 0.1 N HCl)

Summary of Urease Activity Testing Results:

- o Worthington's urease lot 35A616 was active at about 31 units/mg.
- o The enzyme is fully active in Ion Solution II (containing 8 ppm K^+, 5.0 ppm Ca^{++} and 1.2 ppm Mg^{++}) plus 8 ppm Na^+.
- o The enzyme does function nicely in phosphate buffer at 25°C.
- o The enzyme does not function without pH control.
- o The enzyme is inactive in Ion Solution II plus 8 ppm Na^+ with no buffer or pH control at 25°C.
- o The enzyme is active in Ion Solution II plus 8 ppm Na^+ in the presence of the resin, with pH control at 25°C. 98% hydrolysis was obtained in twenty minutes in two low volume tests.
- o The enzyme is only slightly less active at 16.5°C. 96% hydrolysis was obtained in twenty minutes in one low volume test.
- o The antibiotics kanamycin and tetracycline HCl effect enzymatic hydrolysis by urease in 20 minutes by about 9%. This small amount of loss is probably acceptable if the antibiotics must be used for bioburden control.
- o Urea is not absorbed or effected by being in contact with ANGC-101 ion exchange resin.

10 Day Simulation of 30 Day Experiment

Objective: The objective of the ten day simulation of a 30 day experiment was to determine the capability of the free urease/ion exchange resin system to maintain an OFO compatible environment. This is defined as maintaining the recirculated water below toxic limits of urea and ammonium ion.

Discussion of Factors Influencing Test Design: In this test the waste excretion rates discussed by Dr. R. Husted (1) (i.e. 28 mg urea and 19 mg NH_4Cl/day/two 350g bullfrogs) determined urea and ammonium chloride addition rates. To simulate a 30 day experiment in 10 days the urea and NH_4Cl concentrations were tripled to 84 mg urea and 57 mg NH_4Cl per day. Free urease enzyme was added when the assayed urea concentration approached 50 mg/liter and periodically after that. Urease requires pH control in unbuffered water, so a buret containing 0.1 N HCl was set up directly over the Lauda bath.

Although an ammonium ion trapping ability of 7 to 9 mg N/g was found for the resin in columns 7 and 8, the figure of 5.7 mg N/g from Dr. Husted was used for sizing the resin bed in this test. Estimated resin requirement on that basis would be 95.0g. A safety factor of 26% was added, bringing the total resin up to 120 g prior to washing the resin to remove the fines.

Description of Test: The following is a list of guidelines that were used in setting up this test.

maximum allowable urea 50 mg/liter

urea generation rate (2-350g frogs) 28 mg/24 hours

ammonium ion generation rate (2-350g frogs) 19 mg/24 hours(NH_4Cl)

ammonium trapping ability of resin 5.7 mg N/g resin

recirculation rate 300 ml/min

temperature of water 16.5°C

initial cation concentrations (8 ppm K^+, 8 ppm Na^+, 5.1 ppm Ca^{++}, 1.2 ppm Mg^{++})

oxygenated with sparger

sample daily

assay urea, NH_4^+, NO_3^-, pH, Na^+, K^+, Ca^{++}, Mg^{++}, and bioburden

maintain data log

A Lauda constant temperature circulating water bath was used in this test to maintain the water temperature at 16.4°C and circulate the water at 360 ml/min. through a flowmeter, a column of ANGC-101 ion exchange resin, and back into the Lauda bath. The column was constructed of glass, had the configuration shown in Figure 16, and was packed with washed resin. 120 g of ANGC-101 was washed in 1 liter of double distilled water three times and then transferred to the column. On the inlet end of the column a glass grid supported the resin over a layer of polyester filter floss. More filter floss and a stainless steel screen in the top of the column prevented the resin from moving from the column into the Lauda bath.

Oxygen gas was sparged into the Lauda bath to maintain dissolved oxygen levels. The temperature probe of a digital thermometer was placed in the bath. Additions of urea, ammonium chloride, urease enzyme, 0.1 N HCl, and Kantrex antibiotic were made directly to the Lauda bath. A total elapsed time meter was used to keep track of sampling times and when additions were made to the system. Equipment layout for the 10 day simulation is shown in Figure 17.

The Lauda bath was decontaminated at the start of this test by circulating water in the bath at 95°C for one hour. The test was initiated by placing 3915 ml of Ion Solution II 98 ppm K^+, 5.1 ppm Ca^{++} and 1.2 ppm Mg^{++}) plus 8 ppm Na^+ into the system, activating the Lauda, and going through the daily routine of observations, sampling, assaying, making additions, adding urease and 0.1 N HCl for pH control and filtering the water through 0.22 Millipore filter membranes or adding Kantrex for bioburden control as necessary. Samples were withdrawn from the system before any additions were made.

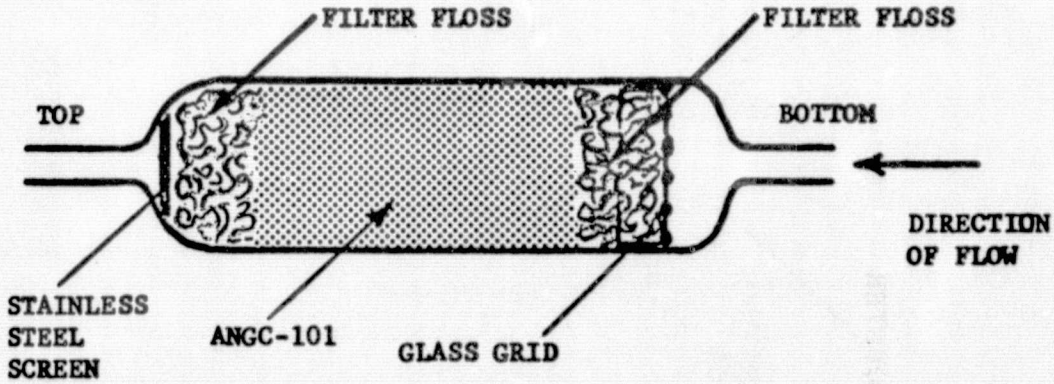

Figure 16 **Ion-Exchange Resin Column**

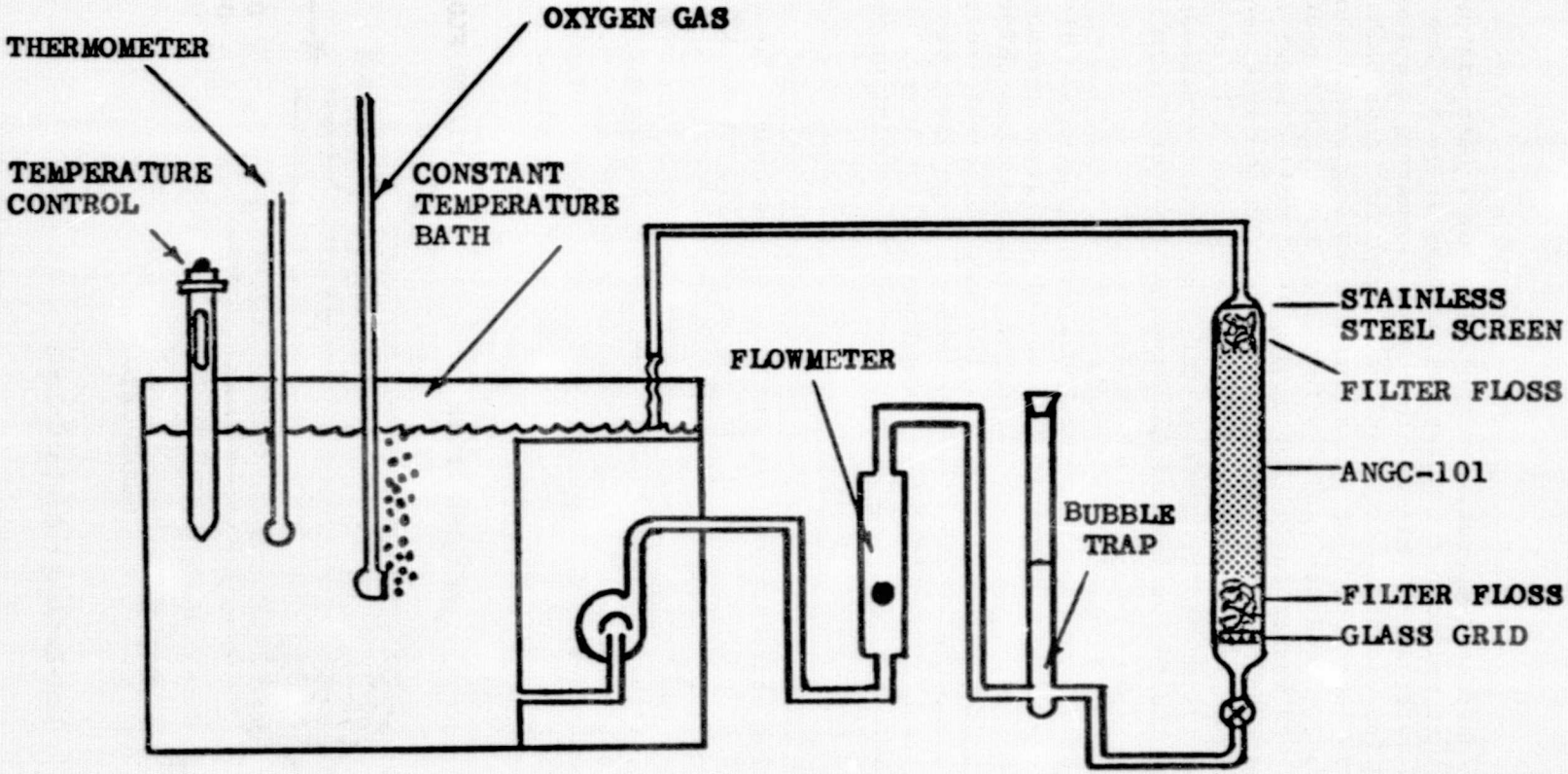

Figure 17 Schematic Diagram for the 10 Day Simulation of a 30 Day Experiment.

Test Results and Discussion: A complete log of all data gathered in the 10 day test is included in the appendices. Specific subtopics of system performance, such as maintenance of an "OFO" compatible environment, column behavior, and bioburden control, are discussed separately on the following pages. Most tables and figures are grouped at the end of this section for readability.

pH: the pH tolerance range of bullfrogs is 6.5-8.5 according to National Academy of Sciences (NAS) guidelines (4). Figure 18, a graph of the pH during the 10 day test clearly shows the pH remained in a range of 6.8-7.2.

Nitrate: NAS guidelines for water supplies for amphibians mention that nitrates and nitrites should not exceed 0.3 ppm (4), however, it is not clear exactly what is meant. Do they mean ppm NO_3^-, ppm sodium nitrate, or what? As a point for comparison, the mean concentration of NO_3^- for all lake and stream water in North America is 1.0 ppm (10).

Figure 19 shows a slight accumulation of nitrate over the ten days of test up to 0.9 ppm NO_3^-.

Urea: A 50 ppm toxicity limit for urea was specified by Catherine Johnson of Ames Research Center on April 19, 1974 in a letter to Dr. R. Husted (1). Figure 20 shows the accumulation of urea in the system from the periodic urea additions and the influence of free urease enzyme additions. Additions of free urease with pH control were able to control urea concentrations. A total of 843.3 mg of urea was added and 776.7 mg was hydrolyzed by a combination of autolysis and enzymatic hydrolysis. Table 18 details an analysis of urea conversion in the 10 day test. Figures 22 through 26 show the pH record during urease additions and pH control by acid addition.

Ammonium Ion: No toxic concentration has been specified. NAS guidelines state that concentrations of 0.24 ppm of free ammonia are detrimental to fish and may also affect amphibians (4). The concentration of ammonium ion at pH 7.0 and 25°C that is in equilibrium with 0.24 ppm of free ammonia is 45.08 ppm. There is a table in the appendices showing these ratios for pH 6-8 and 15-25°C. Samples were basified to convert all ammonium ions to free ammonia for assay with the Orion 95-10 ammonia-gas electrode (see methods of analysis in Appendices). The concentration of ammonium ion never exceeded 5.08 ppm during the test, hence, never coming close to the presumed toxic ammonia concentrations. Figure 21 is a graph of ammonia assay data during the test. In Table 19 data and calculations show that 120 g of ANGC-101 (prewashed weight) absorbed 483.16 mgN (as NH_4^+) giving an overall ammonium ion trapping capacity of 4.03 mg N/gram of resin.

Cations: The minimum allowable cation concentrations have been discussed on page 12. Figures 27 and 28 show the nature of the cation changes taking place in the circulated water during the test. It should be noted that water samples withdrawn for assay were replenished with ion solution identical to the starting solution. Na^+ steadily increases throughout the test. K^+ was never below 0.1 ppm, staying well above the 0.003 ppm minimum. Ca^{++} assayed under the detection limit of 0.1 ppm Ca^{++} on the 2nd day of test, meaning it could have had a concentration of anywhere between 0.1 and 0.0 on the 1st, 5th, 8th and 11th day Ca^{++} was at least 0.2 ppm, again, well above the 0.006 ppm minimum.

Temperature and Water Circulation Rate: The temperature of the Lauda was slightly under the planned 16.5°C, averaging 16.38, with a high of 16.5 and a low of 16.2°C. The water flow rate was adjusted to a setting of 6 on the flowmeter giving a circulation rate of 360-370 ml/minute.

Bioburden Control: Attempts at bioburden control proved rather difficult in this test. On the third day 0.15 ml of Kantrex (kanamycin) was added to the bath and on the 4th, 5th, and 6th days 0.2 ml was added. As Figure 29 indicates the microorganisms that developed in the 10 day test are quite resistant to kanamycin. Filtration of the water through Millipore 0.45μ or 0.22μ membrane filters seemed to help. There does not seem to be an obvious reason for the downward trend during the last two days. Microorganisms from this test and several others have been partially characterized in terms of differential counts and antibiotic sensitivities. From details of that characterization which are found in the Appendices it appears that antibiotics would not be an efficient method of bioburden control.

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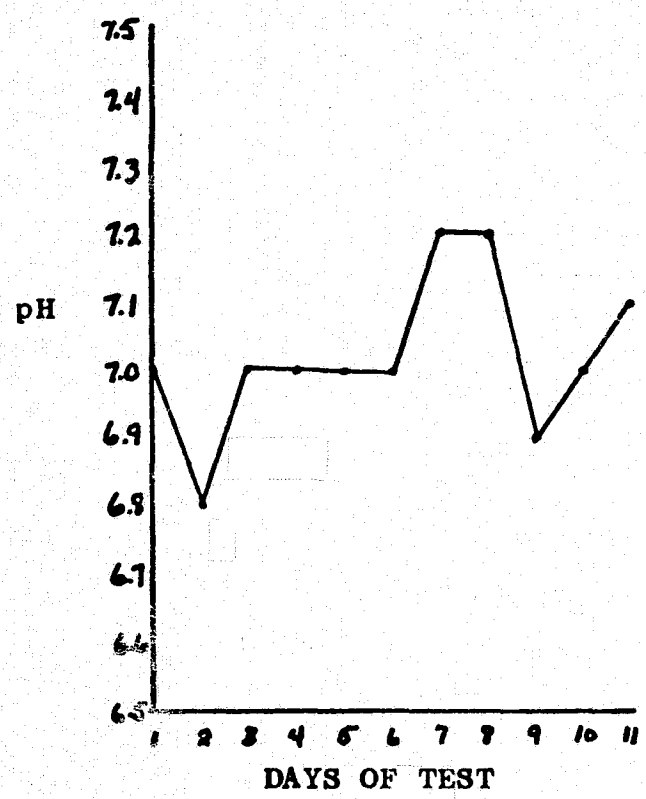

Figure 18 pH in the 10 Day Simulation of a 30 Day Experiment

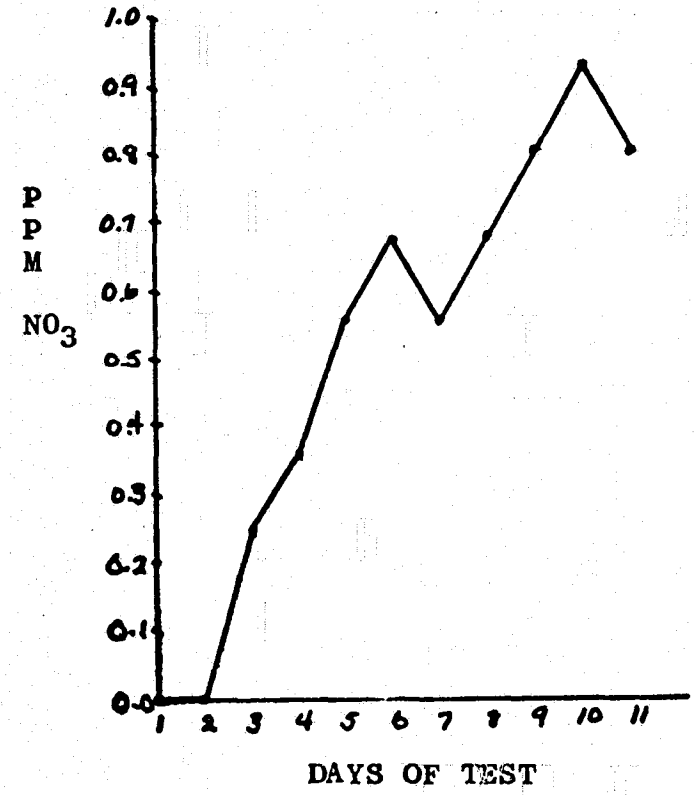

Figure 19 Nitrate Concentration in the 10 Day Test.

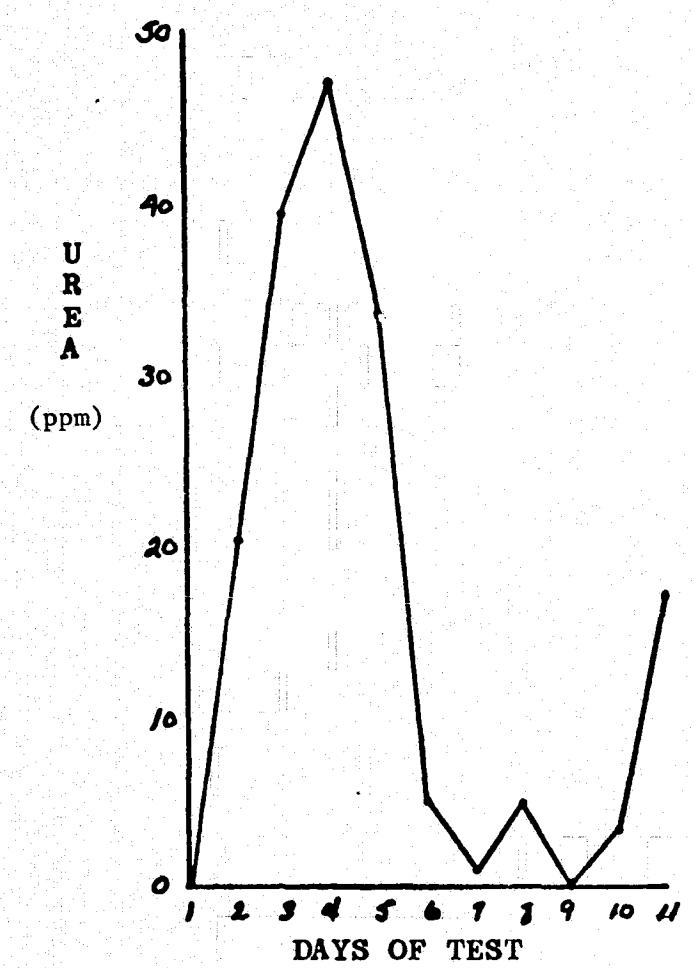

Figure 20 Urea Concentrations in the 10 Day Test

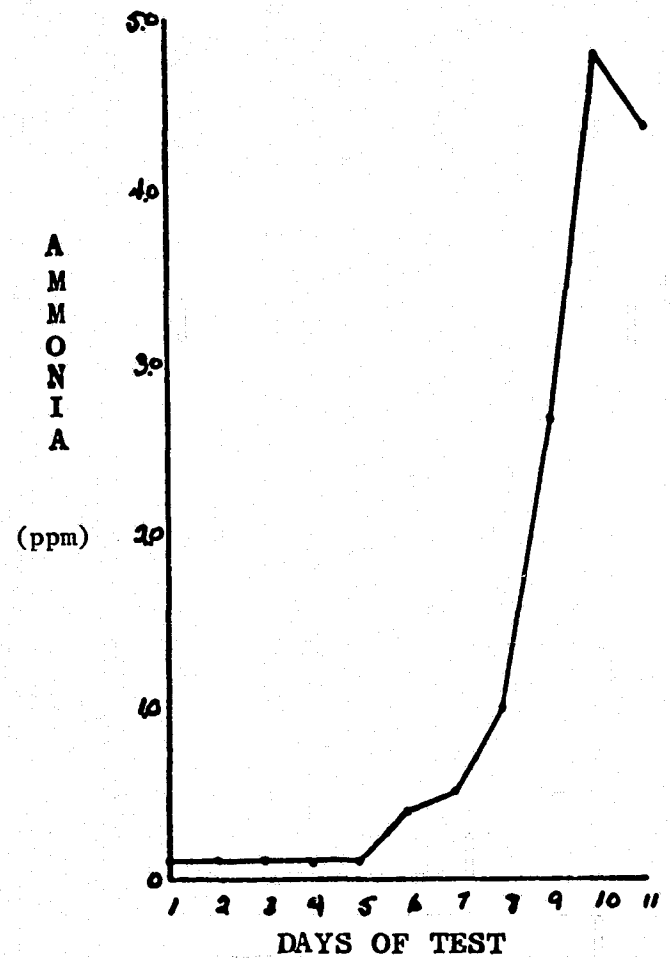

Figure 21 Ammonia Assay Data for 10 Day Test.

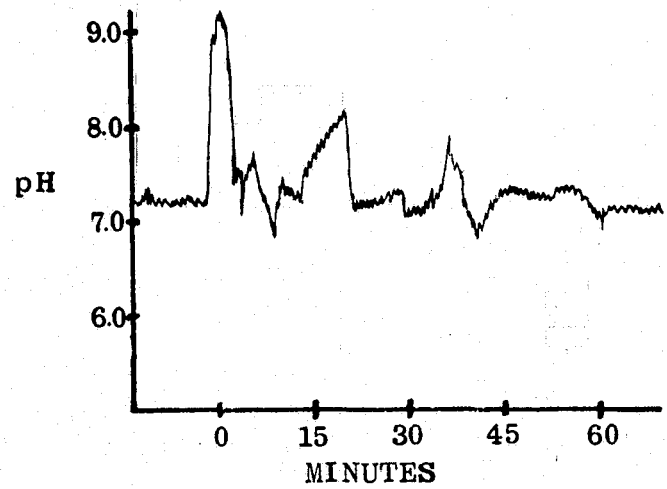

Figure 22 Urease Addition 99.5, 77.1 mg enz.,31.45 ml 0.1 N HCl.10 Day Test.

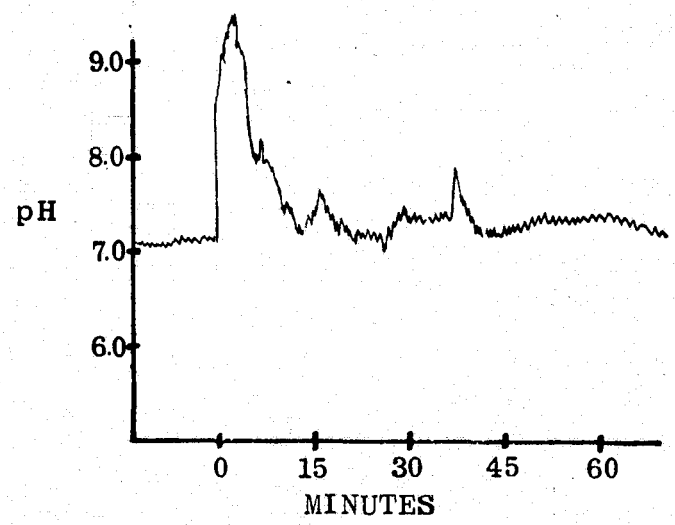

Figure 23 Urease Addition 73.2, 53.7 mg enz.,22.13 ml 0.1 N HCl.10 Day Test.

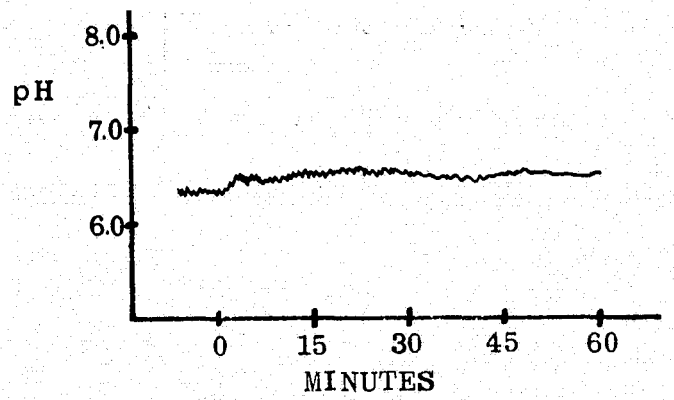

Figure 24 Urease Addition 123.2, 75.8 mg enz.,no acid. 10 Day Test.

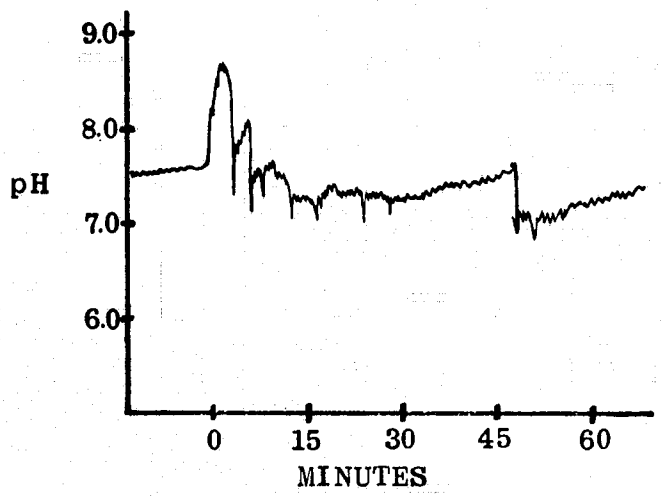

Figure 25 Urease Addition 147.0, 75.0 mg enz.,17.70 ml 0.1 N HCl. 10 Day Test.

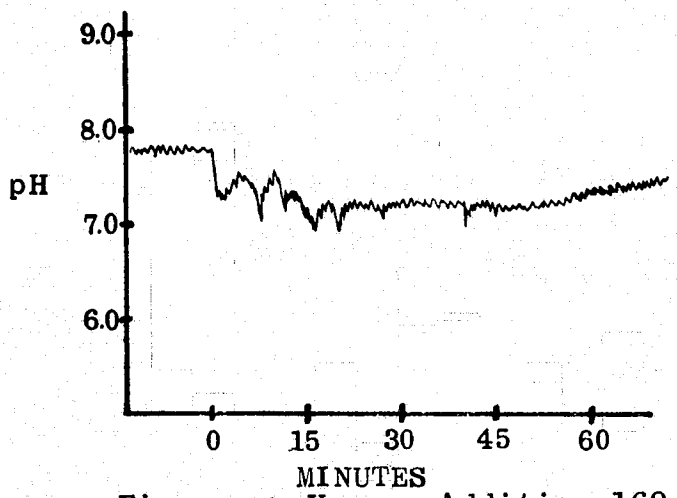

Figure 26 Urease Addition 169.8, 76.2 mg enz.,28.66 ml 0.1 N HCl. 10 Day Test

Table 18 Evaluation of Urea Conversion in the 10 Day Test

DAY	mg UREA ADDED	ppm ADDED DAILY [1]	ASSAYED ppm UREA [2]	UREA CONCEN-TRATION BEFORE UREASE [3]	ppm UREA HYDROLYZED [4]	mg UREA HYDROLYZED [5]	UREASE ADDITIONS	ml 0.IN HCl	ml ACID/ mg UREA H [6]	MICRO-ORGANISMS/ ml
1	85.4	21.81	20.0	21.81	1.8	7.0			0	0
2	83.7	21.38	39.2	41.38	2.18	8.5			0	1.4×10^3
3	83.9	21.43	47.0	60.63	13.63	53.4			0	$>3.0 \times 10^4$
4	84.9	21.63	33.5	68.63	35.13	137.5	53.7	22.13	6.21	4.1×10^6
5	84.0	21.46	5.0	54.96	49.96	195.6	77.1	31.45	6.22	4.7×10^5
6	84.5	21.58	<1.0	26.58	26.58	104.1	75.8	0.0	0	2.1×10^6
7	84.3	21.53	5.0	21.53	16.53	64.7	75.0	17.70	3.66	7.2×10^5
8	84.1	21.48	0.0	26.48	26.48	103.7	76.2	28.66	3.62	3.1×10^5
9	84.2	21.51	3.3	21.51	18.21	71.3			0	4.8×10^5
10	84.5	21.58	17.0	24.88	7.88	30.9			0	3.7×10^4
	843.3					776.7				

1 mg urea added ÷ 3.915

2 Shifted up 1 day since urea added one day before assay

3 Example 20 + 21.38 = 41.38
39.2 + 21.43 = 60.63

4 Urea concentration - assayed ppm urea

5 ppm Col 5 x 3.915ℓ = mg

6 Hydrolyzed

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Table 19 Analysis of Ammonium Ion Absorption in 10 Day Test

DAY	mg NH_4Cl ADDED	mg NH_4^+ ADDED [1]	mg UREA HYDROLYZED [2]	mg NH_4^+ FROM HYDROLYZED UREA [3]	TOTAL mg NH_4^+ ADDED [4]	ASSAYED mg/1 NH_3	ASSAYED mg NH_4^+/1 [5]	TOTAL mg NH_4^+ IN SYSTEM [6]	mg NH_4^+ ABSORBED [7]	mg N ABSORBED [8]
1	57.3	19.28	7.0	4.20	23.48	<0.1	0	0	23.48	18.26
2	57.0	19.18	8.5	5.09	24.27	<0.1	0	0	24.27	18.87
3	57.3	19.27	53.4	32.01	51.28	<0.1	0	0	51.28	39.88
4	56.8	19.11	137.5	82.40	101.51	<0.1	0	0	101.51	78.95
5	57.2	19.24	195.6	117.24	136.48	<0.1	0	0	136.48	106.15
6	57.1	19.21	104.1	62.40	81.61	0.4	0.42	1.644	79.97	62.20
7	56.8	19.11	64.7	38.78	57.89	0.5	0.53	2.075	55.82	43.42
8	59.9	20.15	103.7	62.16	82.31	1.0	1.06	4.150	78.16	60.79
9	57.4	19.31	71.3	42.74	62.05	2.7	2.86	11.197	50.85	39.55
10	56.8	19.11	30.9	18.52	37.63	4.8	5.08	19.888	17.74	13.80
11								18.239	1.65	1.28
										483.16

1 $\text{mg } NH_4Cl \times \frac{18 \text{ mg } NH_4^+}{53.5 \text{ mg } NH_4Cl}$

2 From Table Analysis of Urea Conversion in 10 Day Test

3 $\frac{\text{mg Urea}}{60.06 \text{ mg/m mole urea}} \times \frac{2\text{m mole } NH_4^+}{1\text{m mole urea}} \times \frac{18 \text{ mg } NH_4^+}{\text{m mole } NH_4^+}$

4 From added NH_4Cl and hydrolysis of urea

5 $\text{mg } NH_3 \times \frac{18 \text{ mg } NH_4^+}{17 \text{ mg } NH_3}$

6 mg NH_4^+/ x 3.915 liters

7 Total NH_4^+ added - NH_4^+ in solution

8 $\text{mg } NH_4^+ \times \frac{14 \text{ mg N}}{18 \text{ mg } NH_4^+}$

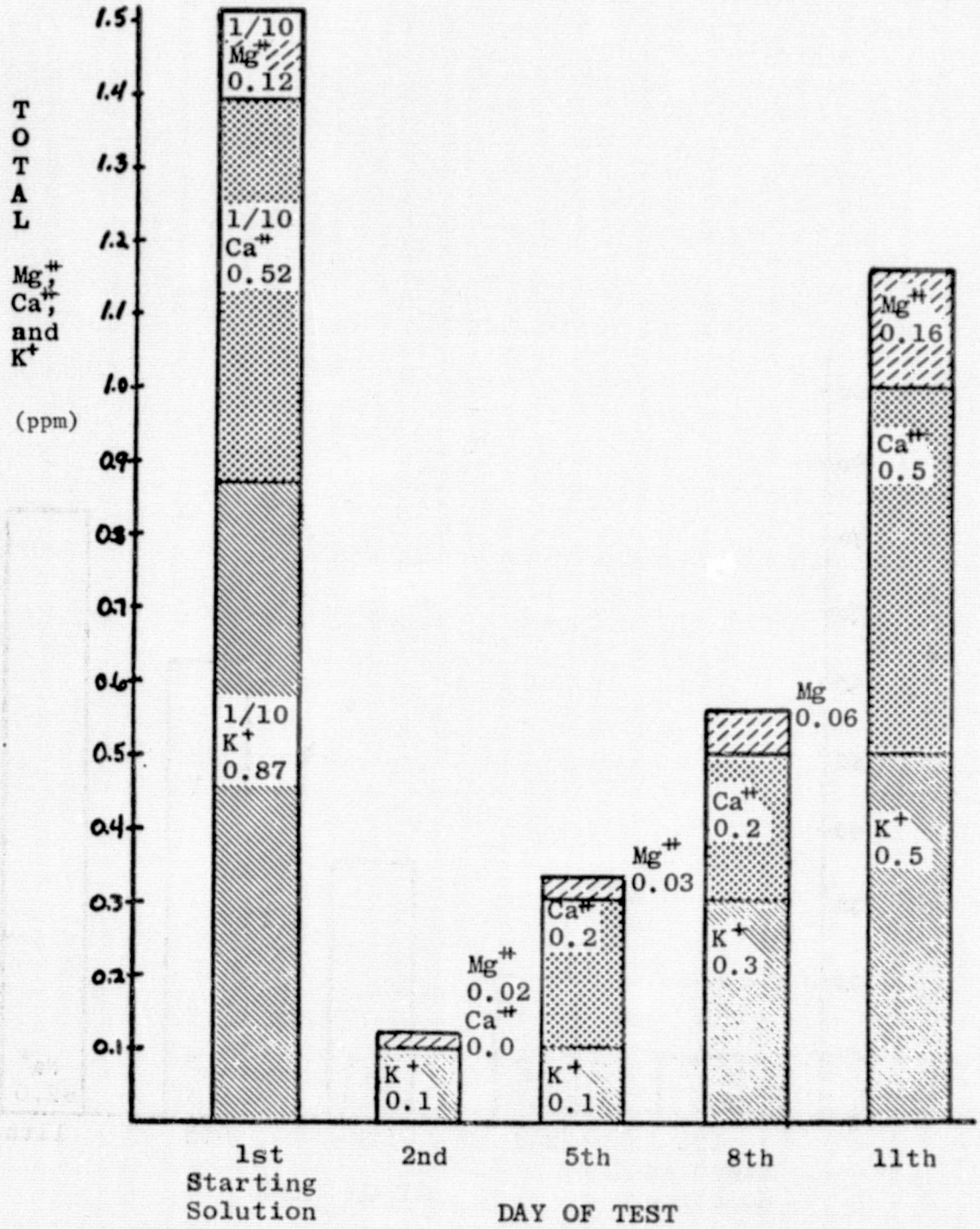

Figure 27 Mg^{++}, Ca^{++}, and K^{+} Concentrations in Solutions from the 10 Day Simulation of a 30 Day Experiment.

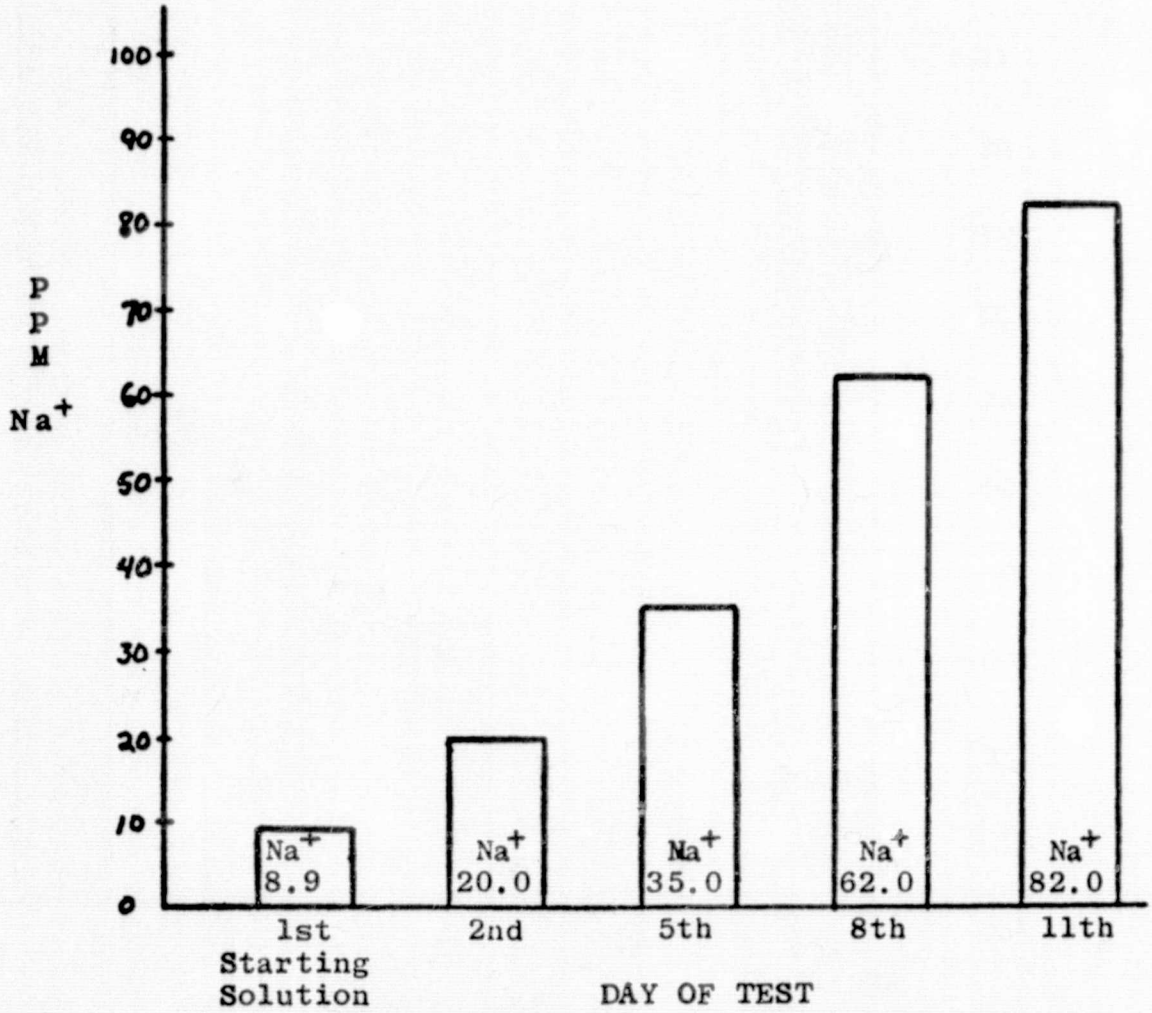

Figure 28 Sodium Concentrations in Solutions from the 10 Day Simulation of a 30 Day Experiment.

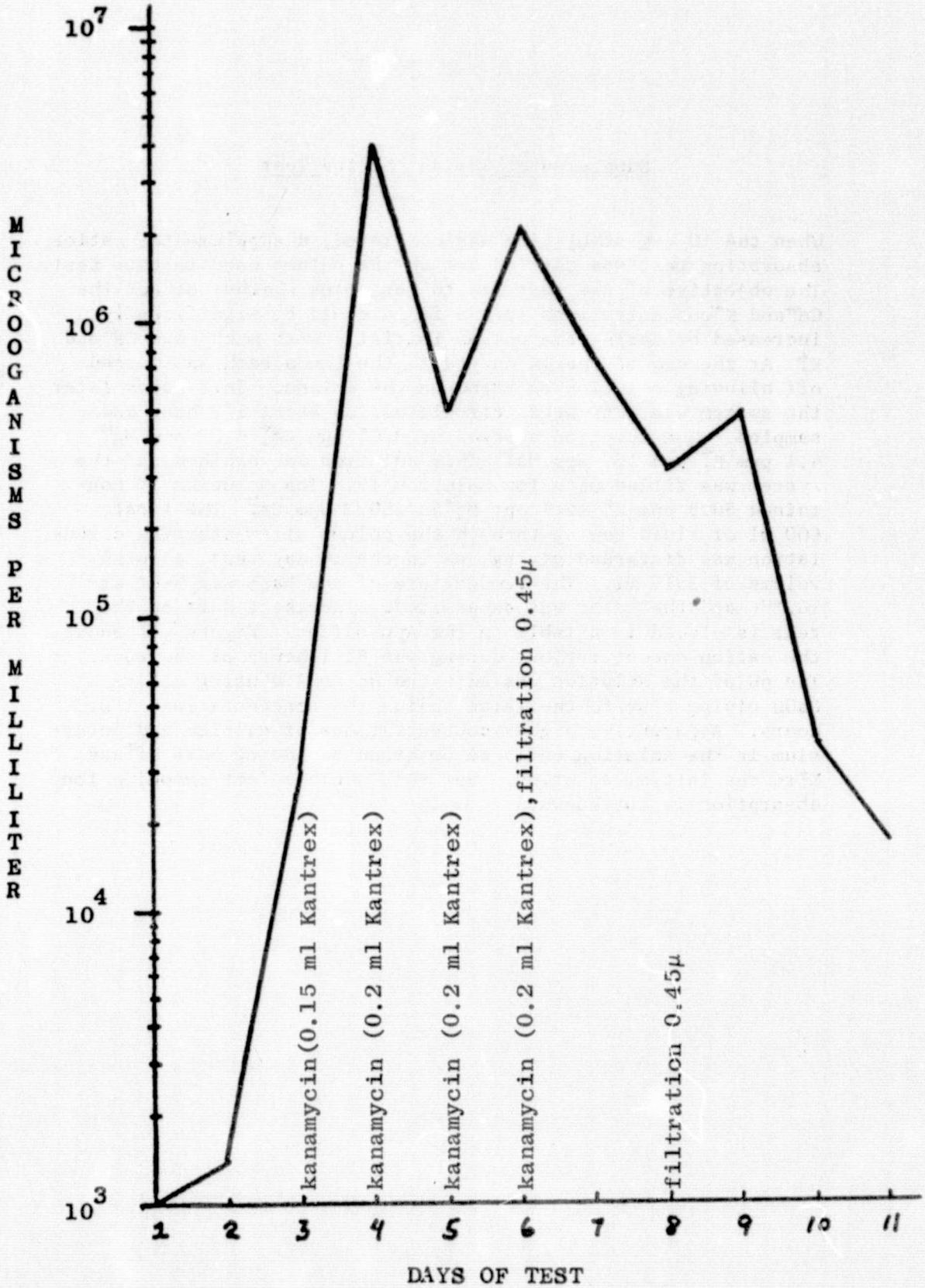

Figure 29 Microbial Burden in the 10 Day Simulation of a 30 Day Experiment.

Supplemental Cation Loading Test

When the 10 day simulation was completed, a supplemental cation absorbtion test was carried out on the column used in that test. The objective of the test was to determine whether or not the Ca^{++} and K^{+} concentrations in the fluid could be significantly increased by dosing the column initially with much more Ca^{++} and K^{+}. At the end of the 10 day test, the Lauda bath was turned off allowing solution to stand on the column. 36.8 hours later the system was activated, circulated for about 1/2 hour and sampled. The solution assayed at 0.02 ppm Ca^{++}, 0.03 ppm Mg^{++}, 4.1 ppm K^{+}, and 127 ppm Na^{+}. This solution was drained and the system was filled with Ion Solution IV. Ion Solution IV contained 80.5 ppm K^{+}, 12.1 ppm Mg^{++} and 50.2 ppm Ca^{++}. The first 600 ml of fluid coming through the column after starting circulation was discarded giving, as in the 10 day test, a total volume of 3915 ml. The temperature of the bath was kept at 16.3°C and the water was oxygenated. Tabulated data on the test is placed in a table in the Appendices. Figure 30 shows the cation concentrations during the 81.1 hours of the test. The pH of the solution was adjusted up to 7.0 using dilute NaOH giving rise to the large sodium ion concentration at 0.3 hours. Apparently, higher concentrations of calcium and potassium in the solution could be obtained by adding more Ca^{++} and K^{+} to the initial solution. How this would effect ammonium ion absorption is not known.

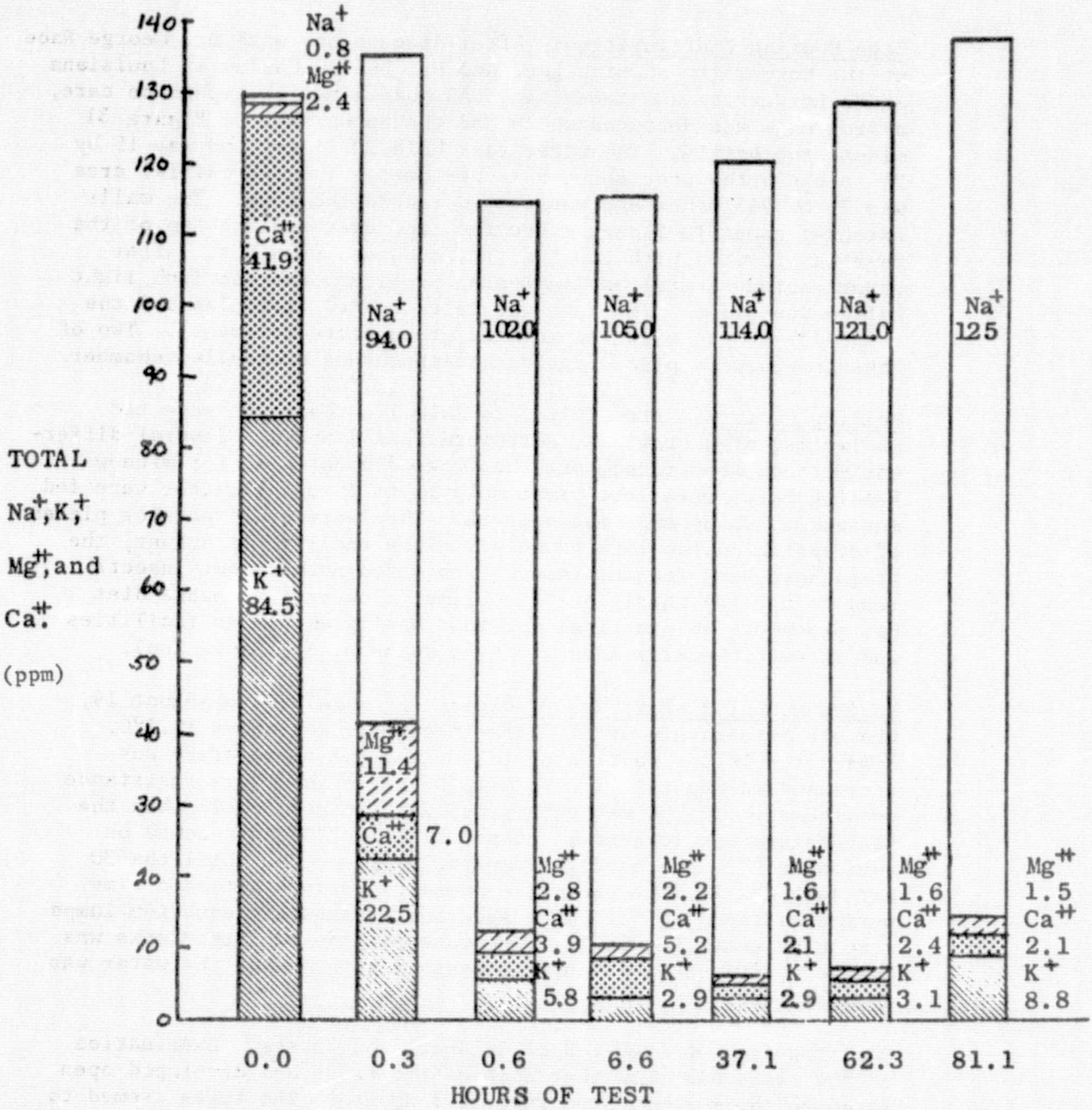

Figure 30 Cation Concentrations in Solutions from the Supplemental Cation Loading Test-10 Day Test.

TASK 5

30 DAY SYSTEM PERFORMANCE TEST

Frog Care and Maintenance

Cage Housing Configuration: After discussions with Dr. George Nace at the University of Michigan, and Dr. Dudley Culley at Louisiana State University and consulting NAS guidelines on amphibian care, a frog cage was designed which had the appearance of Figure 31 minus the baffle. The water tank held 20 liters and was 15 by 24 inches with water about 5 inches deep. The terrestrial area was 21 by 24 inches and was sloped toward the tank. The walls extended about 16 inches above the land area and the top of the cage was covered with one-quarter inch mesh fish net. After experiencing several environmental problems, the ten inch light baffle was added. The tank was latex coated fiberglas and the remainder of the cage was mottled green plate (styrene). Two of these cages were placed inside a temperature controlled chamber.

Feeding History: From July 2 to July 11, the frogs were fed mealworms, nightcrawlers, earthworms and minnows. Several different methods were tried, but the frogs did not care for minnows. Feeding rates were low. From July 11 to August 17, they were fed mealworms, worms and grasshoppers. They were still passing pieces of crayfish on the 19th of July. Since the 18th of August, the frogs have been fed only mice. Frogs fed nothing but insects tend to develop nutritional problems (rickets). A mouse diet has proved to be practical for zoos having amphibian facilities and is nutritionally adequate (Tom Johnson, St. Louis Zoo).

Environmental History and Problems: From July 2 to August 19, the air temperature of the walk-in chamber was about 17.2°C. Toward the latter portion of this time, the temperature was increased to about 22.2°C to help increase the frogs resistance to disease. 22.2°C was maintained until September 18 when the temperature was lowered in steps until 16.7°C was reached on September 27. The average temperature from then until the 30 day test was 17.3°C. Relative humidity during that same time period averaged 50.5%. Wide spectrum fluorescent aquarium lamps were used until August 22. The water in the 20 liter tanks was changed every other day until August 8 after which the water was changed every day.

One frog died on August 8 of unidentified causes. Examination of the frogs did show that some of the frogs had developed open sores on their feet, hind legs, and snouts. The sores seemed to be the result of mechanical abrasion from digging at the sides of their tank. This is not normal and indicated some facet of their environment was overstressing them.

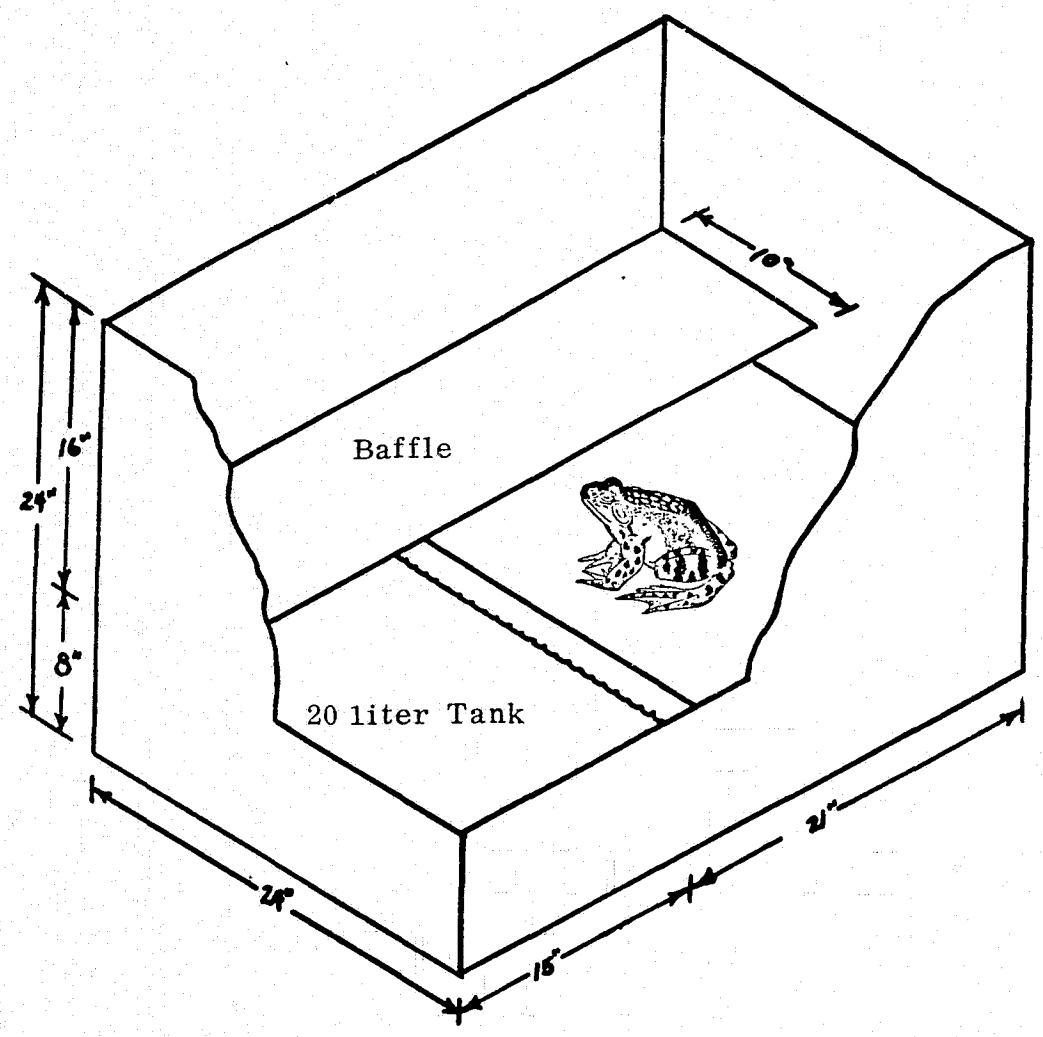

Figure 31 Cage and Tank Configuration for Routine Frog Care

A summary of environment and nutritional factors influencing the frogs up to August 18 is listed below:

- Temperature 17.2°C
- Water was changed every other day up to August 8 and every day thereafter
- Blower noise in the chamber was 72-74 db
- There were six frogs/20 liter tank with a 21 x 24 inch terrestrial area
- Wide spectrum fluorescent aquarium lamps provided lighting. Photo period was not uniform, being on a 8-5 period daily and was mostly off during weekends.
- The frogs would not eat insects dusted with dolomite (as a calcium supplement)

After consultation with several expert herpetrologists, the most likely source of the problem was tentatively identified. The environmental stresses of being transported from a pond in California to a radically different laboratory environment at a higher altitude caused the frogs to be insecure and seek a place to hide. Since a hiding place was not provided, the frogs dug at their tanks causing the sores and providing entry for infection. The following corrective actions were taken to reduce environmental stress on the frogs as much as possible.

- The temperature was increased to about 22.2°C from 17.2°C since the frog's natural immunity is largely temperature dependent(4). 17.2°C is an environmental stress compared to the frog's normal optimum range of 20.0-23.9°C.

- A timer was installed to control the photoperiod to a 12-hour day-night cycle. Ordinary fluorescent lights were used instead of wide spectrum lights to avoid photosensitivity problems related to the antibiotic therapy program that was carried out.

- Translucent baffles were installed in the cages to provide hiding places for the frogs.

- Sound absorbing materials were added to the walls of the chamber to reduce blower noise.

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The frogs were placed on a 7-day antibiotic therapy program for treatment and as a precautionary measure. Three of the frogs with the worst sores died during the treatment. The treatment consisted of the above environment changes plus oral administration of a dose of tetracycline-HCl[1] two times a day with a stomach catheter[2]. The dosage, 5 mg/30 g of body weight, and duration of treatment was based on the recommendations of the National Academy of Sciences guidelines on amphibian care and management (4). The treatment was carried out from August 23 to August 29, 1975, and no food was given during treatment. A mouse diet was resumed on August 29, 1975.

Of the treated frogs, besides the three that died during treatment, two did not respond well to treatment and also eventually died.

Characterization of Frog Waste Water: Assays were performed over a 10 day period on water from the tanks in the frog cages. During this period, the air temperature in the chamber was about 22.2°C and the tap water in the tanks was changed every day after sampling. All data gathered is placed in the Tables section of the Appendices.

For a 10 day period at 22.2°C, eight bull frogs (ranging in weight from 360-650 g each) ate 26 mice for an average intake of 0.30 mice on the left and 0.33 mice/day on the right. The frogs excreted a total of 2.302 g NH_4^+, 9.952 g urea, and other waste products into the water in their cages during the 10 day period. The mice did contribute to these figures somewhat with their own waste products before they were eaten. It should also be understood that the mice were not of uniform weight (ranging from 6 weeks old to adult). The excretion data demonstrate that one mouse should, roughly, contribute 88.53 mg NH_4^+ (263.7 mg NH_4Cl) and 382.7 mg urea to the system when digested by a frog.

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[1] Rachelle, Tetrachel-Vet-102, soluble tetracycline HCl powder.

[2] Approximately six inches of a Robinson #8 Frech catheter with two eyes was attached to a cannula with a 5 ml syringe (Ed Schmidt of the Denver Zoo demonstrated the technique).

Thirty Day System Performance Test

Objective: The primary objective of the thirty day system performance test was to determine if the waste water treatment system could maintain a viable environment for two frogs for thirty days. A secondary objective was to determine if the environment remained suitable for activity of the enzyme urease.

Test Design: As mentioned elsewhere in this report, the frog's intake of food was drastically reduced when the temperature in the environmental chamber was lowered to 16.5°C. In an attempt to reestablish the vigorous feeding of the aclimatization period, the air temperature was increased to 21.5°C while the water in the temperature controlled bath remained at 16.5° and the water in the frog cage was about 17.5°. The food intake improved but remained sluggish.

The ammonia in the water resulted from urea and ammonium compounds eliminated by the frogs. Urea (28 mg/24 hrs 2 frogs) was expected to contribute 15.9 mg ammonia (in salt form) and 6.02 mg ammonia was expected to arise from ammonium salts directly (1, p. 2). The total ammonia excreted was expected to be 21.6 mg/day. At the pretest feeding rate of 0.3 mouse/frog day and at an estimated 83.6 mg ammonia/mouse and 382.7 mg urea/mouse the ammonia burden was expected to be as high as 300.5 mg/frog day or 601.0 mg/day for two frogs. For 14 days prior to test, the mouse intake was only 0.06 mouse/frog day or 20% of the above for a total of 120.2 mg NH_3/day.

The ANGC-101 has a stated capacity of 12 mg of ammonium N/g of ion exchange resin (dry wt). Previously (1, p. 19) we found the resin capacity to be 5.3 mg N/g. Using the latter figure and 120.2 mg NH_3/day the total amount of resin required was expected to be 560.3 g. The column was sized to contain in excess of 200 g and provision made for periodic changing as needed.

A coarse filter was placed in line between the frog water outlet and the inlet to the temperature controlled water circulation (see Appendix on Filtration). The purpose of this filter was to prevent mouse feces, hair from digested mice, and shedded frog skin from entering the resin packed column and possibly plugging it.

The system was provided with positive pumping through the flowmeter, bubble trap, and column but gravity flow from the tank in the frog cage, through the coarse filter and back into the temperature controlled bath.

Test Description: The frogs (Figure 32) were maintained in a cage (Figure 33) inside a temperature controlled Percival R chamber. The lights to the chamber were controlled by a timer to give a photoperiod of 12 hours light and 12 hours of darkness.

The noise from the compressor and blower were measured at 72 decibels (see Appendices). The blower was surrounded with noise absorbing material except for space necessary for proper operation. A rug was hung from the wall to cut down sound reflection and sound absorbing foam was placed around the edge of the cage. The effect was to reduce the noise level somewhat but not more than 5 decibels.

The air temperature was controlled at 21.5°C. The relative humidity was uncontrolled but measured daily, ranging from 18 to 66 and averaging 41.5% relative humidity.

The water in the test system had a total volume of 6162 ml. There were 3200 ml in the constant temperature bath and 2400 ml in the tank in the test cage. The remaining volume was due to the flowmeter, bubble trap, column and connecting tubes. The flow rate was set for 360-370 ml/min. Figure 34 shows a schematic of the whole system.

A bubble trap was placed in line between the flowmeter and the column. It was especially useful during start-up in preventing air bubbles from entering the column. When the test was set up, Ion Solution V was used to fill the tanks and to replace solution lost as samples. Double distilled water was used to replace evaporation losses. Ion Solution V contained no Na^+, 16 ppm, K^+, 1.2 ppm Ca^{++} and 10.0 ppm Mg^{++}.

Mice were added to the frog cage at the end of each day and uneaten mice were returned to their cages at the beginning of the following day. The mice were not fed while in the frog cage. The frogs were also fed mouse puree to maintain an input of food (see Appendices).

The resin used in the column was washed with distilled water and decanted (see Appendices for description of how each column was prepared). The treatment removed very fine particles of resin which tended to give a higher back pressure and more rapid plugging of the column if not removed.

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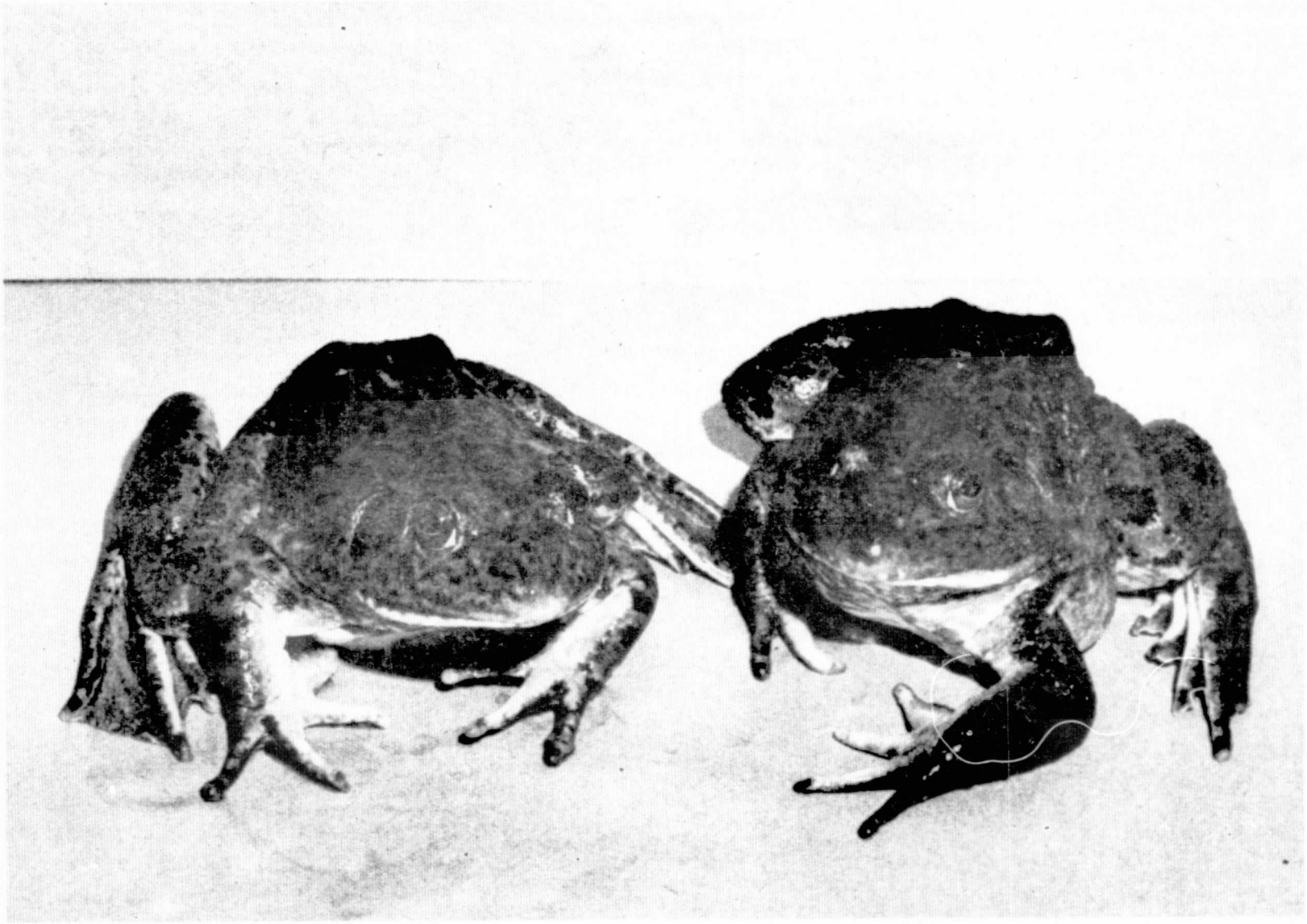

Figure 32 Bullfrogs (Rana catesbeiana) in the 30 Day System Performance Test

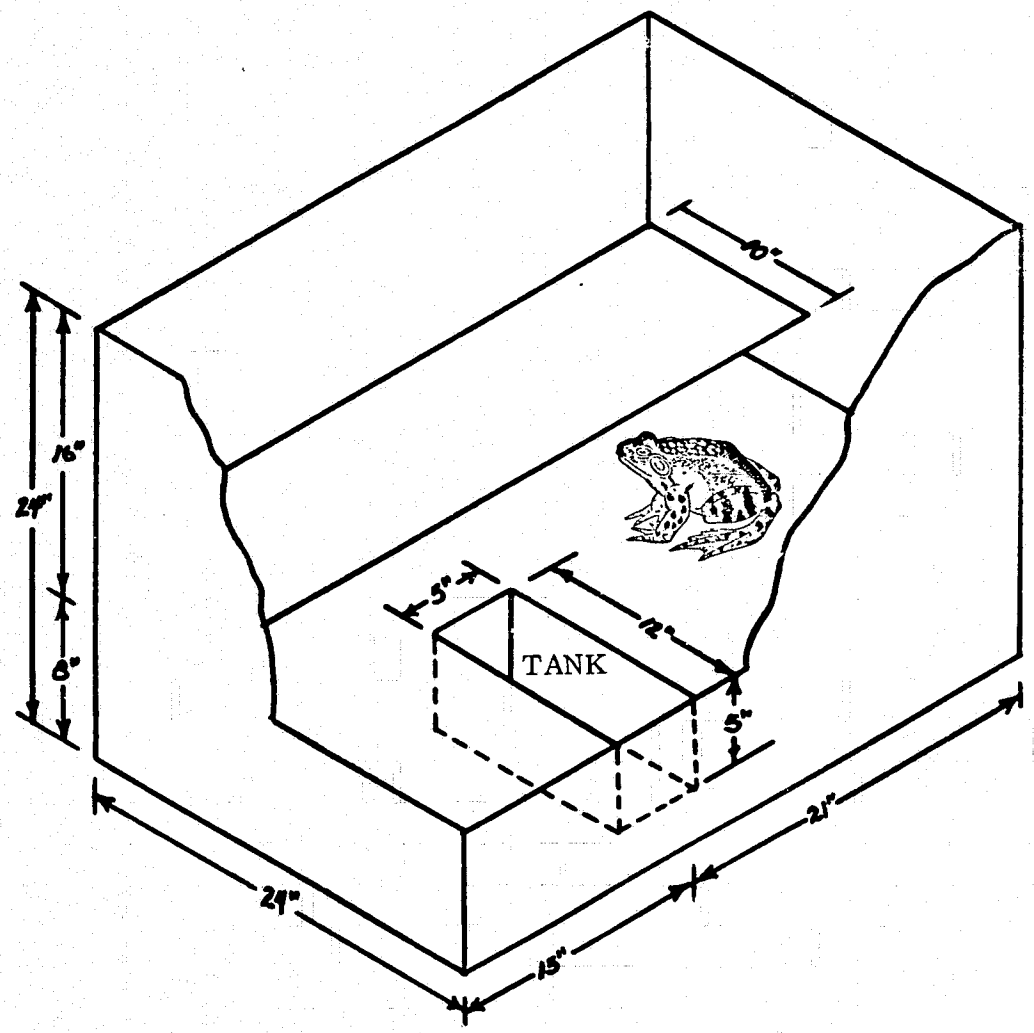

Figure 33 Frog Cage and Tank for the 30 Day System Performance Test

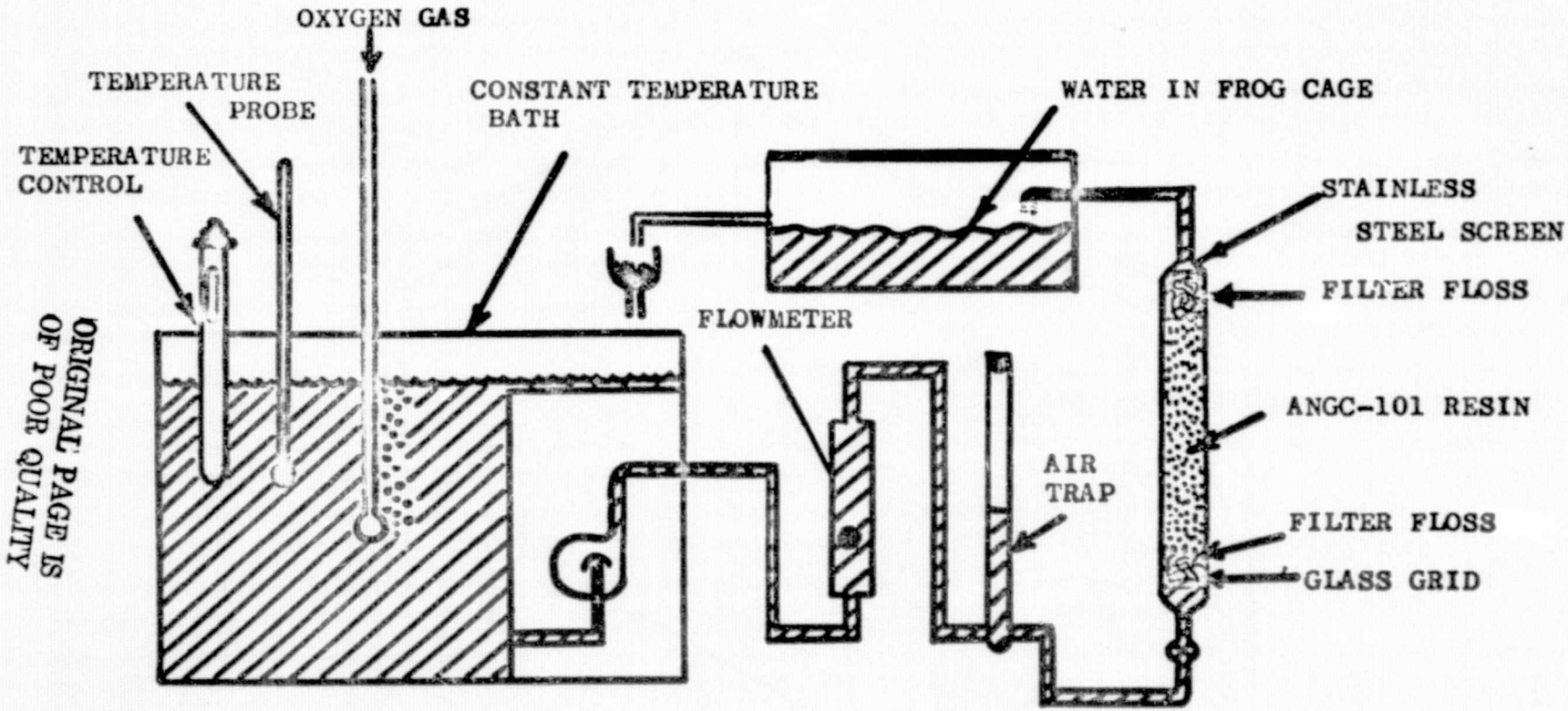

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Figure 34 Schematic Diagram of 30 Day System Performance Test

Oxygenation was accomplished by bubbling oxygen gas through the water in the controlled temperature bath. Circulation through the system was sufficiently rapid, and stirring inside the temperature controlled bath was sufficiently vigorous that additional oxygenation was not necessary.

The pH of the system was measured by a Beckman Zeromatic pH meter and displayed on a recorder. The pH was recorded as needed especially during urease and acid additions.

The urease was weighed on the Ainsworth balance and added as a dry solid to the water in the temperature controlled bath. The oxygen stream was turned off during the addition and neutralization with acid to avoid foaming and denaturation of the enzyme.

The four control frogs were housed in a cage the same size and shape as the two test frogs, except the tank in it held 20 liters of water (see Figure 31). The air temperature was the same but the water temperature was not controlled. The water was aerated with a six inch air stone but was not oxygenated. Both groups were fed mice and mouse puree at the same rate and were weighed on the same days.

Test Conduct: The thirty day test was initiated the first time on 10/28/75. After 66.5 hours, the test was terminated because more than half the water in the system had escaped. The frogs entering the water caused it to overflow. The problem was corrected and the test restarted on 10/31/75.

Daily observations included total elapsed time, air temperature, frog water temperature, controlled bath temperature, per cent relative humidity, oxygen flow, circulation flow, and volume of sample removed. The oxygen flow was not measured because most of the oxygen delivered to the system escaped but rather the flow was inspected to be sure it was on and bubbling through the water. The water circulation flow was measured with a flow meter and held at 360-370 ml/min.

The assays performed daily included pH, total microbial count, urea, ammonia, and nitrate. Dissolved oxygen was measured occasionally. The cations Ca^{++}, Mg^{++}, K^{+}, Na^{+} were assayed from frozen samples after the test. Samples were selected to include one every three days plus additional samples before and after column washes and column changes.

The frogs were weighed and given a cursory health examination every three or four days. Total coliform count was made every day at the beginning of the test but terminated after 12 days when the results were found to be uniformly negative.

Any additions (e.g., urease, acid, water) to the system were noted when they occurred and treatment of the water was described as performed.

The land area of the frog cage was swabbed down daily with a paper towel wet with test system water and rinsed into the system water with a small portion of double distilled water.

Test Results and Discussion: A complete listing of all data gathered is placed in the tables section of the appendices. For readability, most figures are grouped at the end of this section. The ANGC-101 ion exchange resin was effective in removing ammonium ions from the circulated water (see Figure 35). A total of 608 g dry resin was used in the column. The concentration remained low until day 14 and then began moving up slowly until day 17. At that point the resin was saturated and the concentration shot up to 10 ppm. A resin change brought the ammonium ion concentration down to below the measurable limit but, often in only four days, the concentration began to rise again. On day 12, the column became clogged and had to be disassembled, the resin washed and the floss changed. Observations and assays were made on both the column and the aliquots of wash water. A description of these is included in the appendices. Removal of the resin from the column and beaker washing with distilled water reduced the ammonium ion level only slightly after which the concentration continued up. Another change of resin reduced both the ammonium ion concentration and the pH.

Ammonium Ion Toxicity: Although the toxic limit of ammonium ion has not been specified, 0.24 ppm of free ammonia is detrimental to fish and may also affect amphibians (4). At pH 7.0 and 25 °C, there must be 45.08 ppm NH_4^+ in equilibrium with 0.24 ppm free ammonia (see Table in Appendices). The amount of free ammonia present in the test water was usually below this limit. Even on day 26 when the pH had risen to 7.6 ($\frac{NH_4}{NH_3} = 44.57$) and total ammonium ion concentration was 4.0 ppm, it was below the limit of 11.33 ppm. On day 27, the resin was changed because the toxic limit was exceeded when the assayed total ammonia concentration reached 8 ppm (samples were basified to convert all ammonium ion to ammonia). At pH = 8, the ratio is

17.74 to 1 allowing a total ammonia concentration of 4.5 ppm which is over the limit of 0.24 ppm. On changing the resin, the pH dropped to 7.0 and the assayed total ammonia concentration also dropped to 0.2 ppm (0.21 ppm NH_4^+).

pH: The rise in pH was useful in signalling the end of useful resin life. Acid was added concurrently with each addition of urease which makes the pH graphs somewhat erratic. These pH graphs are shown in Figures 36 through 41. The trend (Figure 42) indicates a general rise in pH from day 0 to 16. On day 16, 18.73 ml of 0.1 N HCl was added to the system to bring the pH to 6.9. Urease was not added. The acid was added only to reduce the pH. Nevertheless, the pH had risen to 7.7. On day 18, the pH had dropped slightly because of acid addition following urease treatment on the 17th. Only after resin change did the pH stabilize near 7. The pattern was repeated during the next cycle of resin loading.

Urea: On the graph of urea concentration, urease is effective in reducing the urea concentration with the singular exception of day 22. It has been noted earlier that urease was effective only in the presence of buffer or in the presence of resin. If either is absent, the enzyme is rapidly denatured and the enzyme fails to hydrolyze urea and acid is not consumed. On day 22, the urease treatments did result in 40.52 ml of acid consumption indicating good urease activity. The other explanation is that on day 22 a more than usual amount of urea was added to the water resulting in a higher concentration on day 23. This explanation is plausible because one mouse was eaten by the test frogs on day 17 and both were fed mouse puree on day 21. A higher than average urea excretion rate could be expected.

Bioburden: Methods of bioburden control including coarse filtration followed by either Millipore filtration (0.45μ) or ozonation were effective (see Appendix on Filtration) in reducing the number of bacteria in the recirculating water.

The method for ozone disinfection of the frog water was satisfactory and less time consuming than other methods used (see Appendices). The ozonation of the water at one liter per minute (total O_2 and O_3 gas) for five minutes effectively sterilized the water, however, prolonged treatment as long as forty-five minutes did not materially change the urea, ammonium ion, or nitrate concentration (Table 20).

All of the water could not be removed from the system for bioburden control. The total volume of the system was estimated as 6162 ml and 6000 ml typically was treated. The treated water was added to the system and pumped into the surge tank and ion exchange column. The first 1500 ml was collected and treated because the total count of microorganisms in the forefraction as measured on 11-10-75 as shown in the table was about twice as high as the filtered water from the system. This source of innoculation was reduced by disinfecting the forefraction but was not eliminated since the column, flowmeter, connecting tubes and other surfaces where growth occurred were not treated.

Bioburden control was necessary on a daily basis. On day 15, it was neglected resulting in a 10^2 increase by day 16 as shown on the graph.

The nitrate ion concentration was not controlled. It was measured daily with an Orion, nitrate specific ion electrode. In dilute solution, such as Ion Solution V, a reading of 8.5×10^{-5} M or 5.27 mg NO_3^-/l was obtained when no nitrate was added. This quantity should be regarded as a blank and subtracted from the readings. From the graph, it is seen that nitrate levels became high on four days ... #9, 10, 25 and 28. On these four days, either urea was high ($>$ 30 ppm) or ammonia was high ($>$ 4 ppm). The high nitrate may be due to microbial conversion of urea and ammonia to nitrate in the well oxygenated system. The nitrate was reduced when the nitrogen sources were removed and the nitrate production rate diminished. This explanation is only partly complete because on other days of high ammonia (e.g., day 18) and high urea (e.g., day 17), the nitrate concentration was not abnormally high.

Metal Ions: The resin seemed to have an affinity for cations in the following order NH_4^+, K^+, Mg^{++}, Ca^{++}, Na^+. The fresh resin absorbed potassium, magnesium and calcium from the water, and liberated sodium ions. As the resin absorbed ammonium ions, the calcium, magnesium and potassium levels in the water increased as well as the sodium level. Also remember the samples were replaced with Ion Solution V. The column is apparently prepared with sodium ion-filled sites. It was unnecessary to add sodium to the water (Ion Solution V) used in the thirty day test.

Feeding Behavior: One mouse was eaten by one of the two test subjects on day 2 of the test and one on day 17. Mouse puree (1/2 oz. each dose) was given to each frog on days 7, 10, 14, 21, and 26.

TABLE 20 INFLUENCE OF OZONATION ON FROG WATER PARAMETERS

DATE	OZONATION	TIME (MIN.)	UREA (ppm)	NH_4^+ (ppm)	NITRATE M	MICROORGANISMS/ml
10-31	Before	0	12	$\ll 0.1$	3.6×10^{-5}	2.6×10^{6}
-	After	45	12	$\ll 0.1$	4.4×10^{-5}	-0-
11-7	Before	0	2	0.1	6.6×10^{-5}	3.6×10^{5}
-	After	5	2	0.1	6.6×10^{-5}	-0-
11-10	Before	0	32	< 0.1	6.2×10^{-4}	7.0×10^{4}
-	After	5	30	< 0.1	6.2×10^{-4}	-0-
	Forefraction	-	-	-	-	1.45×10^{5}

Urease Contact Test: Two frogs were placed in a bell jar with 2000 ml water and 200 mg urease was added. After 2 1/2 hours, the frogs exhibited normal behavior and had their eyes open above and below the water on occasion. There appeared to be no adverse affects from having the frogs in contact with a relatively high concentration of urease.

Mouse Excretion Test: One adult mouse was placed in a pyrex jar with a wire screen over the top. After 22 hours, the mouse was removed and 50 ml of glass distilled water was added to dissolve urine and feces in the jar. After the sides were washed down, the solution was filtered through Whatman filter paper and assayed. Urea excretion equaled 152.2 mg, ammonia equaled 0.195 mg or the equivalent of 0.614 mg NH_4Cl, and approximately 0.45 mg nitrate ion.

Frog Weight and Condition: Figures 50 through 55 show the changes in the weight of the two frogs in the 30 day test (1 and 4) and the four control frogs (2, 3, 6 and 7). Eating a mouse or urinating before or after weighing can cause a 20-30 g change in a frog's weight so interpretation of this data should be done with care. The weight of each frog was averaged for three time periods during the test (Table 21). Comparison of weight during the first third to second or third third did not turn up any weight differences gain or loss greater than 25 g indicating there were no differences which could not be accounted for by the natural variability of the weights from urination and/or eating.

TABLE 21 AVERAGE FROG WEIGHT DURING THREE TIME PERIODS

TIME PERIOD DAYS	TEST		CONTROL			
	#1	#4	#2	#3	#6	#7
1-6 3 Measurements	493	471	549	514	651	653
7-19 2 Measurements	496	450	562	539	665	639
20-33 3 Measurements	475	428	556	541	642	641

Conclusion: The test system as described maintained a habitable environment for the test frogs and had no adverse effects on them. Urease was effective in reducing urea concentrations and remained active for a sufficiently long period of time reduce the concentration to acceptable levels. Potassium concentrations remained above the minimum allowable concentration of 0.003 ppm during the entire test. There were several days during which the calcium concentration was below a detection limit of 0.1 ppm and could have been anywhere between 0 and 0.1 ppm.

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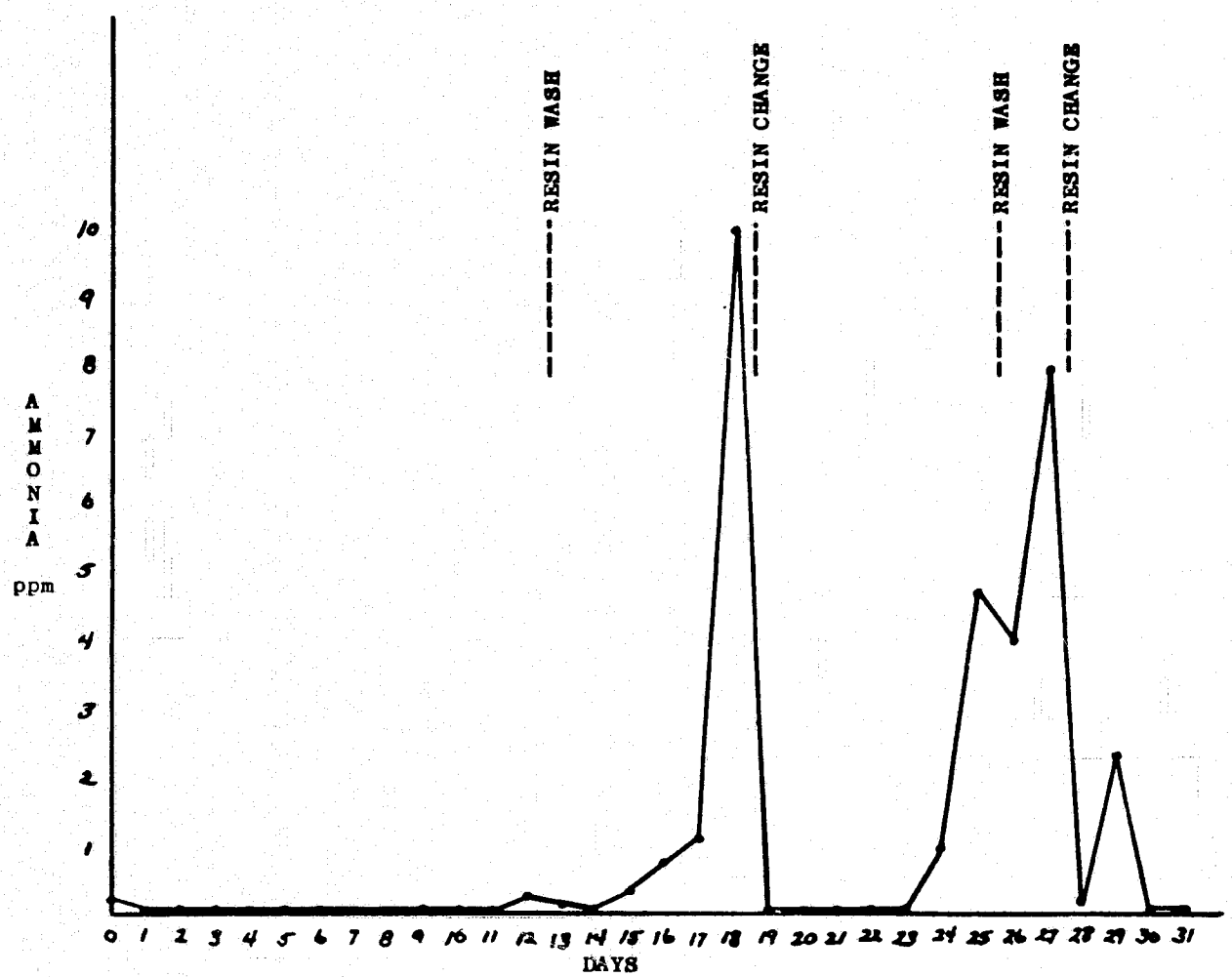

Figure 35 Ammonia Concentrations in the 30 Day Test

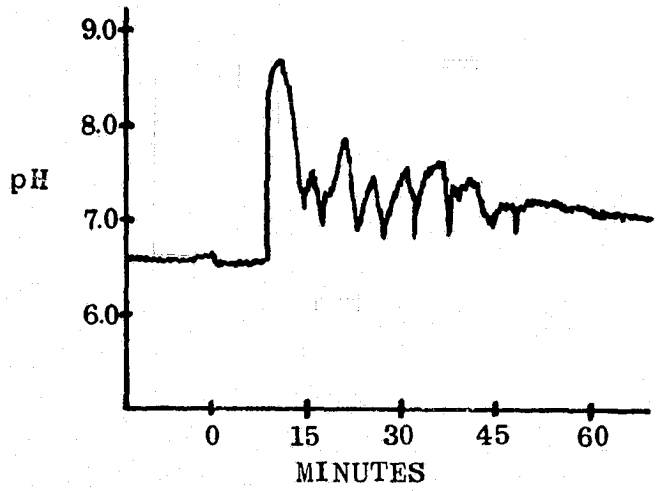

Figure 36 pH During Urease Addition, 127.2 hr. (97.5 mg Urease, 16.85 ml 0.1 N HCl)

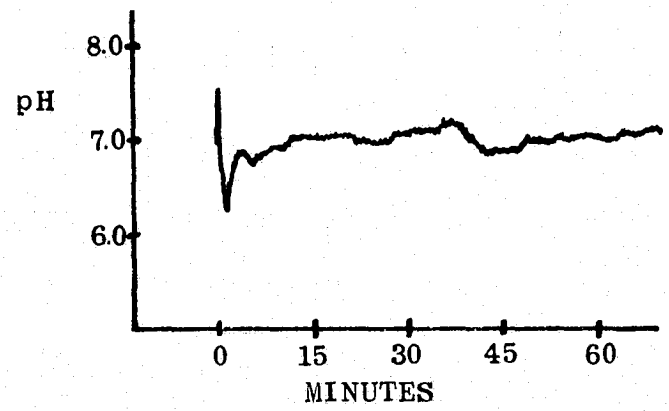

Figure 37 pH During Urease Addition, 216.7 hr. (106.7 mg Urease, 20.37 ml 0.1 N HCl)

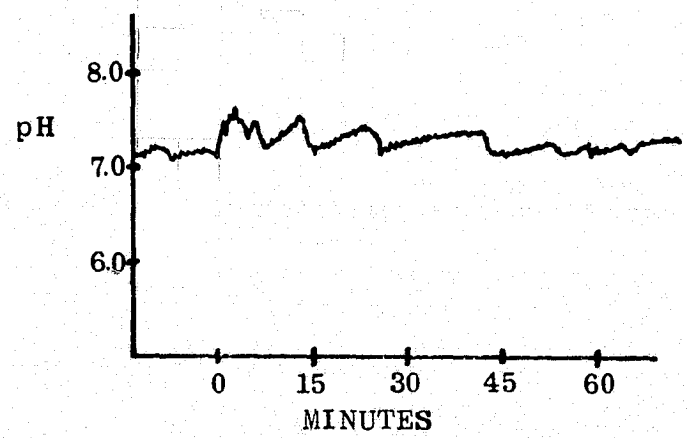

Figure 38 pH During Urease Addition, 266.1 hr. (101.6 mg Urease, 15.5 ml 0.1 N HCl)

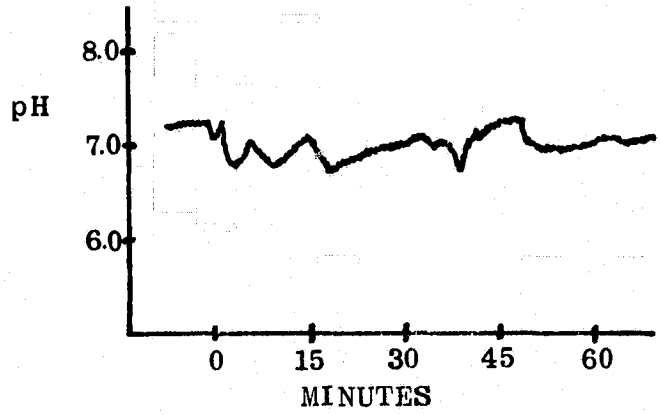

Figure 39 pH During Urease Addition, 408.4 hr.
(103.3 mg Urease, 40.28 ml 0.1 N HCl)

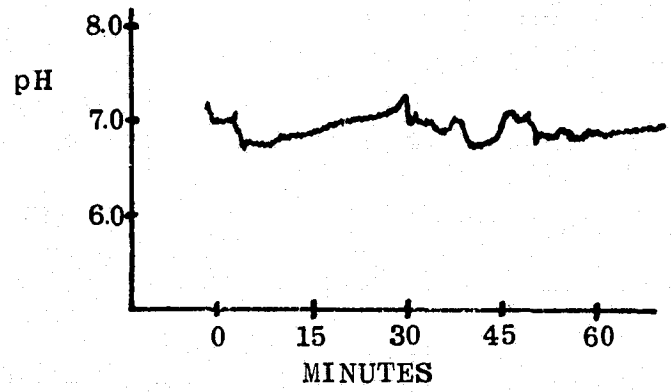

Figure 40 pH During Urease Addition, 481.7 hr.
(204.6 mg Urease, 49.70 ml 0.1 N HCl)

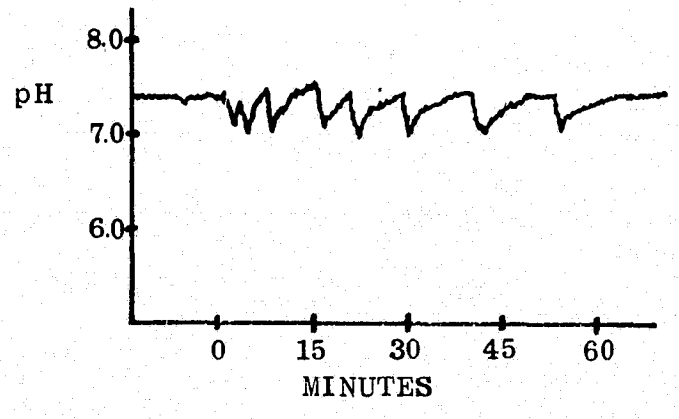

Figure 41 pH During Urease Addition, 745.0 hr.
(95.5 mg Urease, 68.25 ml 0.1 N HCl)

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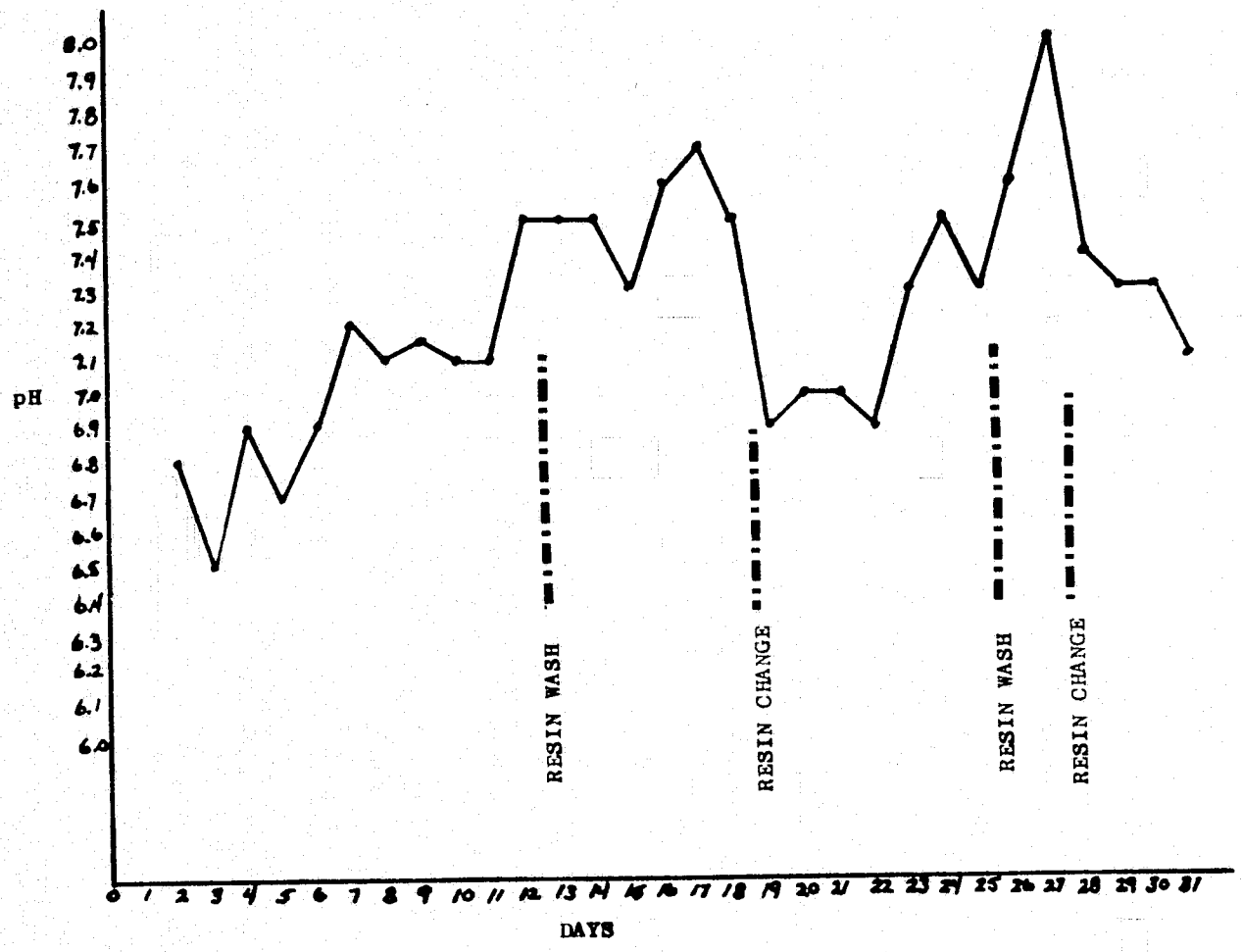

Figure 42 pH in the 30 Day Test.

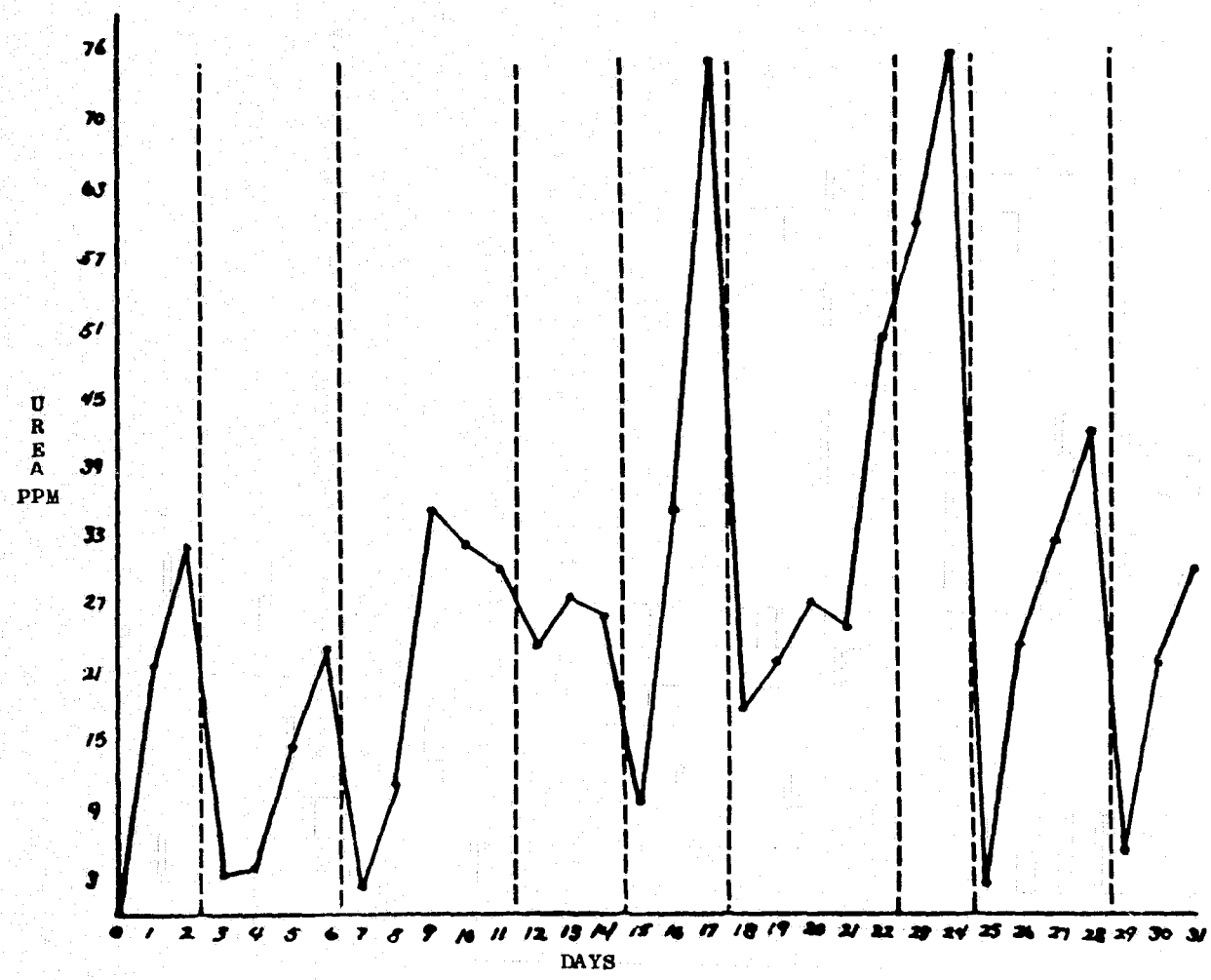

Figure 43 Urea Concentration in the 30 Day Test (urease additions shown with dotted vertical lines)

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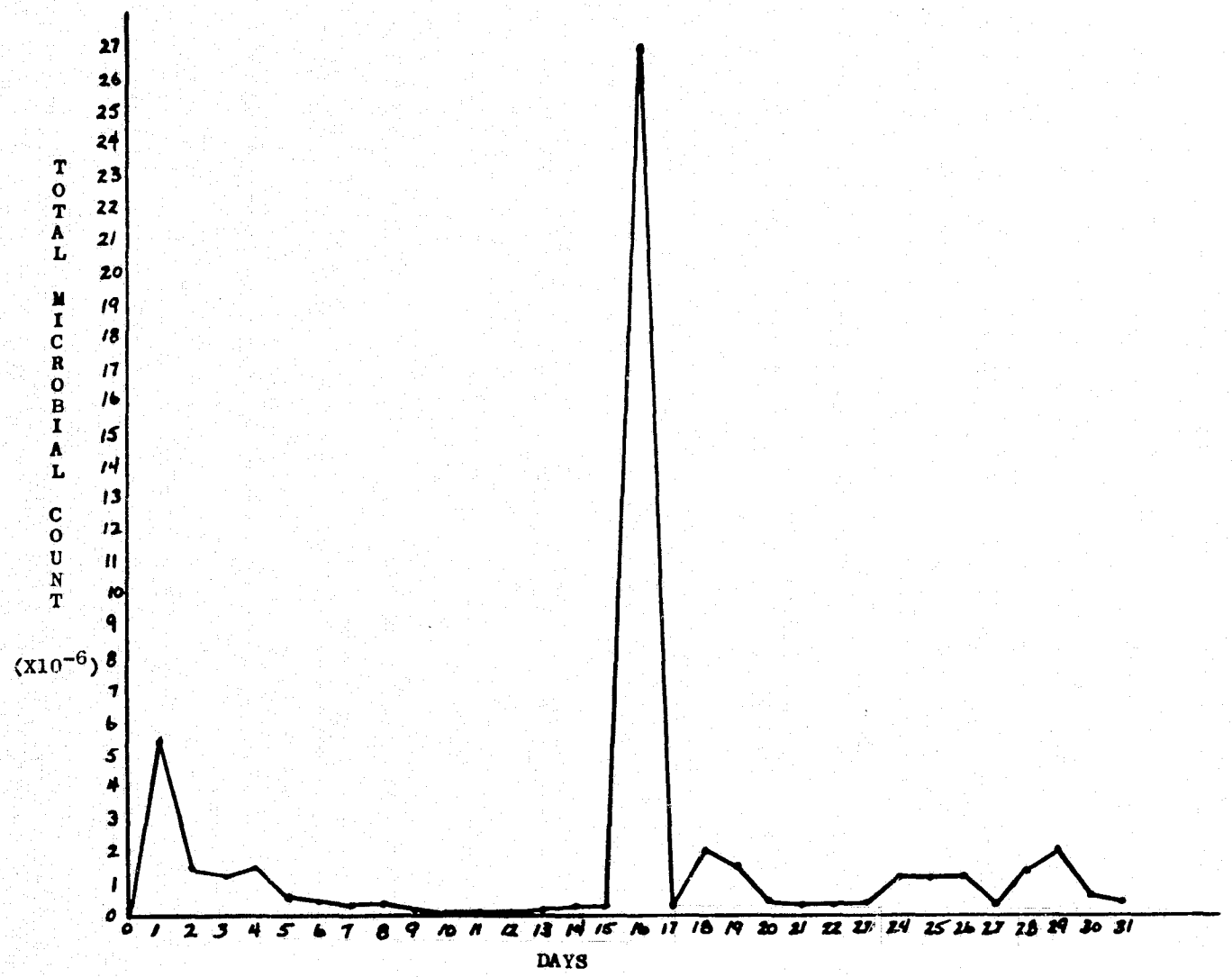

Figure 44 Bioburden in the 30 Day Test

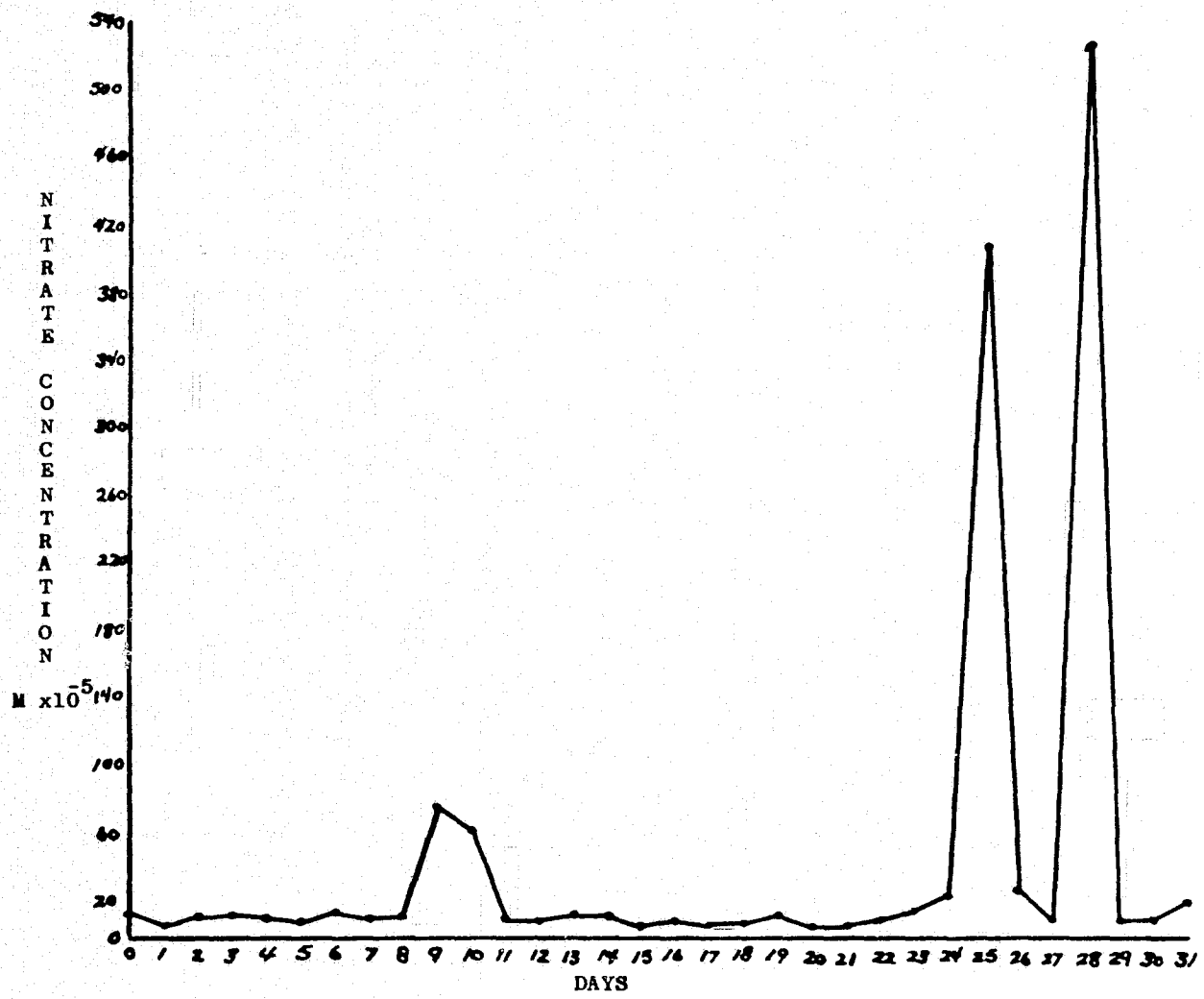

Figure 45 Nitrate Concentration in the 30 Day Test

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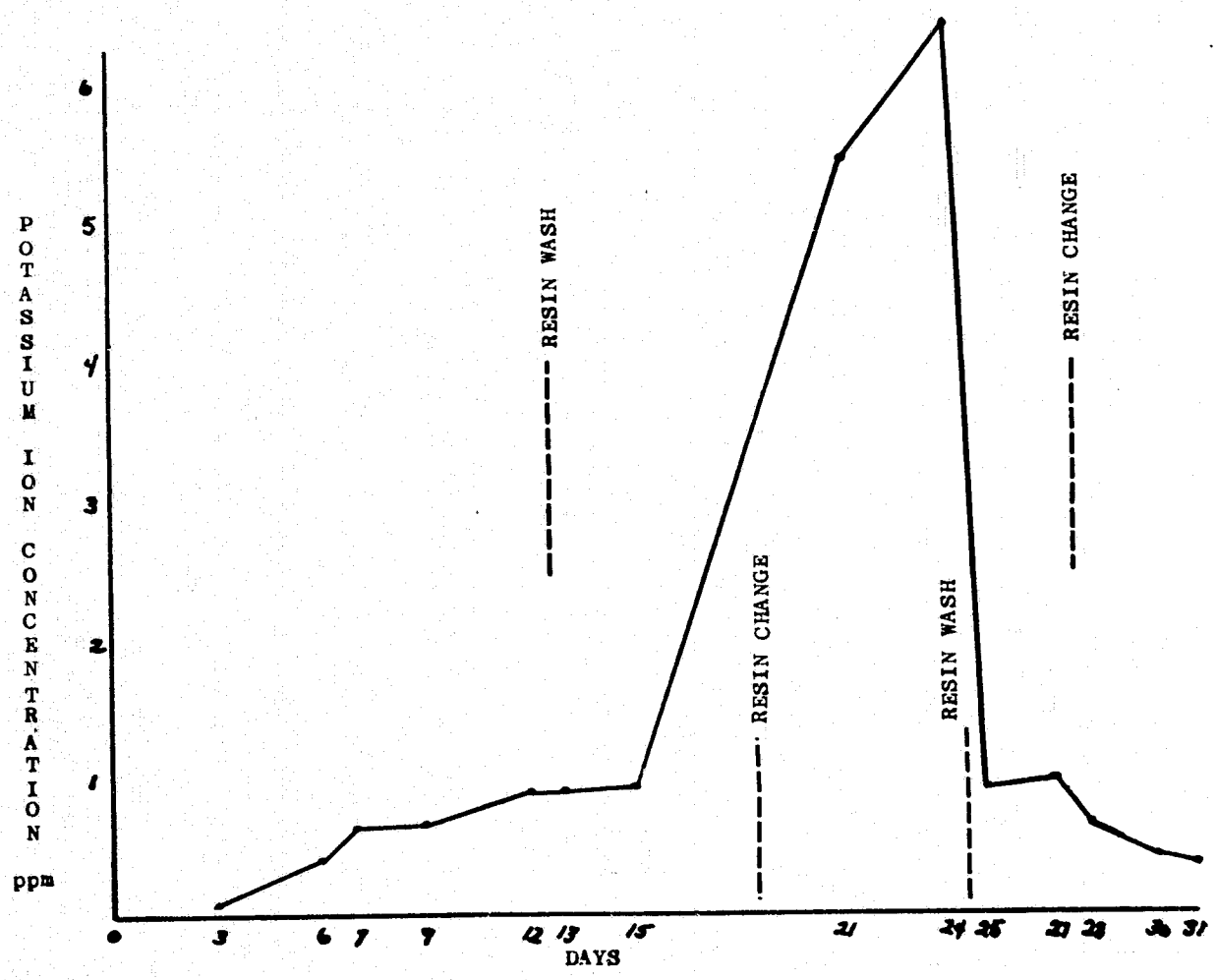

Figure 46 Potassium Ion Concentration in the 30 Day Test

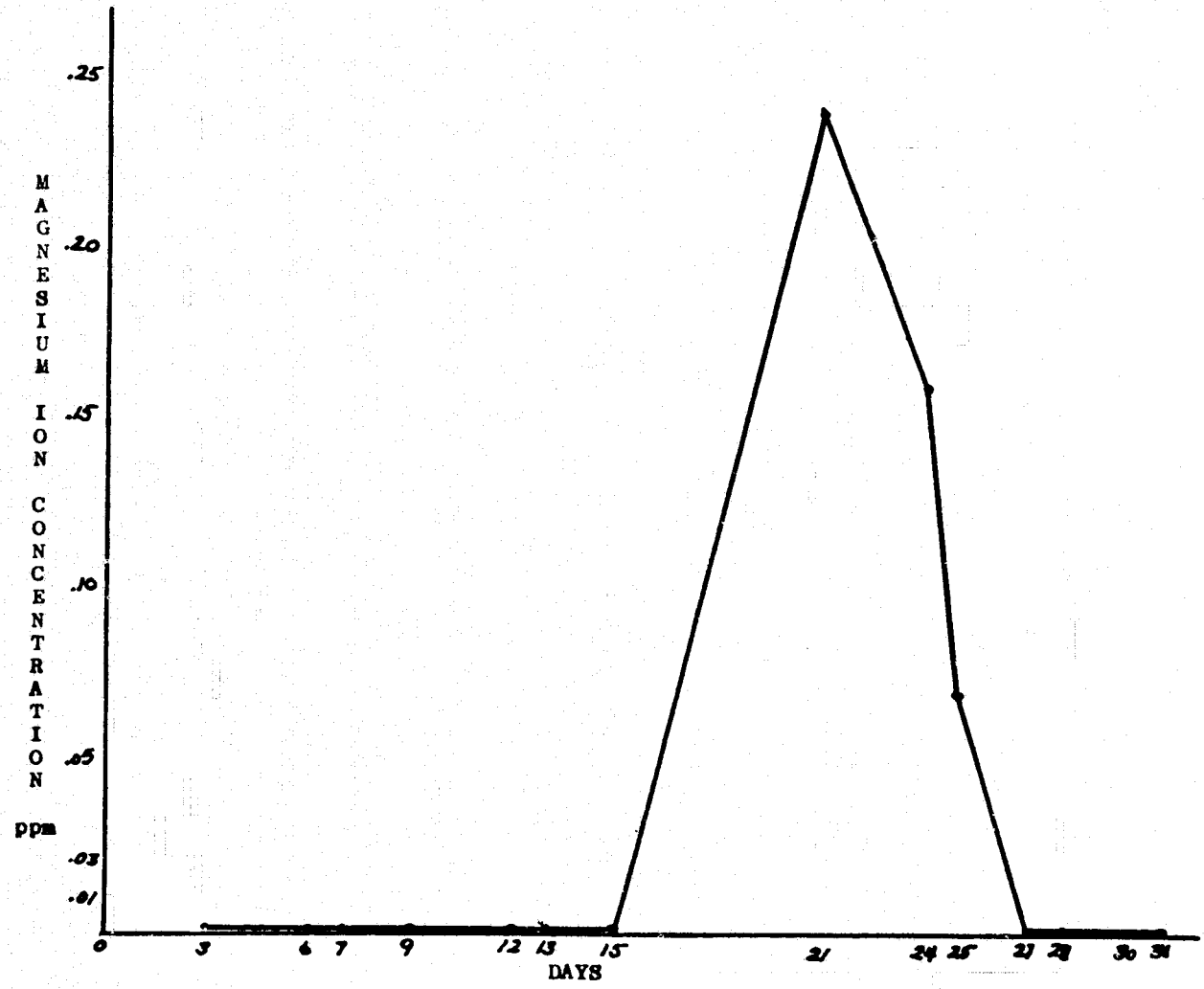

Figure 47 Magnesium Ion Concentration in the 30 Day Test

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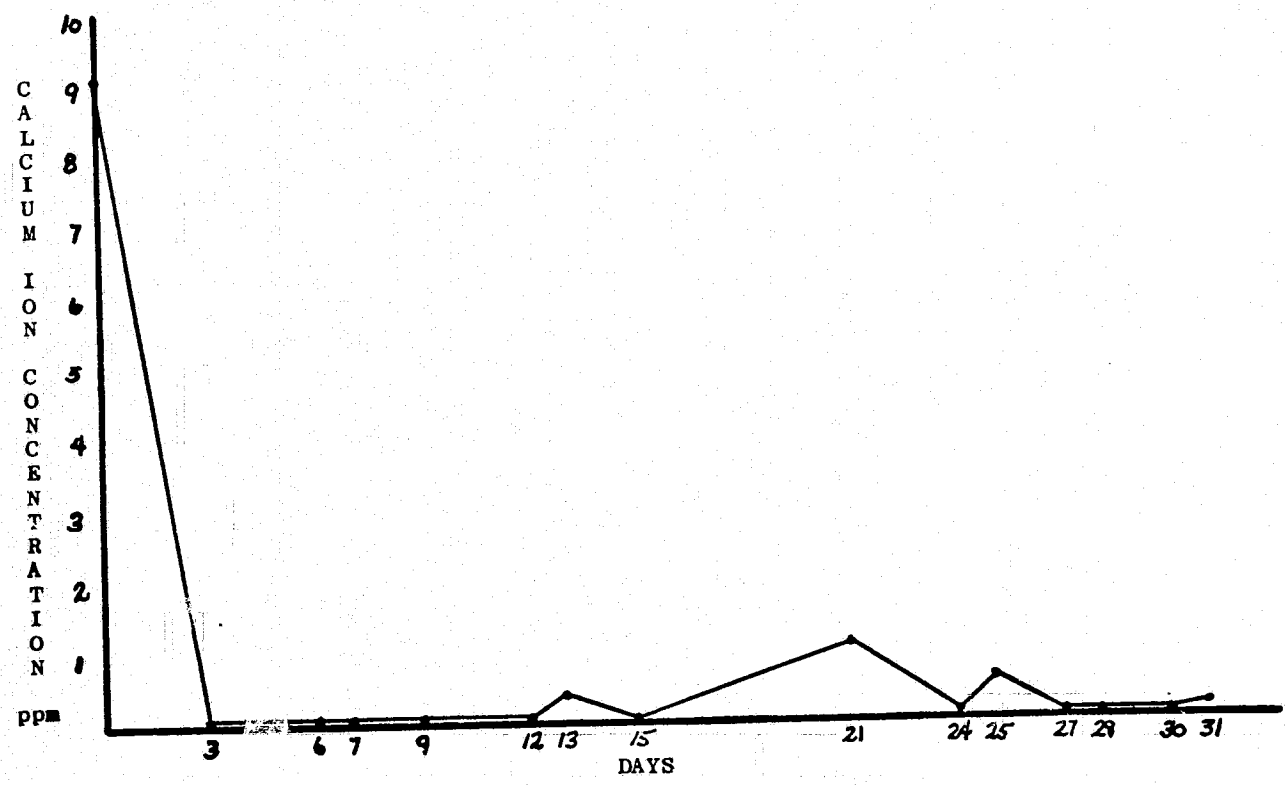

Figure 48 Calcium Ion Concentration in the 30 Day Test.

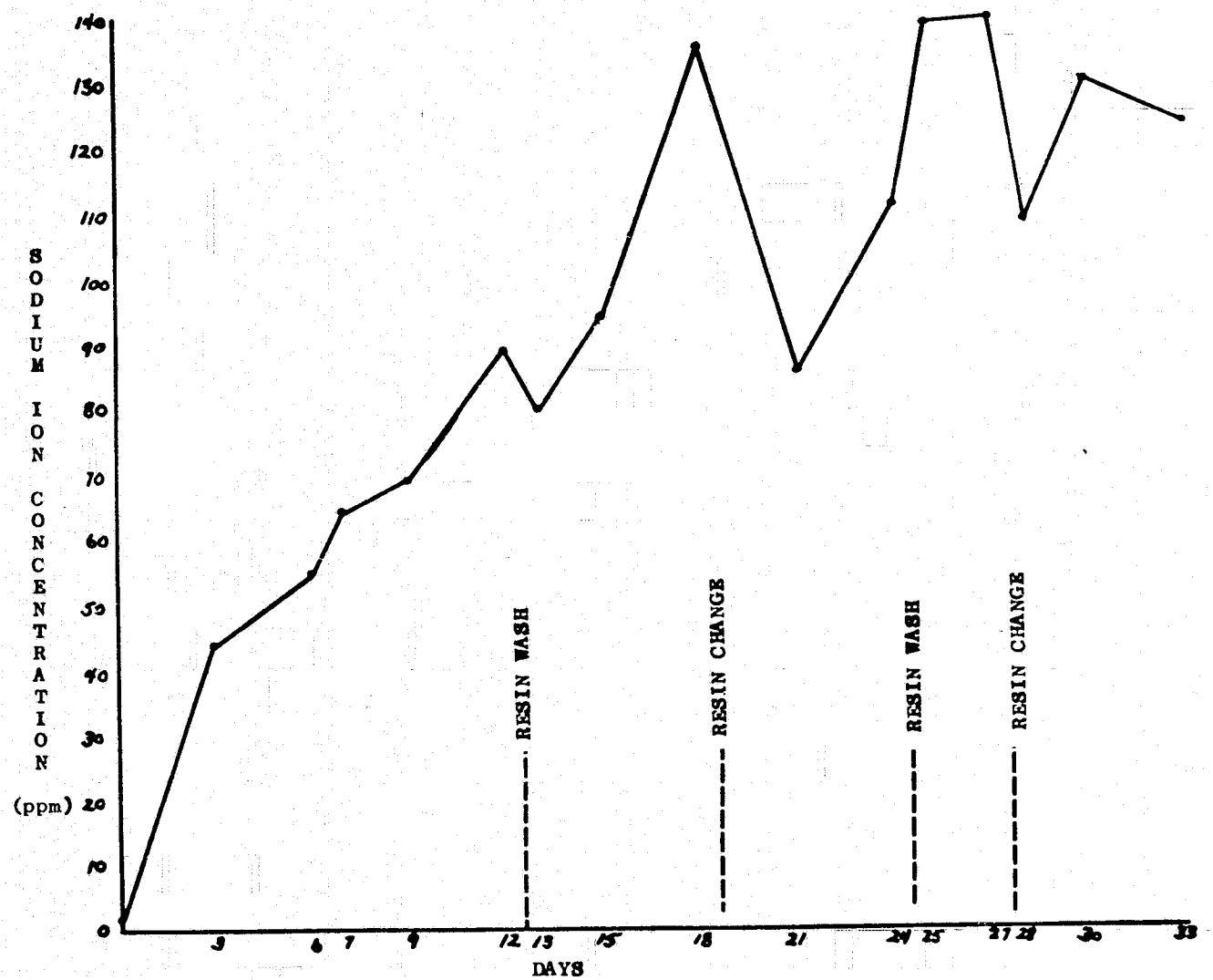

Figure 49 Sodium Ion Concentration in the 30 Day Test

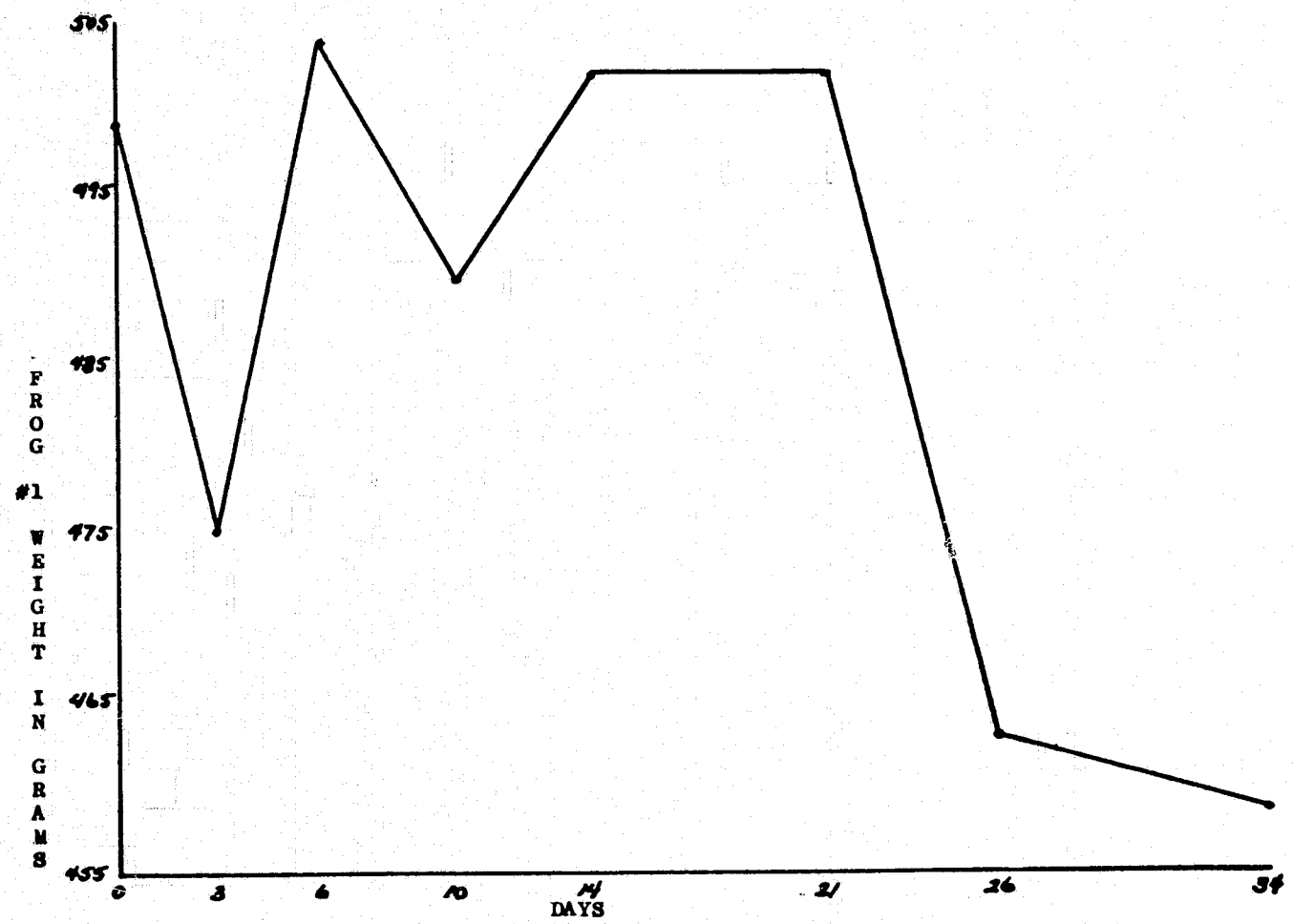

Figure 50 The Weight of Frog #1 in the 30 Day Test in Grams.

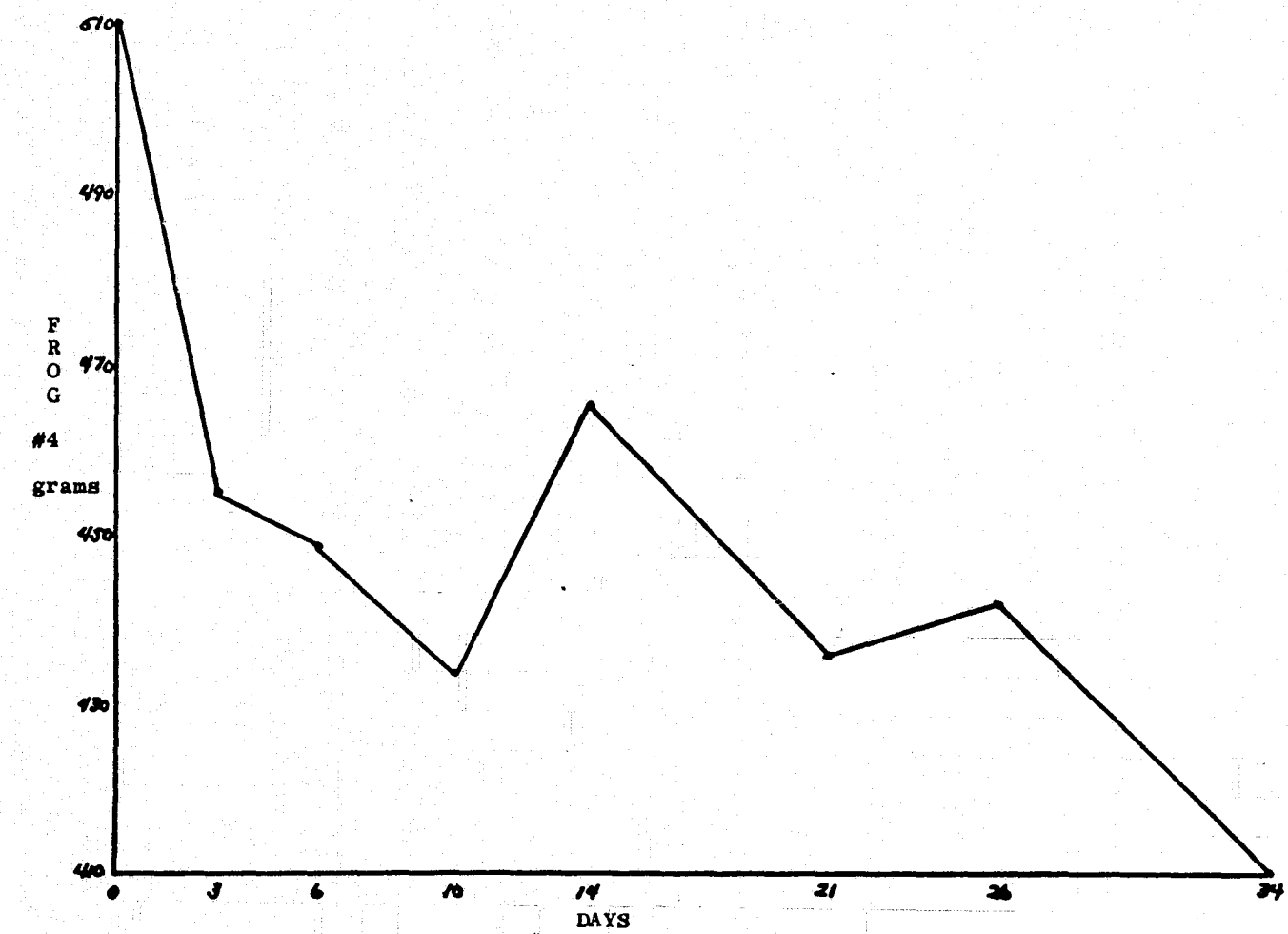

Figure 51 The Weight of Frog #4 in Grams During the 30 Day Test.

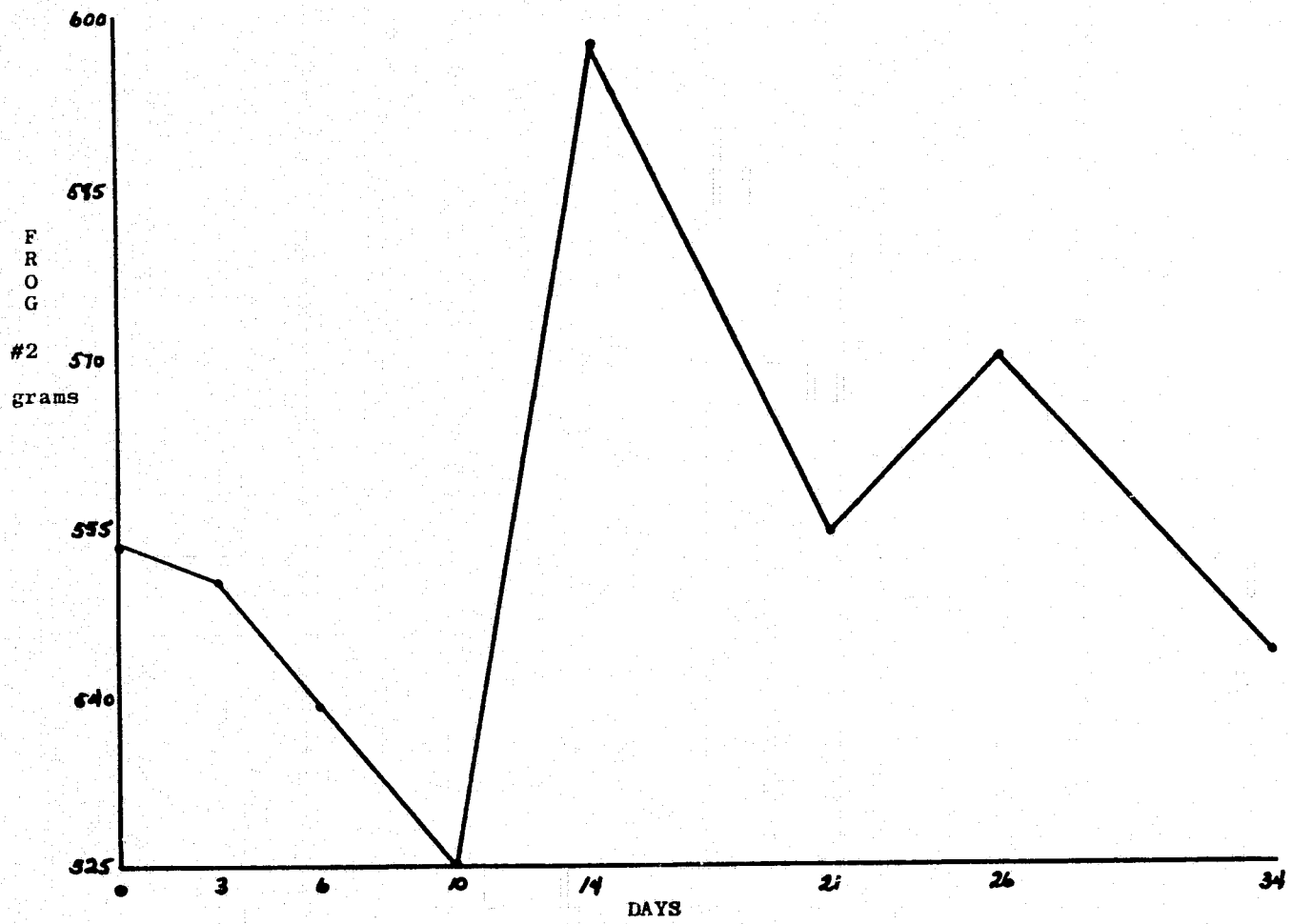

Figure 52 The Weight of Frog #2 in Grams During the 30 Day Test.

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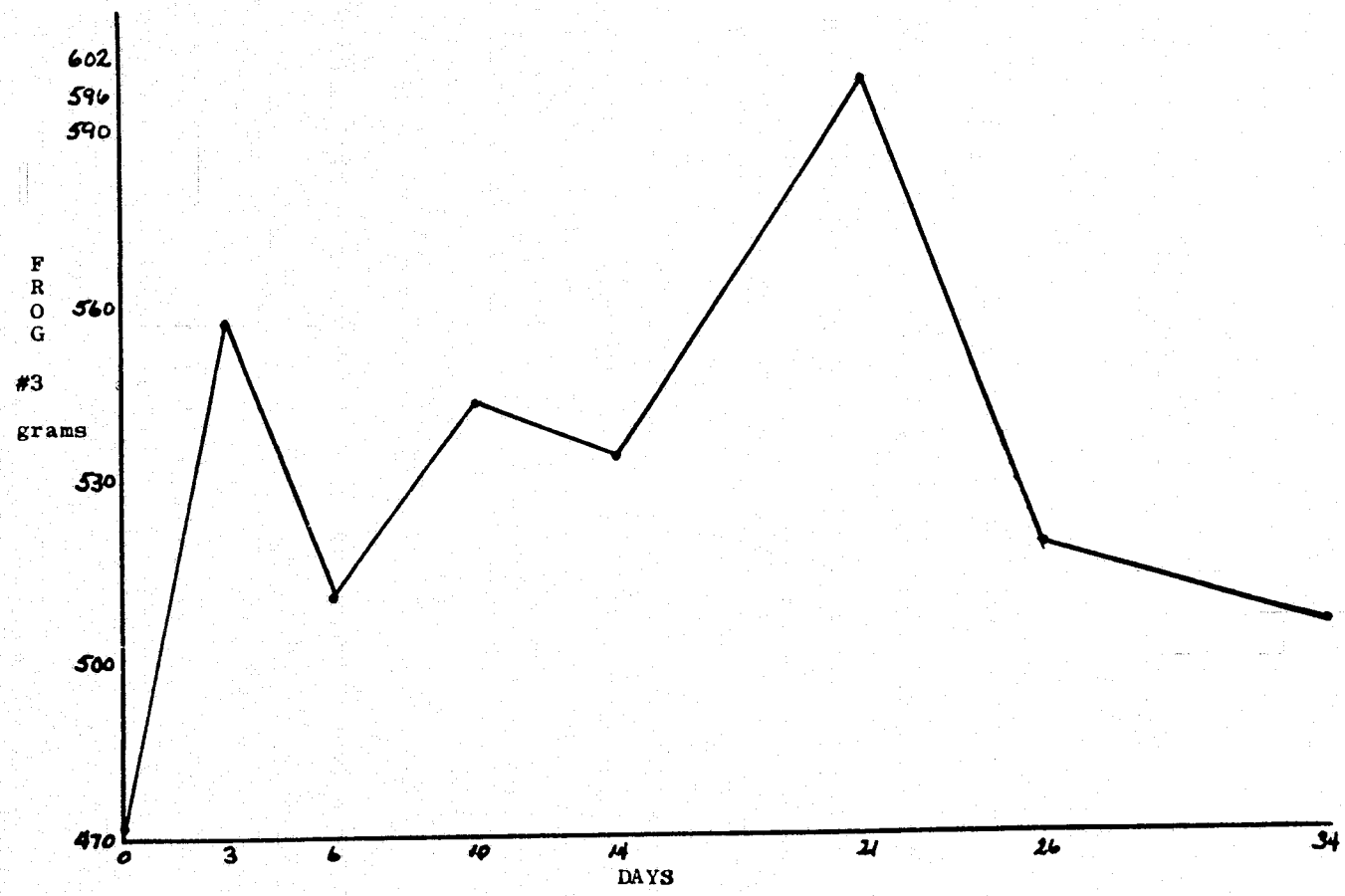

Figure 53 The Weight of Frog #3 in Grams During the 30 Day Test

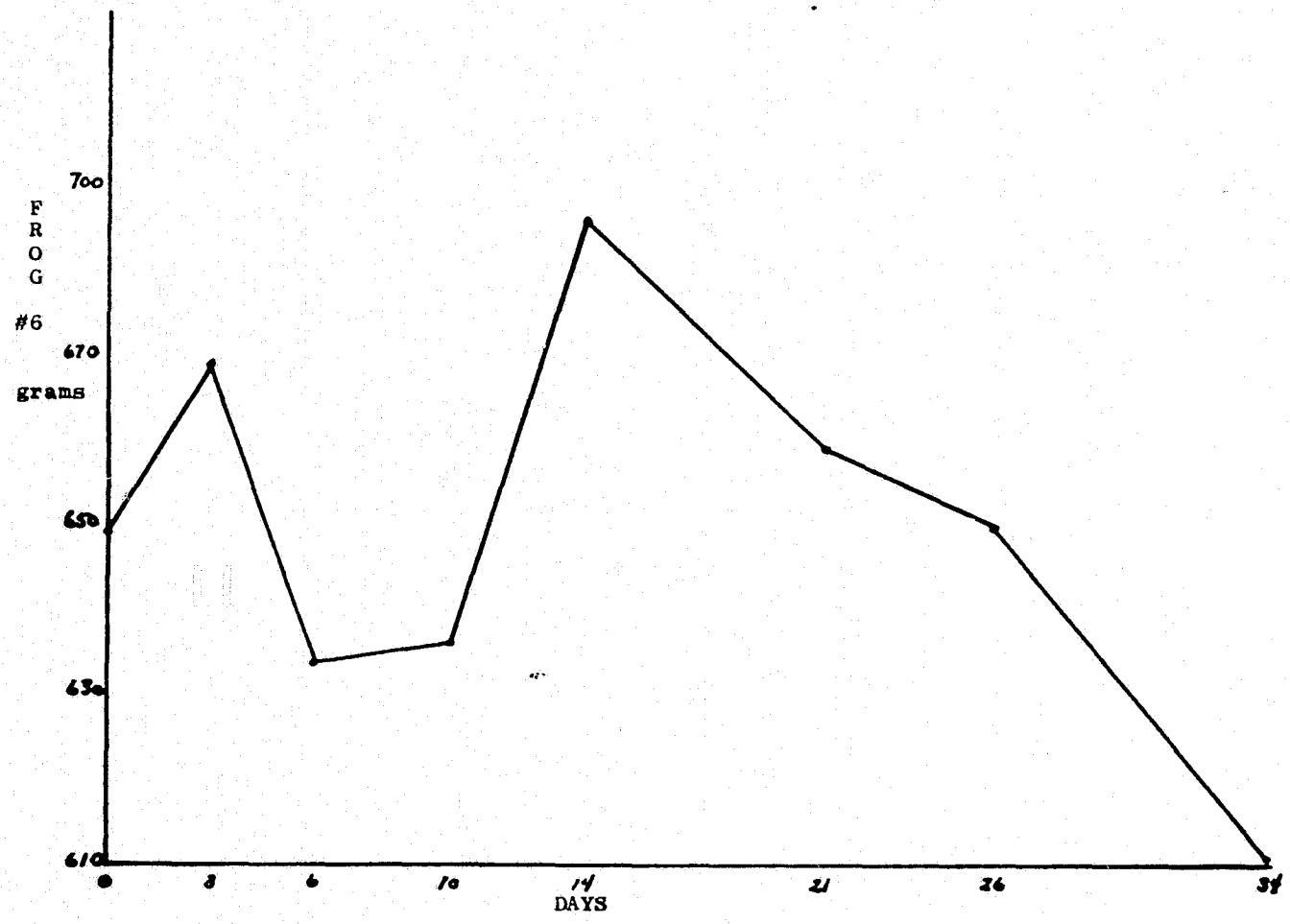

Figure 54 The Weight of Frog #6 in Grams During the 30 Day Test.

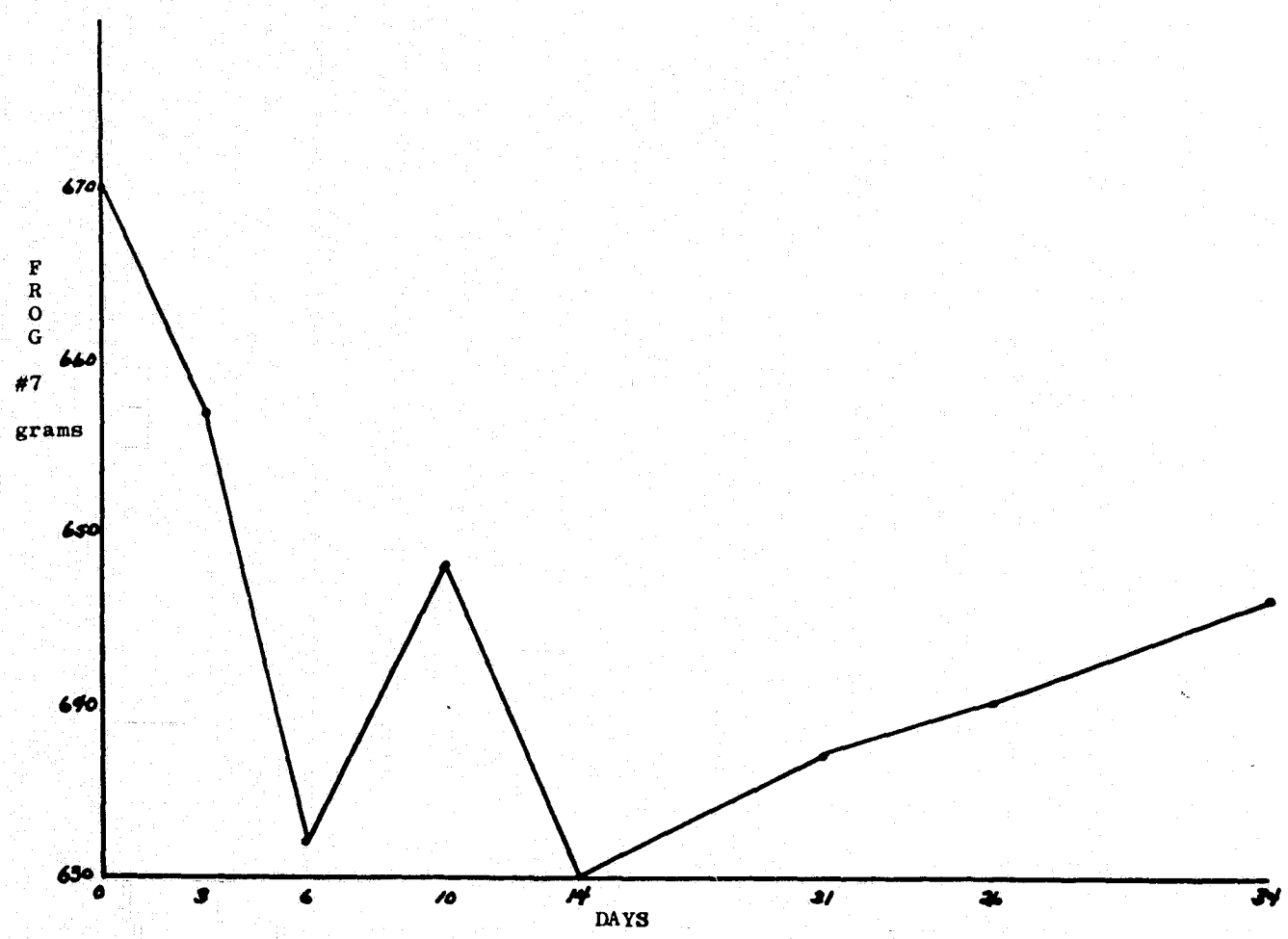

Figure 55 The Weight of Frog #7 in Grams During the 30 Day Test.

CONCLUSIONS TASKS 4 AND 5

The objective of this program was to determine the feasibility of using free urease enzyme and ANGC-101 ion exchange resin to remove urea and ammonium ion for space system waste water applications, specifically, the prevention of urea and ammonia toxicity in a 30 day Orbiting Frog Otolith (OFO) flight experiment.

Three questions were posed in the statement of work.

1. Is free urease a detriment to the frogs or vise versa?

2. Will free urease be effective in the presence of ANGC-101 ion exchange resin?

3. Can resin be pre-loaded with required cations in order to maintain the required cationic balance and remain active for ammonium ion?

In Task 4 it was determined that ANGC-101 ion exchange resin has a preferential affinity for NH_4^+, K^+, Mg^{++}, Ca^{++} and Na^+ in that order in solutions of relatively low cation concentration. If the solutions are made up in buffer the relative affinity changes depending on what ionic species are present. Other substances such as urea or other excreted waste materials present in the water also seem to alter the resin's affinity for cations.

To answer question three, the concept of preloading the resin required modification. It was demonstrated during Task 4 that the initial ion concentration in a solution was reduced roughly by a factor of ten very quickly and then slowly increased during the test. If the criteria of minimum allowable concentration is used to judge whether or not the resin maintains the required cationic balance, it does maintain it. Since the resin has a preferential affinity for ammonium ion over others, it retains affinity for ammonium ion even when partially loaded with other cations. Ammonium ion seems to displace other ions on the resin.

In 0.05M sodium phosphate buffer containing a relatively low concentration of cations, the resin had the ability to absorb 5.8 mgN/gram of resin. In the same ion solution without buffer the resin absorbed 7 to 9 mgN/gram of resin. At the

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end of the 10 day simulation of a 30 day experiment the resin was still functional and had absorbed 4.03 mgN/gram of resin. It may be that the ammonium ion absorption capacity determined by flooding the resin with an excess amount of ammonium ion cannot be validly applied to situations where low concentrations are loaded onto the resin over a period of time or it may be in this case that the column had not reached its functional saturation.

During the urease activity testing in Task 4 it was shown that in unbuffered ion solutions free urease actually requires both pH control and ANGC-101 to function properly, answering positively question number two.

Question number one was answered in Task 5. Testing during Task 5 demonstrated no short term gross toxicity of urease to the frogs. Frogs placed in distilled water containing 100 mg urease/liter for 2 hours did not display any symptoms of irritation, often having their eyes open below the surface of the water and otherwise behaving normally. The two test frogs were exposed to a recirculated water containing low levels of active urease for thirty-days. The frogs were in and out of the water at night during the dark portion of the photoperiod. Based on these observations and the fact that there were no significant weight change differences between the test and control frogs, no adverse effects can be attributed to the urease. Free urease is not a detriment to the frog. If other more subtle biochemical effects are suspected and are considered critical to the OFO experiment, specific investigations should be conducted.

Free urease added directly to the Lauda bath in the 30 day test was able to effectively hydrolyze urea with pH control. The urease continued to function at a low rate for at least 24 hours after addition. This observation and the fact that other unidentified metabolic wastes excreted into the water simply accumulated during the test indicates that frogs and their waste products are not significantly detrimental to urease function.

The usefulness and feasibility of using free urease and ANGC-101 ion exchange resin to prevent urea and ammonia toxicity has been demonstrated. Investigation and analysis of methods for automating the system for use in a flight package is warranted. Specific questions which need to be addressed

include:

1. How much urea and ammonium ion are excreted by bullfrogs fed an OFO diet?

2. What would be the most effective practical method for bioburden control?

3. What method should be used to add free urease to the system?

4. How can pH control be accomplished?

5. How can urease additions and pH control be automated and logically controlled?

6. How can the whole system be combined for a functional semi-automated breadboard?

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APPENDIX 1

EQUIPMENT

1. Lauda Temperature Controlled, Circulating, water bath Type K2-R
   Mess-Gerate Werk Lauda
   Dr. R. Wobser KG
   W. Germany

2. Ammonia gas electrode and Beckman research pH meter
   Beckman Research pH meter.
   Orion ammonia electrode model 95-10

3. Nitrate electrode and specific ion meter
   Ionalyzer, model 404, Orion Research 11 Blackstone St., Cambridge, Mass. 02139
   Nitrate Ion Electrode model 92-07, Orion Research

4. Aminco Bowman Spectrophoto fluorometer Model #4-8202
   American Instrument Co.
   Silver Spring, Maryland and x-y recorder
   Photomultiplier Tube type 1P21 Catalog #C57-62140 #10-267

5. Jarrell Ash Atomic Absorption Unit

6. Beckman zeromatic pH meter, Model 96

7. flowmeter, Fischer-Porter tri-flat, stainless steel ball, 3/8" diameter

8. ozone generator: Welsback model 408

9. High capacity millipore filter holder.
   Millipore Corporation 142 mm filter holder
   200 psi inlet 100 psi differential
   (Used in this work with water pump suction. Ambient pressure)

10. Mettler Balance P1000N
    Mettler Instrument Corp.
    Highstown, New Jersey

11. Environmental Chamber: Percival, Walk-in type, Boone, Iowa

12. Digital Thermometer: Digitec, United Systems Corp., Dayton, Ohio Model #1501

13. Right-A-Weigh Balance, Wm. Ainsworth & Sons, Inc., Denver, Colo.

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APPENDIX 2

MATERIALS

1. Atomic Absorption and Flame Emission Standards:
   $MgSO_4$, Anhydrous, Baker Analyzed, Assay 100.0%, Lot #44660
   KCl, crystal, Baker Analyzed, Assay 99.7%, Lot #44572
   NaCl crystal, Baker Analyzed, assay 99.7%, Lot #323652
   $CaCl_2$, Anhydrous, Granular, Baker Analyzed, 97.6% Lot #432956

2. NH_4Cl for NH_4^+ standards and ammonium ion additions to test:
   Baker Analyzed, Granular, 99.8% pure, Lot #43792

3. Buffers
   Na_2HPO_4 MW 141.965 Anhydrous, Baker Analyzed, 99.8%, Lot #401250
   NaH_2PO_4 MW 137.998, Baker Analyzed, 99.3%, Lot #334185
   KH_2PO_4 MW 136.09, Mallinckrodt, analytical reagent
   K_2HPO_4 MW 174.18, Baker Analyzed, 99.8%, Lot #6308

4. Nitrate Standards: Sodium Nitrate, crystal, Baker Analyzed, 99.8%, Lot #91621

5. Urea for urea standards and additions to tests: Baker Analyzed m.p. 132.0 - 133.5°C Lot #45145

6. Urea Assay reagents:
   2,3 - Butanedione monoxime, J. T. Baker Co., m.p. 73-75°C, Lot No. 1-3805.
   Concentrated sulfuric acid, Baker Analyzed, 95.7%

7. Bioburden assay materials:
   Trypticase Soy Agar, BBL Cockeysville, Md 21030, Lot H5D AJR
   Levine's Eosin - Methylene Blue Agar, Difco Laboratories, Control 564961
   Ethyl Alcohol, J. T. Baker, Anhydrous, Reagent, Denatured, Lot No. 44533
   Petri Plates, 100x15 mm Van Lab Sterile Disposable
   Disposable Pipets, 7503 Falcon Plastics, 1950 Williams Dr., Oxnard, CA 93030

8. Ion exchange resin: Ionac, ANG C-101 50-80 mesh, Baker Analyzed, Lot #44030

C.2

9. Filter floss, polyester, Metaframe, A Mattel Co., "Wonderwool" for Aquarium Filtration

10. Tetracycline: Rachelle, Tetrachel-Vet-102, Tetracycline HCl 700 Henry Ford Ave., Long Beach, Calif. 90801

11. Whatman Filter paper #1, #30 and #40, made in England, W&R Balston Ltd.

12. Millipore filters, Lot 340773 0.45 μ 142 mm diameter

13. Antibiotic sensitivity discs: Multidisc for ten antibiotics, Consolidated Labs, Inc., Code No. 11-160T and 11-1600

14. Chemware Fluorocarbon, Filter Membranes of Zitex, Chemplast, Inc. Wayne, New Jersey, 07470 150 mm diam.

15. Jack Bean, Urease enzyme
    Baker Chemical Company
    Worthington Biochemical Co., Lot URC 44E040
    Worthington Biochemical Co., Lot URC 35A616

16. Kantrex, Kanamycin sulfate injection (1g per 3 ml), Bristol Laboratories, Syracuse, New York

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APPENDIX 3

METHODS FOR PREPARATION OF REAGENTS, STANDARDS AND SOLUTIONS

1. Standards for atomic absorption and flame emission analysis:

   a) 1001.0 ppm Mg^{++}: 4.95105 g $MgSO_4$/liter for 1000 ppm

   b) 1006.9 ppm Ca^{++}: 2.78595 g $CaCl_2$/liter for 1000 ppm

   c) 1000.4 ppm Na^{+}: 2.54997 g NaCl/liter for 1000 ppm

   d) 1004.2 ppm K^{+}: 1.91254 g KCl/liter for 1000 ppm

   e) Other concentrations prepared by appropriate dilution using Class A volumetric pipets

2. Standards for ammonium assay:

   a) 100 ppm NH_3: 0.3145 g NH_4Cl/liter (actually 99.96 ppm)

   b) Other concentrations were prepared by appropriate dilution. Standards for the 30 day test were pipeted and frozen until used.

3. Standards for nitrate assay:

   a) A solution 0.1 M $NaNO_3$ was prepared by dissolving 0.8500 g sodium nitrate in double distilled water and making up to 100 ml.

   b) Other concentrations were prepared by appropriate dilution. Standards for the 30 day test were pipeted and frozen until used.

4. Standards for urea assay:

   a) 1000 ppm urea: 1 g urea/liter

   b) Other concentrations were prepared by appropriate dilution. Standards for the 30 day test were pipeted and frozen until used.

5. Ion Solution I:

   K^{+} 8 ppm (0.0153 g KCl/liter)
   Na^{+} 931 ppm (Counting 923 from 0.025M sodium phosphate buffer 0.0204 g NaCl/liter)
   Ca^{++} 48.3 ppm (0.1338 g $CaCl_2$/liter)
   Mg^{++} 11.8 ppm (0.0582 g Mg SO_4/liter)

6. Ion Solution II:

K^+ 8 ppm (0.0153 gKCl/l)
Mg^{++} 1.2 ppm (0.0059 $gMgSO_4$/l)
Ca^{++} 5 ppm (0.0139 $gCaCl_2$/l)

7. Ion Solution III: (for columns 5.1 and 6.1)

Mg^{++} 0.52 ppm
Ca^{++} 1.1 ppm
Na^+ 160 ppm

8. Ion Solution IV (for supplemental cation loading test on 10 day column)

K^+ 80.5 ppm (0.7058 gKCl/4.6 liters)
Mg^{++} 12.1 ppm (0.2754 $gMgSO_4$/4.6 liters)
Ca^{++} 50.2 ppm (0.6400 g $CaCl_2$/4.6 liters)

9. Ion Solution V: (for 30 day test)

K^+ 16 ppm (0.3060 gKCl/10 liters)
Mg^{++} 1.2 ppm (0.0590 $gMgSO_4$/10 liters)
Ca^{++} 10.0 ppm (0.2780 $gCaCl_2$/10 liters)

10. 0.05M Sodium Phosphate Buffer

0.0306M Na_2HPO_4 (4.3 g/l)
0.0194M NaH_2PO_4 (2.7 g/l)

11. 0.05M Potassium phosphate buffer

0.0306M K_2HPO_4 (7.0 g/l)
0.0194M KH_2PO_4 (2.6 g/l)

12. 0.02M Potassium phosphate buffer

0.0122M K_2HPO_4 (2.78 g/l)
0.0076M KH_2PO_4 (1.03 g/l)

13. 2,3 - Butanedione monoxime

5 g. 2,3 - Butanedione monoxime and 150 g sodium chloride dissolved in double distilled water and made up to one liter

14. 10M NaOH (400 g/liter)

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APPENDIX 4

METHODS OF ANALYSIS

1. Determination of Urea: Urea was determined fluorometrically using the method of Joon H. Rho. One milliliter of blank, standard or sample was placed in a test tube having a tight fitting plastic cap. 1.6 ml of 2,3-Butanedione monoxime (see appendix on reagents standards and solutions) was added followed by 0.4 ml of concentrated sulfuric acid. The test tube was placed in boiling water bath for 40 minutes, and cooled to room temperature. Since prolonged standing at room temperature made the peak of the 10 ppm standard disappear, solutions were processed in 10-15 minutes from the time they were removed from the bath. Standards having values of 10, 20, 30, 40, and 50 ppm were run with each set of samples.

   After cooling, the liquid was transferred to a 3 ml quartz cuvette, one centimeter square and 3 cm in height. The emission intensity at 515 nm was measured after excitation at 380 nm. Maximum peak height was used for the analyses rather than wavelength because the emission maxima show a slight bathochromic shift at increasing concentrations.

2. Determination of Ammonia: Ten milliliters of standard or sample were placed in a 20 ml beaker with a magnetic stirring bar. The beaker was placed on a magnetic stirrer and the Orion Ammonia-Gas electrode (95-10) was carefully lowered into the liquid to avoid trapping air bubbles. After activating the stirrer, 0.1 ml of 10 M NaOH was added to basify the sample and convert NH_4^+ to NH_3 and a timer was started. Assays on columns (5.1 and 6.1, 7 and 8, and 7.1 and 8.1) and for frog waste water characterization were read 2 minutes after base addition. Assays on the 10 day and 30 day tests were read 3 minutes after base addition. Millivolt readings from samples were compared to a standard curve.

3. Determination of Nitrate: Ten ml of sample or standard was placed in a beaker with a stirrer on a magnetic mixer, the Orion nitrate electrode was carefully lowered into the liquid and the millivolt output determined on a specific ion meter.

4. Determination of Cation Concentrations: Mg^{++} and Ca^{++} were determined using atomic absorption spectrophotometry (Jarrell-Ash). K^+ and Na^+ were determined using flame emission spectrophotometry (Jarrell-Ash). Specific conditions are listed below:

Mg^{++} 2852 A^{o}, H_2 - N_2O, full damping, 18 mm burner height

Ca^{++} 4227 A^{o}, other conditions the same

K^{+} 7665 A^{o}, other conditions the same

Na^{+} 5890 A^{o}, other conditions the same

5. Determination of Bioburden: Materials: (1) dilution tubes containing 9 ml of sterile phosphate buffered saline, (2) Petri plates containing about 25 ml of sterile Trypticase Soy Agar (TSA), (3) ethylalcohol to flame spreader, and (4) spreader, sterile disposable 1.0 ml pipets, Bunsen burner.
Method of assay: (1) a serial dilution of the sample was prepared in phosphate buffered saline (PBS), (2) appropriate 0.1 ml portions of the diluted sample were pipeted asceptically onto TSA and spread with a glass spreader flamed in alcohol, (3) the spread plates were inverted and incubated at 32°C for 24 hours prior to counting the total number of colonies formed.

6. Enzyme Activity Assays: Enzyme activity assays were conducted according to the methods prescribed by Worthington (6) (modified by making the substrate solution in 10^{-3}M sodium EDTA and titrating to pH 5 instead of using indicator). The procedure for conducting a urease activity assay is as follows:

Enzyme: 10 mg of urease is suspended in 10 ml 0.02 M potassium phosphate buffer (pH=7) (Momentary mixing on vortex mixer Vari-whirl from Van Waters and Rogers aided in solubilizing the enzyme).

Substrate: 1.5g urea is dissolved in 40 ml of 0.75 M potassium phosphate buffer (pH 7) containing 18.6 mg Disodium ethylenediaminetetraacetate dihydrate. Quantity sufficient for 50 ml.

Procedure: 1 ml of substrate and 1 ml of 0.02 M potassium phosphase buffer were titrated with 0.10 M HCl to pH 5. Number of ml of acid is "A" in equation below. One ml of enzyme suspension was added to 1 ml substrate at time zero. After 5 minutes, 0.10 M HCl quantity - A was added. Addition continued to pH 5. Total volume = B.

$$\text{Units} = \frac{(B-A)(\text{Molarity of acid})(1000)}{5}$$

One unit consists of a conversion ability of one micromole/minute.

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APPENDIX 5

METHODS OF FILTRATION

1. Routine Prefiltration: Water coming out of the frog cage was passed through a coarse filter before return to the Lauda water bath. The filter was prepared by placing washed filter floss approximately one centimeter thick in a special funnel and covering the floss with a piece of stainless steel screen (0.25 in. mesh). Water from the gravity overflow on the cage tank drained directly into the coarse filter and through into the Lauda water bath.

2. Coarse Filtering: Water in the Lauda water bath and frog cage tank (in 30 day test) were transferred to a separate container and pulled through #1, #30, or #40 Whatman filter paper in a large Buchner funnel with vacuum.

3. Fine Filtration: Water in both the 10 day and 30 day tests was occasionally filtered through either 0.22μ or 0.45μ Millipore filter membranes in 142 mm filter holder as a means of reducing the bioburden (number of bacteria per ml of water).

APPENDIX 6

PREPARATION OF MOUSE PUREE

Four mice (adult) were dropped serially into a Waring Blendor containing 40 ml of distilled water. Another aliquot of 35 ml water was added and the contents of the blendor were transferred to a centrifuge tube. After centrifuging at 2000 rpm for 1 minute, the supernatant including the fat layer was transferred to a syringe and fed to the frogs through a catheter placed into the stomach cavity.

APPENDIX 7

METHOD OF OZONATION

The water was filtered through #1, #30 or #40 Whatman filter paper supported on a Buchner funnel by a Chemware(R) filter disk. The teflon filter disk was used to elevate to paper to prevent plugging and speed filtration.

The filtered water was typically ozonated at 1.0 liter per minute (uncorrected) for five minutes. The water was placed in a 6 liter flask and stirred magnetically while the gas was delivered through a medium porosity glass frit 10 mm in diameter at the edge of the spinbar. The ozone was produced from oxygen without further purification in a Welshack model 408 ozone generator. The gas stream consistently contained at least 5.5% ozone.

The water which was usually cloudy and slightly yellow emerged from ozonation white and clear. Oxygen flow was continued until ozone in the effluent gas was no longer detectable with moistened starch iodide test paper.

APPENDIX 8

METHODS OF COLUMN PACKING IN 30 DAY TEST

Two hundred grams of ANGC-101 in exchange resin (dry weight) were suspended in double distilled water and allowed to partially settle. With the fines still suspended, the supernatant was decanted. The original column used in the 30 day test was prepared from 208 g of resin and decanted five times with 1800 ml of water. The column packed on day 18 of the thirty day test was packed with 200 g (dry weight) of resin and washed one time with 500 ml of water, decanting the fines in the wash water. The column prepared on day 27 was packed with 200 g (dry weight) of resin and washed seven times with 400 ml of water, decanting the fines each time.

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APPENDIX 9

DATA TABLES

Table A1 Assayed Cation Concentration[1] in Solutions from Columns 3 and 4. All Concentrations in ppm.

Element	Starting Solution[2]	100 ml Aliquots			
		1st	2nd	3rd	4th
Mg^{++}	1.2	8.3	5.5	2.2	1.5
Ca^{++}	5.2	7.5	11.2	12.1	11.2
K^{+}	8.4	0.5	1.0	1.0	1.0
Na^{+}	1880	1130	1790	1820	1830

1 K and Na assays were performed using flame emission Spectrometry. Ca and Mg were assayed using atomic absorption spectrometry.

2 Prior to exposure to ANGC-101

Table A2 Assayed Cation Concentration in Solutions from Columns 5 and 6. All concentrations in ppm

Element	Starting Solution[1]	100 ml Aliquots			
		1st	2nd	3rd	4th
Mg^{++}	1.2	0.5	1.7	1.8	1.7
Ca^{++}	4.5	1.1	3.3	3.6	3.7
K^{+}	8.5	0.1	0.2	0.2	0.2
Na^{+}	480	300	460	470	470

1 Prior to exposure to ANGC-101

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TABLE A3 DATA LOG - 10 DAY SIMULATION OF A 30 DAY EXPERIMENT

DAY	DATE	TIME IN HOURS	mg UREA ADDED	mg/l UREA	mg UREASE	TIME OF UREASE ADDITION (Hours)	ml 0.1N HCl	mg NH_4Cl ADDED	mg/l NH_3	ppm NO_3^-	pH
1	10-9	0.0	85.4	0.0	-	-	-	57.3	<0.1	0	7.0
2	10-10	22.2	83.7	20.0	-	-	-	57.0	<0.1	0	6.8
3	10-11	44.2	83.9	39.2	-	-	-	57.3	<0.1	0.25	7.0
4	10-12	70.9	84.7	47.0	53.7	73.2	22.13	56.8	<0.1	0.37	7.0
5	10-13	96.6	84.0	33.5	77.1	99.5	31.45	57.2	<0.1	0.56	7.0
6	10-14	116.8	84.5	5.0	75.8	123.7	-	57.1	0.4	0.81	7.0
7	10-15	142.0	84.3	<1.0	75.0	147.3	17.7	56.8	0.5	0.56	7.2
8	10-16	165.0	84.1	5.0	76.2	170.2	28.66	59.9	1.0	0.68	7.2
9	10-17	188.7	84.2	0	-	-	-	57.4	2.7	0.81	6.9
10	10-18	224.2	84.5	3.3	-	-	-	56.8	4.8	0.93	7.0
11	10-19	240.2	-	17.0	-	-	-	-	4.4	0.81	7.1

TABLE A4 DATA LOG - 10 DAY SIMULATION OF A 30 DAY EXPERIMENT (Cont'd)

DAY	HOURS	TEMPERATURE °C	TOTAL MICROBIAL BURDEN/ml	KANTREX ml	FILTRATION	O_2 FLOW	SAMPLE VOL. REPLENISHMENT [2]	EVAPORATION REP. [3]	PPM			
									Ca^{++}	Mg^{++}	K^{+}	Na^{+}
1	0.0	16.5	0	-	-	OK	100 ml	0	5.2	1.22	8.7	8.9
2	22.2	16.4	1.4×10^3	-	-	OK	100	250	0.0	0.02	0.1	20.0
3	44.2	16.4	$>3 \times 10^4$	0.15	-	OK	100	350	-	-	-	-
4	70.9	16.4	4.1×10^6	0.20	-	OK	100	400	-	-	-	-
5	96.6	16.2	4.7×10^5	0.20	-	OK	100	370	0.2	0.03	0.1	35.0
6	116.8	16.3	2.1×10^6	0.20	0.45	OK	100	110	-	-	-	-
7	142.0	16.4	7.2×10^5	-	-	OK	100	300	-	-	-	-
8	165.0	16.4	3.1×10^5 [1]	-	0.22	OK	100	90	0.2	0.06	0.3	62.0
9	188.7	16.4	4.8×10^5	-	-	OK	100	-	-	-	-	-
10	224.2	16.4	3.7×10^4	-	-	OK	100	470	-	-	-	-
11	240.2	16.4	1.7×10^4	-	-	OK	-	-	0.5	0.16	0.5	82.0

1 1.4×10^4 after filtration and circulation

2 Ion Solution II plus 8 ppm Na

3 Distilled water

TABLE A5 DATA LOG - SUPPLEMENTAL CATION LOADING TEST - 10 DAY SIMULATION

DAY	DATE	HOURS	TEMP. °C	mg UREA ADDED	mg/l UREA	NH_4Cl ADDED	mg/l NH_3	pH	$\underline{M} \times 10^{-4}$ NO_3^-	BIO-BURDEN [4]	Ca^{++}	Mg^{++}	K^+	Na^+
1 [1]	10-20	0.0	16.3								41.9	2.4	84.5	0.8
1 [2]	10-20	0.3									7.0	11.4	22.5	0.8
1 [3]	10-20	0.6		86.2	21	58.1	3.5		1.65		3.9	2.8	5.8	102.0
2	10-21	6.6		84.0	19	57.0	4.8			2.3×10^4	5.2	2.2	2.9	105.0
3	10-22	37.1	16.3		40		5.6	6.8			2.1	1.6	2.9	114.0
4	10-23	62.2			35		6.7				2.4	1.6	3.1	121.0
5	10-24	81.1			31		6.9				2.1	1.5	8.8	125.0

1 Before passage through column

2 After addition of 2 drops dilute NaOH

3 Starting Sample 1st day

4 Total microorganisms/ml

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Table A6- The Ratio of Ammonium Ion to Ammonia at pH 6 to 8 and 15 - 25°C

pH	NH_4^+/NH_3 (15°C)	NH_4^+/NH_3 (20°C)	NH_4^+/NH_3 (25°C)
6.0	1652	1710	1774
6.1	1312	1358	1409
6.2	1042	1079	1119
6.3	827.9	857.0	889.2
6.4	657.7	680.8	706.3
6.5	522.4	540.8	561.0
6.6	415.0	429.5	445.7
6.7	329.6	341.2	354.0
6.8	261.8	271.0	281.2
6.9	208.0	215.3	223.4
7.0	165.2	171.0	177.4
7.1	131.2	135.8	140.9
7.2	104.2	107.9	111.9
7.3	82.79	85.70	88.92
7.4	65.77	68.08	70.63
7.5	52.24	54.08	56.10
7.6	41.50	42.95	44.57
7.7	32.96	34.12	35.40
7.8	26.18	27.10	28.12
7.9	20.80	21.53	22.34
8.0	16.52	17.10	17.74

Table A7- Average Noise Level in Frog Housing

In the walk-in chamher used to house the frogs, compressors and blowers contributed to a noise level which was possibly an environmental stress. For this reason, several actions were taken to reduce blower noise and the residual level of noise was determined using a Bruel and Kjaer precision sound level meter using A weighed scale - slow, on the 70 db range.

The cage farthest from the blower averaged 72.1 db and the one nearest averaged 74.1 db in the cages. Sound Level Readings were taken in each cage.

LEFT CAGE		RIGHT CAGE	
73.0-73.5	72.5-73.5	74.5-75.0	73.0-73.5
70.5-71.0	70.5-71.0	73.5-74.0	73.5-74.0
72.0	72.5-73.0	73.5-74.0	75-75.5

Table A8 Primary Data from 10 Day Frog Waste Water Characterization

DATE	# of mice eaten		Assayed Ammonia conc. (mg/l)[1]		Assayed urea conc. (mg/l)[2]		Total microbial count/ml[3]		
	left	right	left	right	left	right	left	right	Tap Water
9-6-75	2	6	-	-	-	-	-	-	-
9-7-75	0	3	-	-	-	-	-	-	-
9-8-75	1	0	-	-	-	-	-	-	-
9-9-75	1	3	3.9	13.7	24.8	53.0	-	-	-
9-10-75	1	3	6.5	19.4	16.5	42.5	2.8×10^6	2.8×10^6	0
9-11-75	1	3	3.1	11.5	12.0	30.0	3.2×10^6	11.0×10^6	3.5
9-12-75	0	0	3.5	12.8	13.7	41.5	2.5×10^6	6.0×10^6	-
9-13-75	0	2	2.6	9.4	13.0	45.6	0.6×10^6	0.7×10^6	0
9-14-75	0	0	0.6	7.2	3.5	32.5	2.0×10^6	0.4×10^6	0.5
9-15-75	1	3	1.3	4.8	30.0	18.5	0.4×10^6	8.2×10^6	1.5
9-16-75	1	3	1.1	4.4	14.5	25.0	0.7×10^6	1.1×10^6	1.0
9-17-75	0	3	0.9	3.4	10.0	26.5	-	-	-
9-18-75	0	2	0.2	4.8	14.5	30.0	-	-	-

NOTE: Left and right refer to the cages. There were 2 frogs in the left cage and 6 in the right. Air temperature was about 22°C.

1. Assayed using Orion Ammonia-gas electrode, samples are basified to convert all ammonium ion to ammonia. 2. Fluorometric assay procedure as described in last monthly report. 3. Total count on Trypticase Soy Agar.

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Table A9 Analysis of Primary Data from 10 Day Frog Waste Water Characterization: Urea & Ammonium Ion Excretion by Bullfrogs at 22°C

Date	No. Mice eaten		mg NH_4^+ Excreted per Frog[1]		mg Urea Excreted per Frog[2]		Total mg NH_4^+ Excreted[3]		Total mg Urea Excreted	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
9-6	2	6	-	-	-	-	-	-	-	-
9-7	0	3	-	-	-	-	-	-	-	-
9-8	1	0	-	-	-	-	-	-	-	-
9-9	1	3	41.3	48.4	248.0	176.7	83	290	496	1060
9-10	1	3	68.8	68.5	165.0	141.7	138	411	330	850
9-11	1	3	32.8	40.6	120.0	100.0	66	244	240	600
9-12	0	0	37.1	45.2	137.0	138.3	74	271	274	830
9-13	0	2	27.5	33.1	130.0	152.0	55	199	260	912
9-14	0	0	6.3	25.4	35.0	108.3	13	152	70	650
9-15	1	3	13.8	16.9	300.0	61.7	28	101	600	370
9-16	1	3	11.6	15.6	145.0	83.3	23	94	290	500
9-17	0	3	9.5	12.0	100.0	88.3	19	72	200	530
9-18	0	2	2.1	16.9	145.0	100.0	4	101	290	600
					Total		503	1935	3050	6902
					Total		2438		9952	

1. mg $NH_3/\ell \times \frac{18 mgNH_4^+}{17 mgNH_3} \times 20\ell \div$ no. of frogs
2. mg urea/ℓ x 20ℓ ÷ no. of frogs
3. Excretion/frog x no. of frogs

Note: There were 2 frogs on the left, 6 on the right.

TABLE A10 DATA LOG - 30 DAY PERFORMANCE TEXT

DATE	TIME	TEMPERATURE °C			HUMIDITY %
		AIR	WATER	LAUDA	
10/31	73.9	-	-	-	-
11/1	94.3	21.5	17	16.2	66
11/2	122.2	21.5	17.4	16.5	54
11/3	140.9	21.5	17.4	16.5	48
11/4	162.6	21.5	17.5	16.4	48
11/5	187.6	21.4	17.7	16.5	46
11/6	210.5	21.3	16.5	16.4	50
11/7	234.9	20.8	17.5	16.5	45
11/8	262.3	21.2	17.7	16.5	43
11/9	290.7	21.5	17.5	16.2	48
11/10	307.8	21.3	17.8	16.5	42
11/11	330.9	20.8	17.8	16.5	44
11/12	352.9	20.2	18.0	16.5	40
11/13	378.7	21	18	16.4	40
11/14	402.5	21.2	17.8	16.5	40
11/15	426.9	24.6	17.9	16.5	19
11/16	451.4	24.5	17.8	16.5	18
11/17	476.1	21.5	18.5	16.5	20
11/18	498.4	21.5	17.5	16.5	44
11/19	526.6	20.9	17.2	16.4	42
11/20	546.6	21.5	17.8	16.5	40
11/21	570.3	20.5	17.5	16.5	42
11/22	599.7	21.0	18.2	16.5	36
11/23	620.0	21.2	19.0	16.4	40
11/24	642.7	20.0	18.5	16.5	45
11/25	666.4	20.2	17.0	16.3	42
11/26	691.5	21.8	17.0	16.4	43
11/27	716.0	21.5	17.7	16.5	40
11/28	741.8	21.5	17	16.5	40
11/29	765.7	21.5	17.2	16.5	40
11/30	788.2	21.5	17.9	16.5	40
12/1	811.1	21.0	17.5	16.5	40
12/2					
12/3					

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TABLE A11 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	FLOW		pH	D.O.
		O_2	H_2O		
10/31	73.9	✓	6	-	-
11/1	94.3	✓	6	-	10.4
11/2	122.2	✓	5.5	6.8	17.9
11/3	140.9	✓	5.5	6.5	15.8
11/4	162.6	✓	6	6.9	-
11/5	187.6	✓	6	6.7	20
11/6	210.5	✓	6	6.9	20
11/7	234.9	.	6	7.2	-
11/8	262.3	✓	4.5	7.1	20
11/9	290.7	✓	6	7.15	-
11/10	307.8	✓	6	7.1	-
11/11	330.9	✓	6	7.1	-
11/12	352.9	✓	4	7.5	-
11/13	378.7	✓	6	7.5	-
11/14	402.5	✓	6	7.5	-
11/15	426.9	0	4	7.3	-
11/16	451.4	✓	6	7.6	-
11/17	476.1	✓	5	7.7	-
11/18	498.4	✓	Slow	7.5	-
11/19	526.6	✓	6	6.9	-
11/20	546.6	✓	6	7.0	-
11/21	570.3	✓	6	7.0	-
11/22	599.7	0	4	6.9	-
11/23	620.0	0	4	7.3	-
11/24	642.7	✓	4	7.5	-
11/25	666.4	✓	6	7.3	-
11/26	691.5	✓	6	7.6	-
11/27	716.0	✓	6	8.0	-
11/28	741.8	✓	8	7.4	-
11/29	765.7	✓	6	7.3	-
11/30	788.2	✓	6	7.3	-
12/1	811.1	✓	6	7.1	-
12/2					
12/3					

TABLE A12 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	PREVIOUS DAY MICROBIAL COUNT (/ml)	UREA (ppm)	AMMONIA (ppm)	NITRATE M $\times 10^{-5}$
10/31	73.9		0	0.17	7.8
11/1	94.3	-	21.5	<0.1	5.2
11/2	122.2	5.3×10^6	32.	<0.1	8.8
11/3	140.9	1.3×10^6	3.5	<0.1	9
11/4	162.6	1.2×10^6	4	<0.1	7.6
11/5	187.6	1.5×10^6	14	<0.1	5.2
11/6	210.5	5.6×10^5	23	<0.1	8.4
11/7	234.9	5.2×10^5	2	0.1	6.6
11/8	262.3	3.5×10^5	11	<0.1	12
11/9	290.7	4.4×10^5	35	<0.1	79
11/10	307.8	1.5×10^5	32	<0.1	62
11/11	330.9	7.0×10^4	30	0.1	14
11/12	352.9	7.9×10^4	23	0.54	9.8
11/13	378.7	1.2×10^5	28	0.25	12
11/14	402.5	1.6×10^5	26	0.12	14
11/15	426.9	3.3×10^5	10	0.38	5.6
11/16	451.4	2.2×10^5	35	0.78	10
11/17	476.1	2.7×10^7	75	1.2	8
11/18	498.4	1.1×10^5	18	10	8
11/19	526.6	2.0×10^6	22	<0.1	12
11/20	546.6	1.6×10^6	27	<0.1	7.2
11/21	570.3	3.4×10^5	25	<0.1	7.5
11/22	599.7	2.5×10^5	50	<0.1	10
11/23	620.0	1.4×10^5	60	<0.1	19
11/24	642.7	2.5×10^5	75	1.0	25
11/25	666.4	1.2×10^6	2.5	4.7	410
11/26	691.5	1.2×10^6	23.5	4.0	27
11/27	716.0	1.2×10^6	32	8	13
11/28	741.8	4.0×10^5	42	0.2	530
11/29	765.7	1.4×10^6	5.5	2.3	10
11/30	788.2	2.0×10^6	22	<0.1	12
12/1	811.1	6.2×10^5	30	<0.1	21
12/2		2.6×10^5			
12/3					

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TABLE A13 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	EVAPORATION REPLENISHMENT (ml)	UREASE ADDITION (mg)	0.1N HCl ADDITION (ml)	BIOBURDEN CONTROL METHOD
10/31	73.9	-	-	-	-
11/1	94.3	0.0	-	-	-
11/2	122.2	0	97.5	16.85	-
11/3	140.9	100	-	-	Whatman #5
11/4	162.6	700	-	-	O_3
11/5	187.6	300	-	-	O_3
11/6	210.5	200	106.7	20.37	O_3
11/7	234.9	300	-	-	O_3
11/8	262.3	505	-	-	-
11/9	290.7	800	-	-	O_3
11/10	307.8	200	-	-	O_3
11/11	330.9	400	101.6	15.50	O_3
11/12	352.9	300	-	-	Repack Column
11/13	378.7	400	-	-	Filtered 0.45 μ
11/14	402.5	400	103.3	40.28	Filtered 0.45 μ
11/15	426.9	0	-	-	-
11/16	451.4	400	-	18.73	Millipore 0.45 μ
11/17	476.1	600	105.0 99.6	3.80 45.90	O_3
11/18	498.9	300	-	-	Millipore 0.45 μ + O_3
11/19	526.6	200	-	-	O_3
11/20	546.6	1200	-	-	O_3
11/21	570.3	1000	-	-	O_3
11/22	599.7	100	99.9	40.52	O_3
11/23	620.0	400	-	-	O_3
11/24	642.7	-	100.9	51.53	O_3
11/25	666.4	-	-	-	Whatman #1
11/26	691.5	100	-	-	O_3
11/27	716.0	400	-	-	O_3
11/28	741.8	100	99.5	68.25	-
11/29	765.7	-	-	Unknown Amt. added	-
11/30	788.2	200	-	-	O_3
12/1	811.1	-	-	-	-
12/2					
12/3					

TABLE A14 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	CATIONS (ppm)			
		K^+	Na^+	Mg^{++}	Ca^{++}
10/31	73.9	17.3	2.2	0.93	9.2
11/1	94.3				
11/2	122.2				
11/3	140.9	0.10	44	0	0
11/4	162.6				
11/5	187.6				
11/6	210.5	0.43	55	0	0
11/7	234.9	0.64	64	0	0
11/8	262.3				
11/9	290.7	0.64	68	0	0
11/10	307.8				
11/11	330.9				
11/12	352.9	0.83	88	0	0
11/13	378.7	0.82	80	0	0.3
11/14	402.5				
11/15	426.9	0.86	94	0	0
11/16	451.4				
11/17	476.1				
11/18	498.4				
11/19	526.6				
11/20	546.6				
11/21	570.3	5.4	86	0.24	1.2
11/22	599.7				
11/23	620.0				
11/24	642.7	6.4	112	0.16	0.2
11/25	666.4	0.89	139	0.07	0.6
11/26	691.5				
11/27	716.0	0.93	140	<0.01	0.2
11/28	741.8	0.69	108	0	0
11/29	765.7				
11/30	788.2	0.47	130	0.01	0
12/1	811.1	0.44	123	<0.01	0.2
12/2					
12/3					

TABLE A15 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	FROG #1 WEIGHT(g)	FROG #4 WEIGHT(g)	FROG #2 WEIGHT(g)
10/31	73.9	499	510	553
11/1	94.3			
11/2	122.2			
11/3	140.9	475	455	550
11/4	162.6			
11/5	187.6			
11/6	210.5	504	448	539
11/7	234.9			
11/8	262.3			
11/9	290.7			
11/10	307.8	490	434	525
11/11	330.9			
11/12	352.9			
11/13	378.7			
11/14	402.5	502	465	598
11/15	426.9			
11/16	451.4			
11/17	476.1			
11/18	498.8			
11/19	526.6			
11/20	546.6			
11/21	570.3	502	436	554
11/22	599.7			
11/23	620.0			
11/24	642.7			
11/25	666.4			
11/26	691.5	463	442	570
11/27	716.0			
11/28	741.8			
11/29	765.7			
11/30	788.2			
12/1	811.1			
12/2				
12/3		459	405	544

TABLE A16 DATA LOG - 30 DAY PERFORMANCE TEST (Cont'd)

DATE	TIME	FROG #3 WEIGHT(g)	FROG #6 WEIGHT(g)	FROG #7 WEIGHT(g)
10/31	73.9	471	649	670
11/1	94.3			
11/2	122.2			
11/3	140.9	557	669	657
11/4	162.6			
11/5	187.6			
11/6	210.5	510	634	632
11/7	234.9			
11/8	262.3			
11/9	290.7			
11/10	307.8	544	636	648
11/11	330.9			
11/12	352.9			
11/13	378.7			
11/14	402.5	534	694	630
11/15	426.9			
11/16	451.4			
11/17	476.1			
11/18	498.4			
11/19	526.6			
11/20	546.6			
11/21	570.3	598	664	637
11/22	599.7			
11/23	620.0			
11/24	642.7			
11/25	666.4			
11/26	691.5	520	650	640
11/27	716.0			
11/28	741.8			
11/29	765.7			
11/30	788.2			
12/1	811.1			
12/2				
12/3		506	612	646

APPENDIX 10

ANALYSIS OF FAILED COLUMN IN 30 DAY TEST

When the first column in the 30 day test clogged up preventing fluid flow on the 12th day of the test, 352.9 hours into the test, the column was removed and the resin was washed with glass distilled water. The first wash was 740 ml, the second 780 ml, and the third was 600 ml. Observations of the column and assays of the column washes are shown here.

The column had a slightly brownish buildup on the polyester floss on the inlet side of the column. Observation of the floss under a microscope revealed a buildup of slimy material but very little debris on the floss. Large numbers of three types of protozoa were present, (1) clumps of stalked ciliates of the genus Vorticella were attached to the floss in the slimy matrix, (2) many unicellular, colorless, flagellates, and (3) rotifers. The types of organisms found here are commonly found in highly oxygenated activated sludge waste water treatment facilities.

Figure A1 shows a photomicrograph of a stalked ciliate. Figure A2 shows a blurred picture of a rotifer. Resin taken from bottom of the column had the appearance shown in Figure A3 .

The water from the first wash of the resin contained large numbers of the colorless uniflagellates but no stalked ciliates or rotifers. Observation at 1000X revealed large clumps of Gram negative filamentous bacteria and some Gram positive bacteria. A bioassay of the first wash indicated a population of 6.3×10^7 microorganisms/ml of wash water.

The three aliquots of wash water were assayed for cation content and the results are shown in Table A 17.

TABLE A17 CATION CONCENTRATIONS IN WASH SOLUTION FROM THE 1st COLUMN IN THE 30 DAY TEST

ELEMENT	1st WASH	2nd WASH	3rd WASH
K^+	0.94	0.69	0.46
Na^+	32.0	12.5	7.5
Mg^{++}	0.01	0.04	0
Ca^{++}	0	0	0

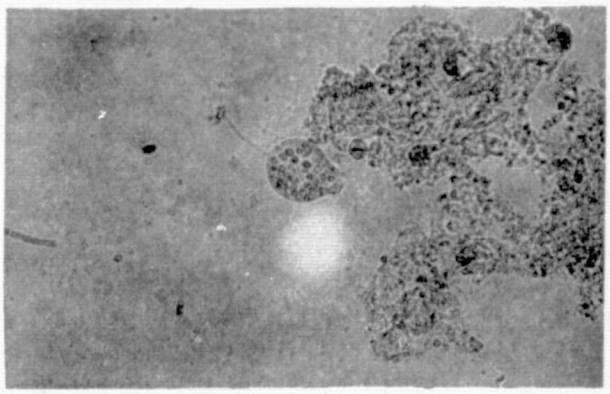

Figure A1 Stalked Ciliate

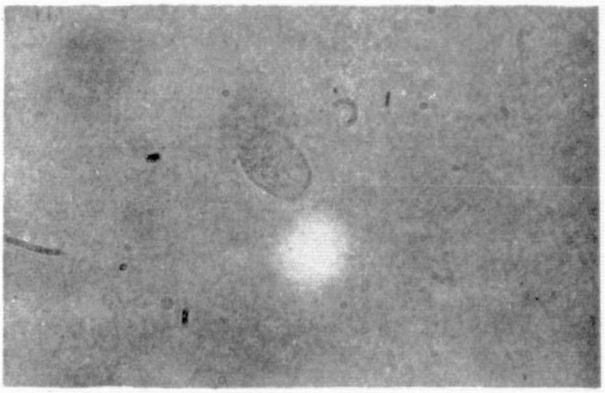

Figure A2 Rotifer

Figure A3 ANGC-101 Ion Exchange Resin

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APPENDIX 11

PARTIAL CHARACTERIZATION OF OFO TYPE MICROORGANISMS

Characterizations are listed according to the test from which the organisms were isolated.

Urease Activity Tests:

Test e: Ion Solution II plus 8 ppm Na^+ at 16.5°C, 40 ppm urea, circulated and oxygenated. The total microbial burden was 2.0 x 10^4/ml after 21 hours, plates were incubated at 32°C for 24 hours. Basic materials and an odor of ammonia were produced during prolonged growth on TSA. Differential counts for seven types of microorganisms are shown below:

1. Creamy tan color, round, 4-8 mm, flat but raised in center, shiny, translucent: 1.1 x 10^4/ml

2. Pale yellow color, round, 3-6 mm, larger colonies have indentation in center, shiny, opaque: 1.6 x 10^3/ml

3. Like 1 but has darker pigment, brownish coloration in the center of the colony, opaque: 4 x 10^2/ml (did not grow on subculture)

4. Tan coloring like 1, convex rather than flat: 5 x 10^2/ml

5. Creamy color, round, 2 mm, translucent: 1.6 x 10^3/ml (did not grow on subculture)

6. White around edges, grey-brown in center, round, 5-10 mm, filamentous (fungi): 6.0 x 10^2/ml

7. Tan color, 2-3 mm, irregular edge, rough, raised: 2 x 10^2/ml

The sensitivity of each of these organisms to kanamycin and tetracycline is shown in the table below. A rating of 0 means there was no visible inhibition and a rating of 5 means a very definite inhibition of growth.

ORGANISM	KANAMYCIN	TETRACYCLINE
1	0	0
2	2	0
4	4	1
6	0	0
7	0	0

Test k: A beaker test like Test j - Ion Solution II plus 8 ppm Na, ANGC-101 at 25°C with pH control. Total burden was 5.8 x 10^2 microorganisms 1 ml after 1 hour. Differential counts for three organisms are listed below:

8. White, round, 1.5 mm, convex, shiny, translucent: 195/ml

9. White, round, 1.5-2 mm, flat, shiny, translucent: 55/ml

10. Creamy, round 4-5 mm, convex, shiny: 25/ml

ORGANISM	KANAMYCIN	TETRACYCLINE
8	5	5
9	5	2
10	0	0

10 Day Simulation of a 30 Day Experiment: See description in Task 4 for details on total bioburden each day. Differential counts are tabulated for four major colony types.

11. Creamy, round, 2-4 mm, entire, shiny, smooth. This organism predominated until 10-16.

12. Salmon color pigment, round, 6-10 mm, entire, shiny. Remained at low level throughout the test.

13. Slightly yellowish pigment, round, 3-5 mm, entire, convex.

14. Creamy, round, 2 mm, raised convex, shiny.

DAY	MICROORGANISMS x 10^4/ml			
	11	12	13	14
10-13	Predominated	6.0	1.5	0.5
10-14	until 10-16	3.0	1.5	1.5
10-15		4.0	1.5	1.0
10-16		4.5	4.5	3.5
10-16[1]		0.5	0.0	0.5
10-17		3.0	5.5	23.0
10-18		1.5	3.5	0.5
10-19		0.5	1.0	0.5

1 after filtration

The antibiotic sensitivities of these organisms were screened using antibiotic multi-discs on TSA spread plates (Organism 14 did not develop on subculture).

ORGANISM	ANTIBIOTIC														
	K	B	A	AL	C	E	P	S	T	L	TE	AM+	CS	N	PB
11	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	3	0	2	0	0	0	0	0	1	0	2	0	0	0	0
13	4	0	0	0	0	0	0	0	1	0	0	0	0	0	4

K	-	kanamycin	T	-	oxytetracycline
B	-	bacitracin	L	-	lincomycin
A	-	chlortetracycline	TE	-	tetracycline
AL	-	novobiocin	AM+	-	ampicillin
C	-	chloramphenicol	CS	-	colistin
E	-	erythromycin	N	-	neomycin
P	-	penicillin G	PB	-	polymyxin B
S	-	dihydrostreptomycin			

30 Day System Performance Test: One unidentified culture (15) from the bioburden studies was assayed as were cultures 11,12, and 13. Colistin and penicillin G had an effectiveness of 1 or 2 for about 2 days then growth developed. All other antibiotics listed were ineffectual. When the first column failed and the resin was washed in glass distilled water, an assay was performed on the first wash. Sensitivity to kanamycin and tetracycline is indicated.

16. Yellow, round, 3 mm, convex, shiny: 11×10^7/ml (K-2, T-2).

17. Creamy, round, 2 mm, convex shiny: 8×10^7/ml (K-0.5, T-0.5).

18. Creamy, round, 3 mm convex but flatter than 16 or 17: 2×10^7/ml (did not develop).

19. Creamy white, round, 4 mm, convex, shiny: 1×10^7/ml (K-3, T-0).