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UTILIZATION OF IMMOBILIZED UREASE FOR WASTE WATER TREATMENT

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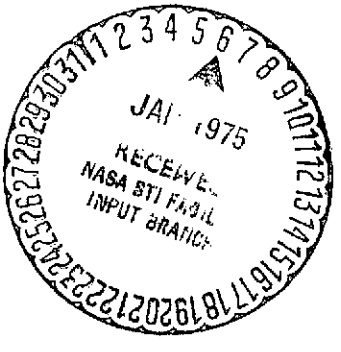

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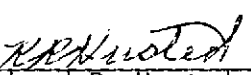

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SUMMARY

The objective of this program was to determine the feasibility of using immobilized urease for urea removal from waste water for space system applications, specifically the elimination of the urea toxicity problem in a 30-day Orbiting Frog Otolith (OFO) flight experiment. Because urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, control of their concentrations within nontoxic limits was also considered. Because the OFO A experiment had methods for controlling the CO_2 levels, only the control of NH_3 levels was addressed in this technical effort.

The results of this study led to the use of free urease in lieu of the immobilized urease for controlling urea concentrations. An ion-exchange resin was used which reduced the NH_3 level by 94% while reducing the sodium-ion concentration only 10%. Further investigation of the efficacy of using free urease and ion-exchange resin for controlling urea and ammonia concentrations was recommended.

CONTENTS

Page

FIGURES

TABLES

SYMBOLS

°C	degrees centigrade
ca.	approximately
CO_2	carbon dioxide
CST	Combined Systems Test
DEAE	diethylaminoethyl
°F	degrees Fahrenheit
g	gram
HCℓ	hydrochloric acid
Hg	mercury
in.	inch
ℓ	liter
M	molar or gram molecular weight/liter of solution
mg	milligram
min.	minute
ml	milliliter
mm	millimeters
mmole	millimole
N	nitrogen
N_2	molecular nitrogen
Na^+	sodium ion
NaOH	sodium hydroxide

NH_3	ammonia
NH_4^+	ammonium ion
NH_4Cl	ammonium chloride
pH	negative log of the hydrogen-ion molarity
psi	pounds per square inch
rpm	revolutions per minute
sec.	second
Torr	mm of Hg
ΔP	differential pressure
µg	microgram
µmole	micromole

INTRODUCTION

This report presents Martin Marietta Corporation's technical effort to demonstrate the feasibility of using immobilized urease for urea removal from waste water for space system application. 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 technical effort was accomplished 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.

TASK 1 - IMMOBILIZED UREASE TECHNOLOGY ANALYSIS

Literature Review

Existing literature on immobilized urease technology was reviewed including articles published from 1969 through 1973. This review indicated that present interest in immobilized urease is in its use in conjunction with ammonia-sensitive specific-ion electrodes for quantifying the presence of urea in solution. Guilbault and Montalvo [1] immobilized urease on nylon netting using an acrylamide gel solution. Modifications of their technique were employed in our immobilized urease preparations and are described in this section under materials and methods.

OFO Circulating Fluid

In order to establish the feasibility of using immobilized urease for controlling urea concentration in the OFO experiment, an environment similar to that expected for the experiment was established. The specifications for the OFO experiment are [2]:

Maximum urea concentration	50 mg/liter
Urea generation rate (two 350-g frogs)	28 mg/24 hrs
Frog container water volume	5 liters
Water temperature	62 $\pm$ 2°F

The composition of frog urine to be simulated was [3]:

Temperature	Urea	Ammonium Ion	Undetermined
11°C	68.2%	21.0%	10.2%
22°C	77.6%	12.2%	10.2%

At 11°C the total composition is only 99.4%; however, this information as well as urea generation rate was used, as shown in Table 1, for determiming the quantities of NH_4^+ and urea excreted daily by the frogs. The conditions employed for simulating the OFO experiment were:

Table 1 - Simulated Frog Excretia Calculations

Temperature (°C)	% Urea	% NH_4^+	% Unknown	% Total
11 *	68.2	21.0	10.2	99.4
22 *	77.6	12.2	10.2	100.0
16.5 (Interpolated)	72.9	16.6	10.2	99.7

Calculations: 28 mg urea is 72.9%; thus, 38.4 mg/day is 100%.

16.6% x 38.4 mg/day = 6.38 mg of NH_4^+/day or

$$\frac{6.38 \text{ mg of } NH_4^+}{\text{day}} \times \frac{53.5 \text{ mg of } NH_4Cl}{18 \text{ mg of } NH_4^+} = 19 \text{ mg } NH_4Cl/\text{day}$$

* "Blood and Other Body Fluids", edited by Altman and Dittmer, published by Federation of American Society for Experimental Biology, 9600 Rockwell Pike, Bethesda, Maryland 20014.

Urea addition	28 mg/24 hours
$NH_4C\ell$ addition	19 mg/24 hours
Liquid volume	4.5 ℓ
Temperature	16.5 $\pm$ 0.5°C (61.7 $\pm$ 0.9°F)
Oxygen pressure	620-700 Torr (12-13.5 psi)
Circulation rate	300 ml/min.
Phosphate buffer	0.05M @ pH 7.0

Materials and Methods

Urease was obtained from different sources both in the immobilized and free states. The greatest quantity of free urease (1 gram) was purchased from Worthington Biochemical Corporation. This urease was designated URC for lyophilized crude urease from jack bean meal. All of our immobilized preparations used this urease. Worthington provided us with two complimentary discs for testing purposes which contained urease immobilized in nitrocellulose. Urease immobilized on DEAE cellulose was purchased from Miles Laboratories under the name of Enzite urease (100 mg). Beckman gave us a 50-mg sample of their jack bean urease which we used in the batch process of urea removal explained in Task 3. The use of Enzite urease is also covered in Task 3.

Urease Assays - The assays for urease activity were conducted under several different conditions with respect to temperature, pH, substrate concentration, buffer concentration, and mixing conditions. However, the majority of the earlier assays were conducted according to the Urease Disc Assay obtained from Worthington Biochemical Corporation.

Urease Disc Assay: Dissolve 1.8 g of urea in 100 ml of 0.05M phosphate (PO_4) buffer, pH 7.0 and add 0.1 ml Hach's solution (Brom Cresol Green-Methyl Red; Hach Chemical Company, Ames, Iowa). The immobilized urease is mounted on the shaft of a 60 RPM motor mounted above a 250-ml beaker containing the substrate solution. The rotating enzyme is lowered into the beaker and 1.0N $HC\ell$ is added dropwise to first purple color; a timer is started and the burette volume is noted. Periodically substrate is brought back to original purple color by addition of acid, and the time and burette volume are recorded. The reaction was run at room temperature ($\sim$23°C). The volume readings are plotted versus time of reading in minutes giving a straight line. The slope of the line

gives millimoles NH_4OH produced per minute and this value divided by 2 is the number of millimoles urea hydrolized per minute.

Variations in Urease Assay: Because of the differences in temperature, pH, and substrate concentrations between the recommended assay conditions and the OFO experiment conditions, certain variations in the assay conditions were incorporated to more closely represent the OFO experiment conditions. At the reduced activities resulting from changes in temperature, pH, substrate concentration and loss of immobilized enzyme, a more sensitive endpoint indicator was used. The color change for the indicator occurred at pH 4.92; therefore a Beckman Research pH meter was used to determine the time at which the pH passed through this end point or any other specified end point after addition of acid.

Immobilization of Urease - Urease was immobilized on nylon netting or glass wool using an acrylamide gel solution and modifications of the procedure described by Guilbault and Montalvo [1].

Tris Buffer: One liter 0.1M Tris buffer at pH 7.0 was prepared and stored in the refrigerator at $\sim 4°C$.

Gel Solution: In 25 ml of Tris buffer is dissolved 0.58 g of N, N'-methylene bisacrylamide, 5 g of acrylamide, 3 mg of $K_2S_4O_8$ and 3 mg of riboflavin. This solution could be stored in the dark at 4°C for a maximum of two days prior to use.

Urease Gel Solution: One milliliter of gel solution is pipetted into small centrifuge tube containing 175 mg of urease. This quantity was later changed to 1 ml/50mg urease because much of the urease did not dissolve. The suspension was stirred for 2 minutes, was let stand for 20 minutes at room temperature, chilled for 10 minutes at 2°C and centrifuged at 3500G for 20 minutes.

Immobilization #1: A piece of nylon was rinsed several times with redistilled water and air dried. The piece of nylon was placed on a slotted 24-mm diameter glass tube and anchored with an O-ring. The excess nylon was trimmed away with a scalpel. Less than 0.3 ml of supernatant from the urease gel solution was placed on the nylon for immobilization. Because oxygen inhibits the polymerization, an anaerobic incubator was evacuated and backfilled with nitrogen twice prior to photopolymerization with a General Electric BBA photoflood lamp. The recommended photopolymerization conditions are 28°C for 60 minutes of illumination.

This immobilization resulted in rapid warming to 45°C and at the end of 30 minutes, the lamp went out. Chilled nitrogen gas was drawn through the incubator continuously to minimize the overheating.

Immobilization #2: Another immobilized urease preparation was made on a 35-mm diameter piece of nylon netting with the polymerization conditions maintained more closely to those recommended by Guilbault and Montalvo [1]. The anaerobic incubator was evacuated to 15 in. of Hg and liquid nitrogen (N_2) was bled in to maintain a pressure of -3 to 3 in. of Hg with the rate of addition dependent on the required cooling to maintain 28, + 1 - 4°C near the polymerization reaction. Approximately 1 ml of urease gel solution supernatant was placed on the nylon for immobilization. Illumination with the BBA lamp was maintained for 60 minutes.

Immobilizations #3 and #4: These immobilizations were accomplished on glass wool. The glass wool was placed in a 50-ml lyophilization flask top (see Figure 1) and soaked in concentrated HCℓ for 5 minutes. The glass wool was rinsed with redistilled water until the influent pH matched the effluent pH and then it was air dried. Approximately 3 ml of the urease gel solution supernatant was used in each immobilization on the glass wool. A Büchi rotary evaporator was used to maintain the oxygen-free environment and temperature requirements for the 60-minute photopolymerization. Nitrogen gas was bled in through a continuous feed tube at ~ 1 ℓ/min. with flask revolving at 60-100 rpm and the outlet was opened to the laboratory vacuum. The flask was bathed in 28°C water.

Results and Discussion

For this section the immobilized ureases are placed in one of three groupings: nitrocellulose entrapment; acrylamide gel entrapment on nylon; and acrylamide gel entrapment on glass wool.

Nitrocellulose Entrapment - This preparation was obtained from Worthington Biochemical in the form of wire-covered discs. The results of the assays are shown in Table 2 and Figures 2 through 5. The end point or equivalence point was a pH of 4.925 and was monitored by the pH meter.

Figure 1 Polymerization Chamber

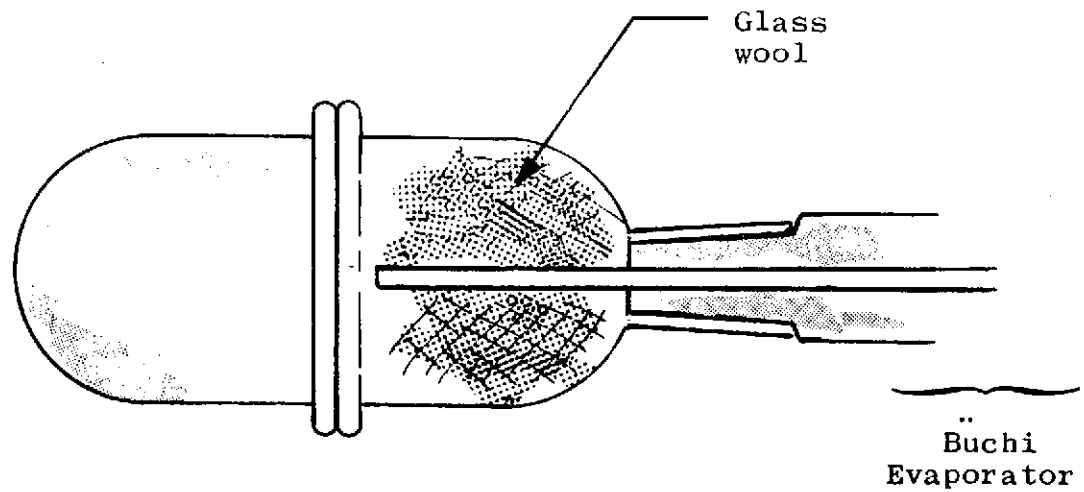

Figure 2 Urease Assay (Nitrocellulose Entrapment)

ASSAY #1

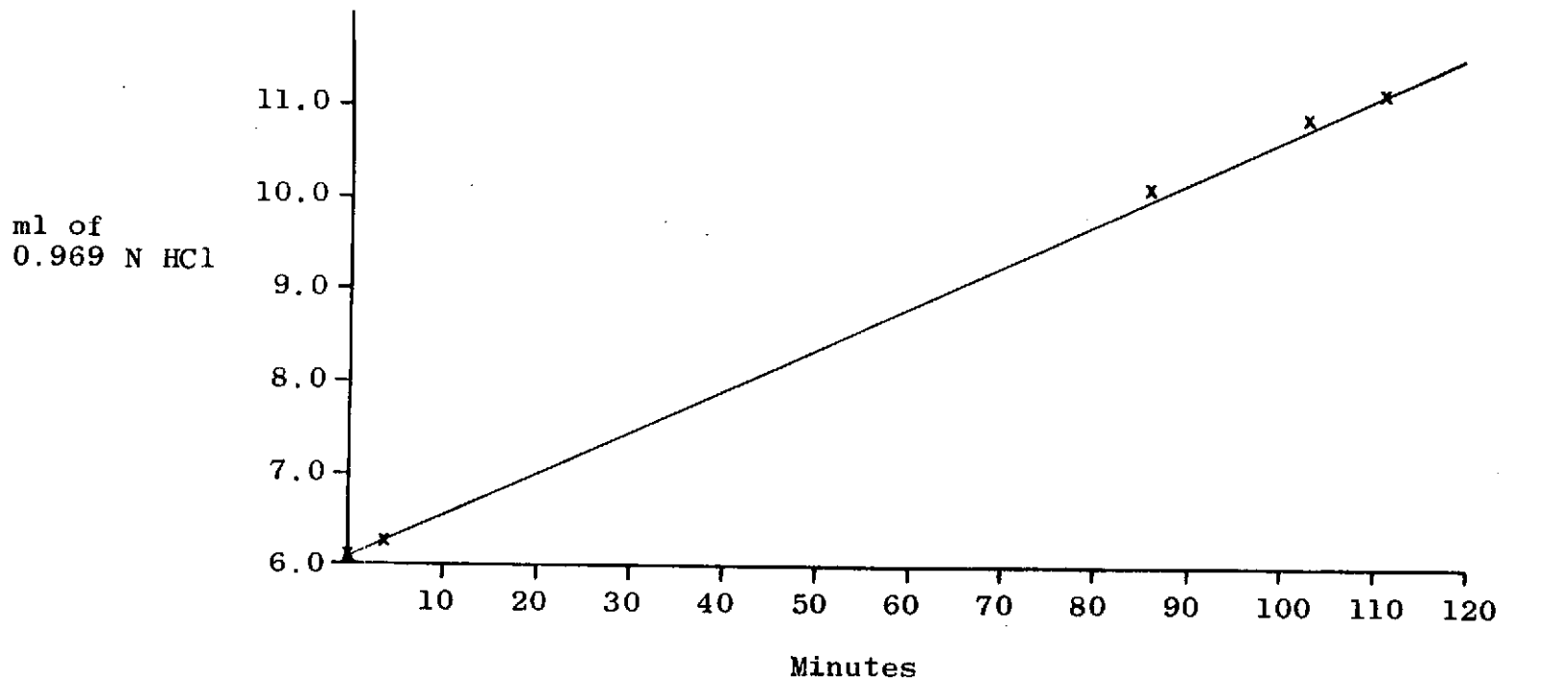

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Figure 3 Urease Assay (Nitrocellulose Entrapment)

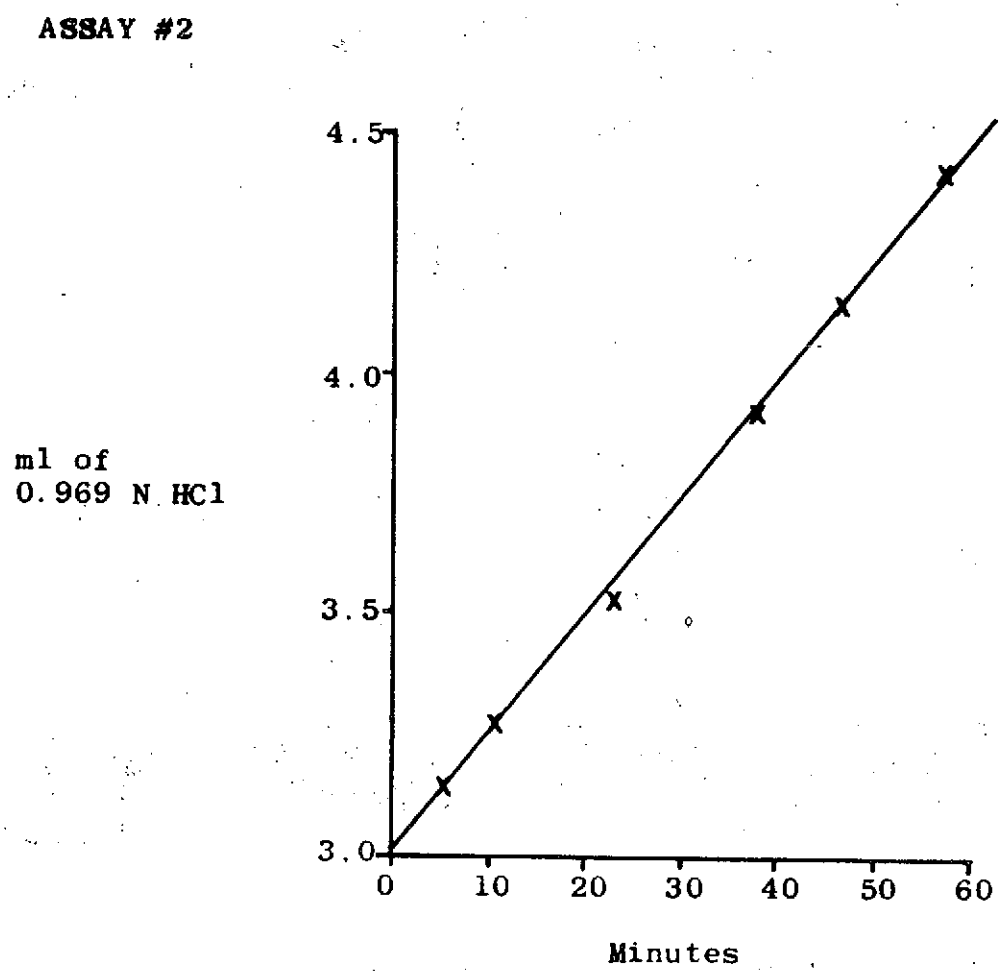


Figure 4 Urease Assay (Nitrocellulose Entrapment)

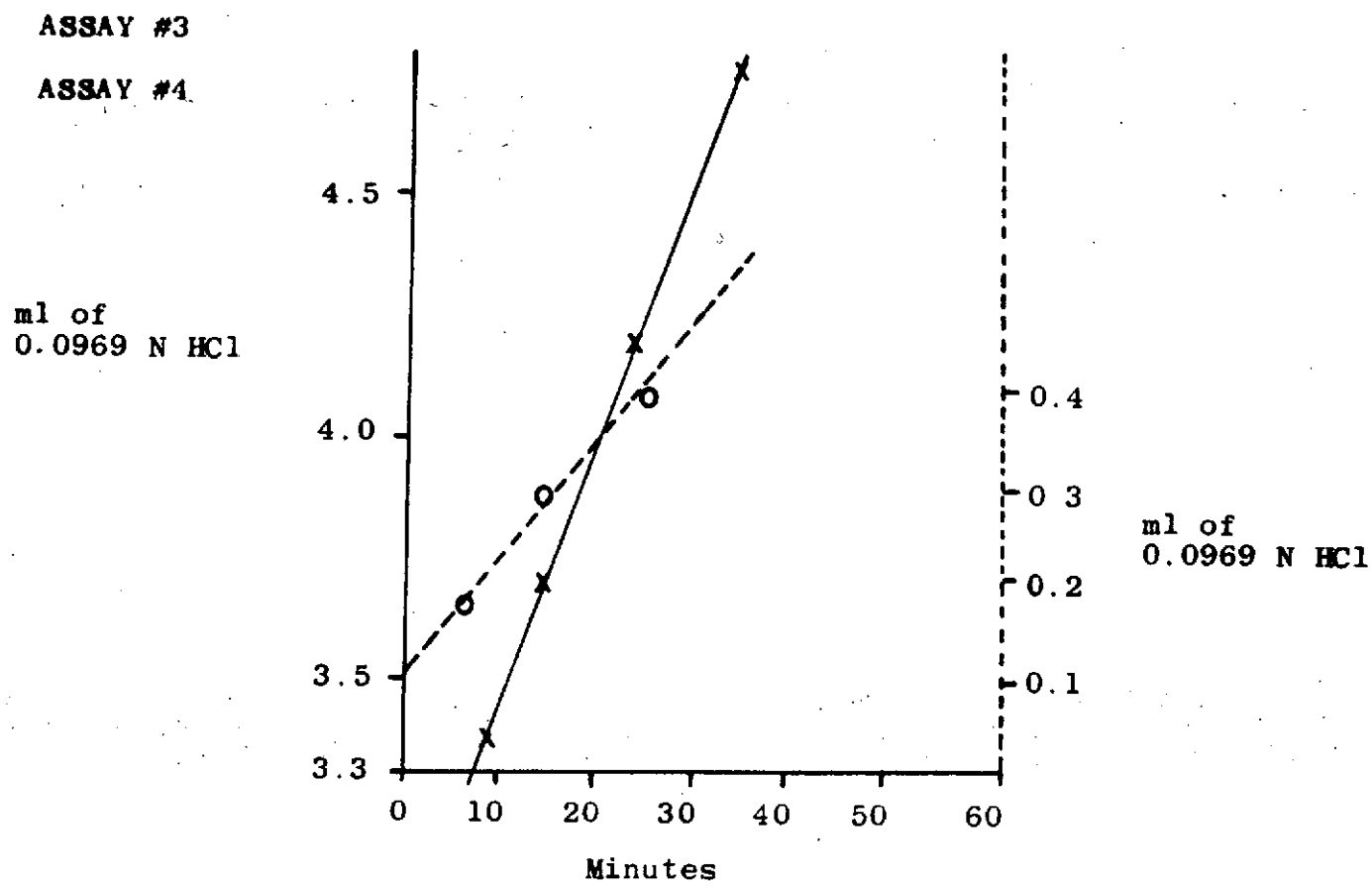
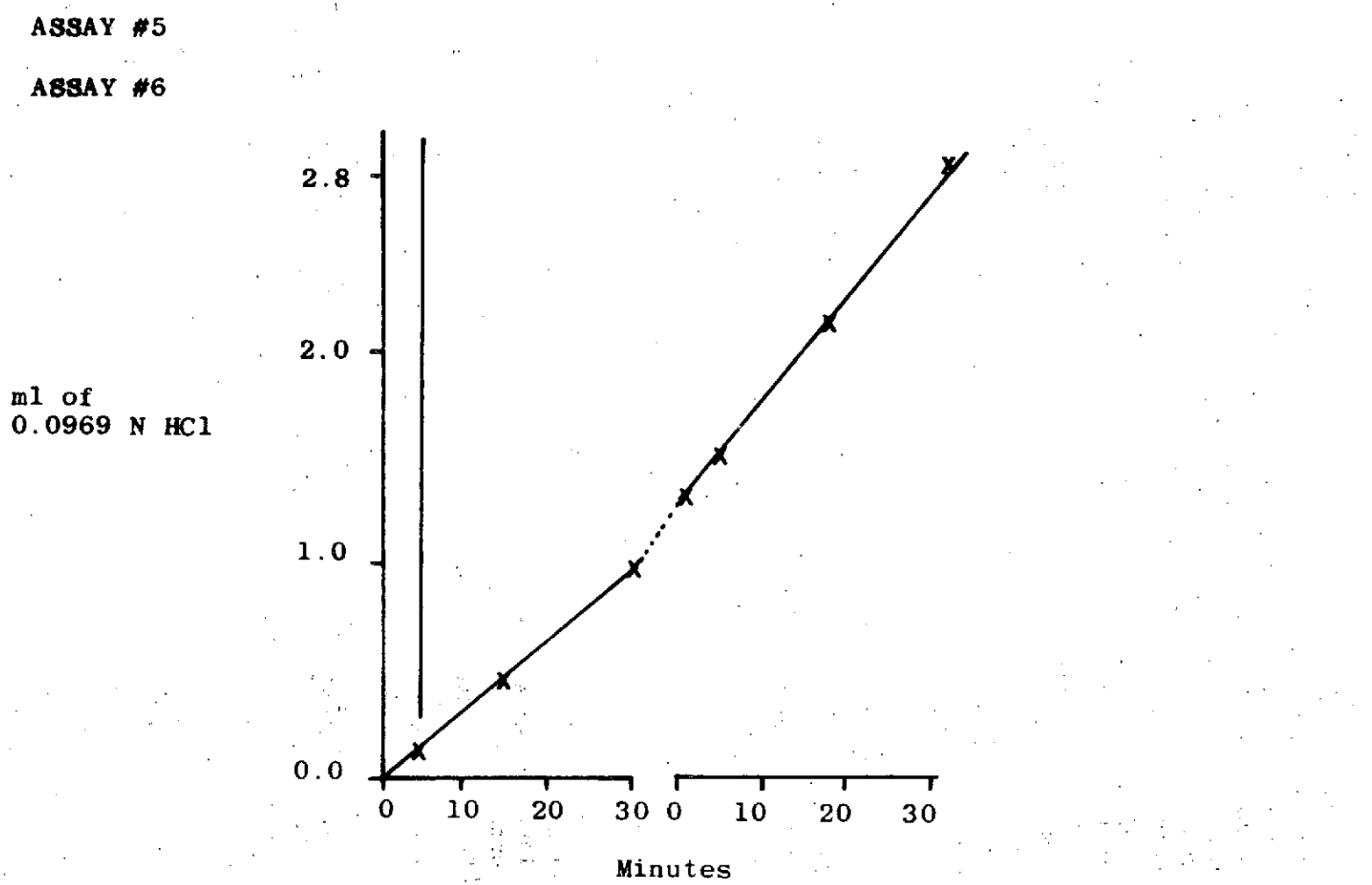


Figure 5 Urease Assay (Nitrocellulose Entrapment)



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Assay No. 1 was run the day before assays 2, 3 and 4; and assays 5 and 6 were run on the following day without changing solutions between assays 5 and 6. Only the temperature was changed. Comparison of assays 1 and 6, and 2 and 5 indicates a loss of almost 90% of the enzymatic activity. During each assay the activity remained constant giving a straight line relationship. This indicates that inactivation is not occurring during the assay. Indeed, comparison of assays 5 and 6 shows the expected activities ratio of 1.4 for an increase of 7° using the relationship of a doubling of enzymatic activity for every 10°C rise in temperature. However, comparison of assays 1 and 2 shows a greater reduction in activity consistent with the loss in activity from one assay to the next resulting from loss of enzyme because of inadequate binding of the enzyme to the disc. During use the discs did lose their yellow hue.

The lowest activity measured is almost a factor of two greater than that required to maintain nontoxic levels of urea for the OFO experiment; however, further comparable losses of activity would be unacceptable. In addition, the lowest substrate concentration used in these assays is $\frac{29.2\ mg}{0.12} \times \frac{M}{60 \times 10^3\ mg/\ell}$ or 4.9×10^{-3}M and the maximum allowable concentration is 50 mg/ℓ or 8.3×10^{-4}M for the OFO experiment. Thus the lowest assay concentration used was almost six times the maximum allowable concentration.

These assays indicated that other immobilized urease preparations would be required for use in the OFO experiment. The variations in temperature and concentrations of buffer and substrate concentration were carried out to demonstrate their effect on the hydrolysis rate of urea.

Table 2 - Urease Assays (Nitrocellulose Entrapment)

Assay Number	Urea Hydrolysis Rate μmoles/min.	Temperature (°C)	Phosphate Buffer Molarity	Urea Added to 100 ml of Buffer
1	21.7	23°C	0.05	1.8 g
2	11.7	16°C	0.05	1.8 g
3	2.69	16°C	7.8×10^{-4}	34 mg
4	0.601	16°C	0.05	29.2 mg
5	1.62	16°C	0.05	1.8 g
6	2.34	23°C	0.05	1.8 g

Acrylamide Gel Entrapment on Nylon - These preparations were immobilizations number 1 and 2.

Immobilization #1: The results of urease assays for this immobilization are shown in Figures 6 and 7. The assay conditions for Figure 6 were 16.5°C, in 0.05 M phosphate buffer and the urea concentration was 50 mg/ℓ (8.3×10^{-4} M). The urease activity (Figure 6) was greater than the rate of urea production in the OFO experiment. Figure 6 shows that at low initial substrate concentration (8.3×10^{-4} M) the activity is reduced with time because of the reduction in substrate concentration with time. Figure 7 shows that saturation of the enzyme with substrate yields an activity of 7.75 μmoles/min. or hydrolysis of urea at the rate of 0.465 mg/min., which is more than 9%/min. of that available in 100 ml of 8.3×10^{-4} M urea solution. Storage of this immobilized urease for 20 days in 0.1 M Tris buffer (pH 7) at 4°C resulted in less than 15% loss of activity (Figure 7). The three sets of data dated 06-21-74 represent three separate assays. Because the data all fall on the same slope, it is concluded that the enzyme has been effectively immobilized.

Immobilization #2: This preparation had the activities shown in Table 3. It is interesting to note that the activity found under recommended assay conditions was approximately 10 times greater than obtained previously; whereas, the surface area for immobilization was increased two-fold and the volume of urease gel solution used was increased three-fold. An increase in the pH of the reacting mixture resulted in a decrease of the activity (Assays 1 - 3), but with a decrease in the pH (Assay 4) the activity increased.

Table 3 - Urease Assays (Immobilization #2)

Assay Number	Temperature (°C)	Phosphate Buffer Molarity	Substrate Molarity	pH	Urea Hydrolysis Rate μmoles/min.
1	18	0.037	0.3	4.5 - 4.6	80.75
2	18	0.05	0.3	4.5 - 4.6	71.25
3	18.5	0.05	0.3	7.0	28.5
4	18.5	0.05	0.3	4.9 - 5.0	54.9
5	18.5	0.05	0.00083	7.0	0.8

Figure 6 Urease Assay (Acrylamide Gel Entrapment)

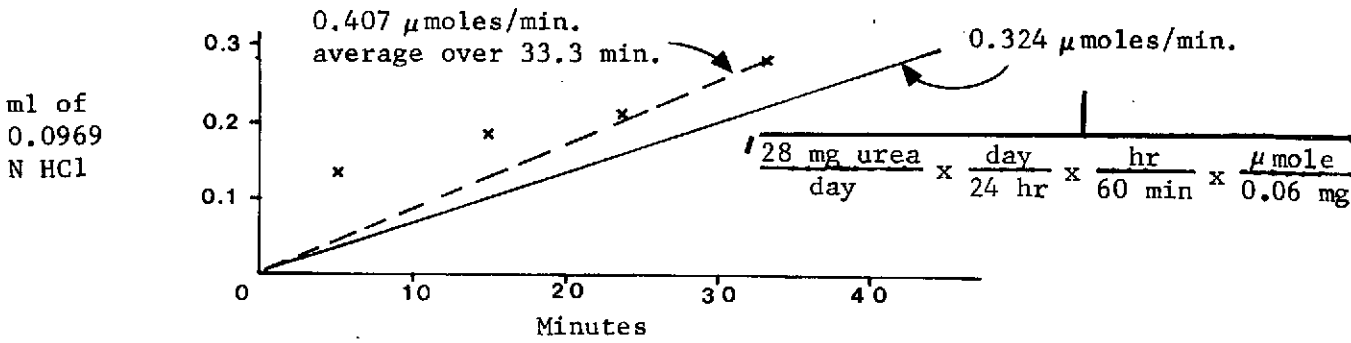
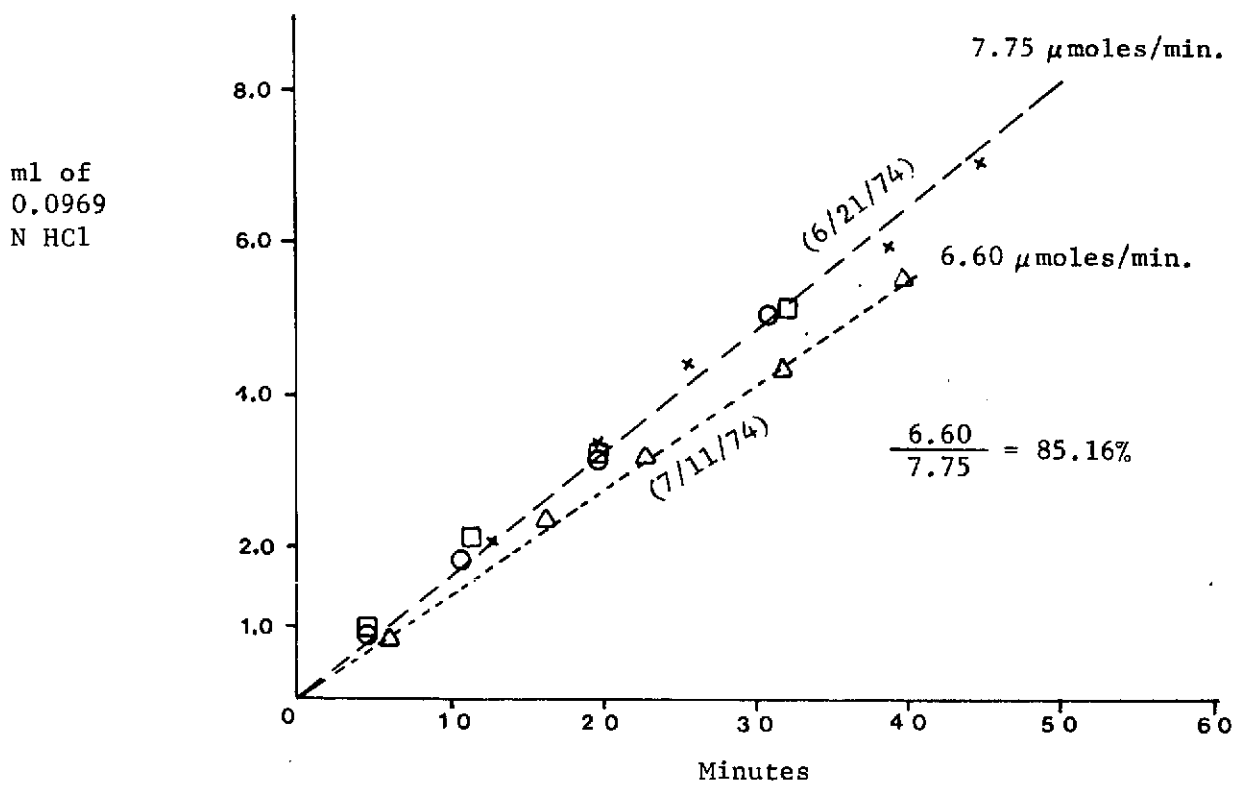


Figure 7 Acrylamide Gel Entrapped Urease Stability



Run 5 was conducted under conditions similar to those used later in the Combined Systems Test. Urea (7.5 mg) in 150 ml of 0.05 M phosphate buffer was circulated at 300 ml/min. through a container housing the urease immobilized on the 35 mm diameter nylon membrane. The pH was maintained near 7.0 using a Beckman Research pH meter for monitoring the pH and equivalence point. Determinations at 16, 22 and 62 minutes into the run resulted in activities of 0.7, 0.9, and 0.8 μmoles/min., respectively. After 60 minutes at 0.8 μmoles/min., 38% of the original 7.5 mg of urea would be hydrolyzed.

Acrylamide Gel Entrapment on Glass Wool - Immobilization of urease on nylon stretched over aluminum supports which were replaced by glass supports following immobilization and equilibration in Tris buffer failed to result in any urease activity. However, immobilization of urease on glass wool previously washed with concentrated HCℓ followed by distilled water resulted in good urease activity. The activities were measured in 0.05 M phosphate buffer with initial urea concentrations of 0.3 M at room temperature (ca. 23°C) and an end point at pH 4.92. The data is shown in Tables 4 and 5. Each assay was conducted by circulating 100 ml of the buffered substrate solution across the immobilized urease at the rate of 300 ml/min. using a parastaltic pump for circulation, except for the last assay in each table which reverted to revolving (60 rpm) the immobilized enzyme in the solution.

The difference in urease activity between assay 1 and assays 2, 3, and 4 of Table 4 was originally thought to be caused by the degradation of the urease from microbial attack; however, assay 5 (130 minutes additional exposure) following a kanamycin application, and assay 6 (2230 minutes total exposure) showed no significant loss in activity compared to assays 2, 3, and 4, although the microbial count was not significantly altered by the kanamycin application. Exposure to kanamycin at 1 mg/ℓ does not appear to inhibit the urease activity. Following assay 7, immobilization #3 was stored in the refrigerator in Tris buffer for 11 days. Assay 8 indicates recovery of some of its activity after cold storage; however, further exposure to the OFO system resulted in further loss of activity (assays 9 and 10). Further cold storage (30 days) resulted in no similar recovery of activity (assay 11).

Immobilization #4 (Table 5) showed similar losses of activity on exposure to the OFO system with no indication of recovery of activity following cold storage. Between assays 3 and 4, 12 and 13, and 13 and 14, immobilization #4 was refrigerated in 0.1 M Tris buffer and showed rather significant losses in activity.

Table 4 - Urease Assay (Immobilization #3)

Assay Number	Immobilized Urease History	Urea Hydrolysis Rates µmoles/min.
1	225 mg/3 ml of gel solution on glass wool, stored in refrigerator in 0.1 M Tris buffer for 11 days, rinsed with 2 ℓ redistilled water prior to assay.	169
2	The above was rinsed after assay with 2 ℓ of redistilled water and placed in the OFO system for 1000 minutes and again rinsed with 2 ℓ of redistilled water prior to assay.	18.8
3	The above rinsed with 0.5 ℓ of redistilled water prior to assay.	14.9
4	The above rinsed with 0.5 ℓ of redistilled water prior to assay.	16.8
5	The above rinsed with 0.5 ℓ of redistilled water subjected to OFO system for 130 minutes and rinsed with 2 ℓ of redistilled water.	14.9
6	The above rinsed with 0.5 ℓ of redistilled water subjected to 1100 more minutes in OFO system, rinsed with 2 ℓ of redistilled water prior to assay.	12.9
7	The above rinsed with 0.5 ℓ of redistilled water prior to assay.	17.0
8	The above rinsed with 0.5 ℓ of redistilled water stored in refrigerator in 0.1 M Tris buffer for 11 days, rinsed with 2 ℓ redistilled water prior to assay.	25.5
9	The above rinsed with 0.5 ℓ of redistilled water, subjected to OFO system for 1000 minutes and rinsed with 2 ℓ of redistilled water prior to assay.	6.14
10	The above rinsed with 0.5 ℓ of redistilled water subjected to OFO system for 1200 minutes and rinsed with 2 ℓ of redistilled water prior to assay.	3.08
11	The above rinsed with 0.5 ℓ redistilled water, stored in refrigerator for 30 days in 0.1 M Tris buffer, rinsed with redistilled water prior to assay.	2.54

Table 5 - Urease Assay (Immobilization #4)

Assay Number	Immobilized Urease History	Urea Hydrolysis Rates µmoles/min.
1	160 mg/3 ml of gel solution on glass wool, stored in refrigerator overnight in 0.1 M Tris buffer, rinsed with 2 ℓ redistilled water prior to assay.	153
2	The above rinsed with 0.5 ℓ redistilled water prior to assay.	170
3	The above rinsed with 0.5 ℓ redistilled water prior to assay.	159
4	The above rinsed with 0.5 ℓ redistilled water, stored overnight in refrigerator in 0.1 M Tris buffer, rinsed with 2 ℓ of redistilled water prior to assay.	77.7
5	The above rinsed with 0.5 ℓ redistilled water prior to assay.	98.9
6	The above rinsed with 0.5 ℓ redistilled water prior to assay.	98.9
7	The above rinsed with 0.5 ℓ redistilled water, stored two days in refrigerator in 0.1 M Tris buffer, rinsed with 2 ℓ of redistilled water, placed in OFO system for 860 minutes, rinsed with 2 ℓ redistilled water prior to assay.	34.2
8	The above rinsed with 0.5 ℓ redistilled water, placed in OFO system for 1200 minutes, rinsed with 2 ℓ redistilled water prior to assay.	19.6
9	The above rinsed with 0.5 ℓ redistilled water prior to assay.	17.9
10	The above rinsed with 0.5 ℓ redistilled water prior to assay.	21.1
11	The above rinsed with 0.5 ℓ redistilled water, placed in OFO system for 1200 minutes, rinsed with 2 ℓ redistilled water prior to assay.	9.69
12	The above rinsed with 0.5 ℓ redistilled water, placed in OFO system for 1200 minutes, rinsed with 2 ℓ redistilled water prior to assay.	4.65
13	The above rinsed with 0.5 ℓ redistilled water, stored 4 days in refrigerator in 0.1M Tris buffer, rinsed with 2 ℓ redistilled water prior to assay.	3.71
14	The above rinsed with 0.5 ℓ redistilled water, stored 32 days in refrigerator in 0.1 M Tris buffer, rinsed with redistilled water prior to assay.	0.84

TASK 2 - AMMONIA REMOVAL ANALYSIS

Materials and Methods

Ammonia Assays - Initial information suggested that by using Beckman's #39626 ammonium specific-ion electrode, the ammonium ion concentration could be monitored continuously without interfering with the experimental parameters. However, this electrode was no longer available so an Orion 95-10 electrode was ordered which measures ammonia gas at pH 11 or higher. The disadvantage of using this electrode is that samples must be adjusted to pH 11 prior to measurement of ammonia concentration. While waiting for the Orion electrode the phenate method recommended for use in detecting ammonia N in the presence of urea N was tried [3].

Phenate Method: The phenate (indophenol blue) method is found on page 232 of *Standard Methods for the Examination of Water and Wastewater*, 13th Edition, 1971. According to Rogers and Pool, readings are taken before and after urease hydrolysis of urea and the difference is used to calculate the urea present in the sample. We obtained urea-N readings (in freshly prepared solutions) comparable to those expected for solutions containing the equivalent amount of ammonia N. Because of our difficulty in distinguishing between urea and ammonia N, this assay was used only in urea-free solutions used in our initial test of the binding capacity of the ANGC-101 ion-exchange resin.

Ammonia Gas Electrode: The Orion 95-10 ammonia-gas electrode uses a hydrophobic gas-permeable membrane to separate the sample solution from the electrode internal solution. Dissolved ammonia in the sample solution diffuses through the membrane until the partial pressure of ammonia is the same on both sides of the membrane. The partial pressure of ammonia is proportional to its concentration in any given sample. Before measurement all solutions were made basic (pH $\geq$ 11) with ammonia-free NaOH. The electrode was used only in aqueous solutions where the sample and standards had the same levels of dissolved species and were at the same temperature. The millivolt potential was measured using the Beckman Research pH meter. A standard curve was plotted on semi-log graph paper with millivolts versus ammonium concentration on the log scale.

Ammonium Removal by Ion Exchange-Resin - Baker's ANGC-101 ion exchange resin is recommended for use in ammonium determinations in urine samples. The material we obtained was reported to trap 12 mg of ammonium N/g of ion-exchange resin (dry weight). Eight grams of the resin was placed in a column (10 x 160 mm) and washed with 1 ℓ redistilled water. The column was then subjected to various loads of $NH_4C\ell$ until ammonium was detected in the effluent using the phenate assay. This was repeated on another column; however, the limit was not determined although the previous loading was exceeded (see Table 6).

Table 6 - Ammonium Trapping

8 grams of Baker's ANGC-101 Ion-Exchange Resin

	NH_3-N Load (mg)	Effluent NH_3-N (mg)
Run 1	40	0.0
	2.2	0.1
	Trapped: 42.1 mg/8g = 5.3 mg/g	
Run 2	20	0.0
	20	0.0
	2	0.0
	5 (0.1)	5 (0.0)
	Trapped: 42.5 mg/8g = 5.3 mg/g	
	Reported: 12 mg/g	

30-Day OFO Requirement:

$$\frac{28 \text{ mg UREA}}{\text{DAY}} \times \frac{28 \text{ mg N}}{60 \text{ mg UREA}} \times 30 \text{ DAYS} \times \frac{\text{g ANGC-101}}{5.3 \text{ mg N}} = 74 \text{ g ANGC-101}$$

$$\frac{19 \text{ mg } NH_4C\ell}{\text{DAY}} \times \frac{14 \text{ mg N}}{53.5 \text{ mg } NH_4C\ell} \times 30 \text{ DAYS} \times \frac{\text{g ANGC-101}}{5.3 \text{ mg N}} = 28 \text{ g ANGC-101}$$

} 102 g ANGC-101

Results and Discussion

The Orion 95-10 ammonia-gas electrode performed quite well for ammonium determinations. However, use of the electrode before and after sulfuric acid hydrolysis on a steam bath for 2 hours proved quite inadequate for quantifying the presence of urea. The technique selected for quantifying urea is discussed in Task 3. The ammonia-gas electrode was selected for monitoring the ammonium concentrations for Task 3.

Initially the ANGC-101 ion-exchange resin was selected for ammonium removal for Task 3; however, during the oral review of Tasks 1 and 2 appreciable concern was expressed that the ion-exchange resin might reduce other cations below their required concentrations. Therefore, the hydrophobic membrane used for the ammonia-gas electrode was selected for testing as a means for ammonia removal. The problem with using the membrane for ammonia removal under the OFO conditions of pH (7.0) and temperature (16.5°C) is that less than 1% of the ammonia nitrogen is present as ammonia. This was determined by using the dissociation constant (K_b) of aqueous ammonia at 15°C [4] and the Henderson-Hasselbach equation [5].

$$pK_b = 4.782$$

$$pH + pOH = 14$$

$$pK_b = pOH$$

$$pK_a = pH$$

$$pK_a = 14 - 4.782 = 9.218$$

$$pH = pK_a' + \log \frac{[NH_3]}{[NH_4^+]}$$

at pH = 7, what is $\frac{[NH_4^+]}{[NH_3]}$?

$$\log \frac{[NH_4^+]}{[NH_3]} = 9.218 - 7.0 = 2.218$$

$$\frac{[NH_4^+]}{[NH_3]} = 165$$

$$\frac{[NH_3]}{[NH_4^+] + [NH_3]} = \frac{1}{165 + 1} = 0.6\%$$

In addition, the solubility of ammonia in water is 0.497 g/ml [4] or 29 M and the accumulation of ammonia over 30 days in the OFO experiment would only approach 10^{-2} M.

$$\frac{\left(\frac{28 \text{ mg Urea}}{\text{day}} \times \frac{2 \text{ moles } NH_3}{60{,}000 \text{ mg Urea}} + \frac{19 \text{ mg } NH_4C\ell}{\text{day}} \times \frac{\text{Mole } NH_3}{53{,}500 \text{ mg } NH_4C\ell}\right) 30 \text{ days}}{5\ell}$$

$$= 7.7 \times 10^{-3} \text{ M } NH_3$$

The hydrophobic Millipore membrane (3.5-cm diameter) supported on a glass frit passed 5 ml of air/sec at ΔP of 4 Torr. A fresh membrane wetted at ΔP of 320-326 Torr and a previously wetted membrane passed water at ΔP of 155 Torr. For Task 3 the membrane was operated at ΔP of 80-100 Torr; however, after a few days the membrane wetted and became nonfunctional as a liquid-gas separator. The operation of this membrane at a lower ΔP to prevent wetting or the use of some other membrane as a liquid-gas separator for ammonia removal from the OFO experiment would probably be inadequate, because of the relatively low accumulation of ammonia species compared to their solubility (7.7×10^{-3}M/29M = 1/3766).

TASK 3 - COMBINED UREA/AMMONIA REMOVAL SYSTEMS TEST

Materials and Methods

Urea Assay - The technique selected for quantifying urea is that reported by Joon H. Rho [6], "Direct Fluorometric Determination of Urea in Urine." The reaction products of urea with diacetyl monoxime in acid solution reportedly fluoresce at 410 and 525 nm with excitation at 380 nm. We doubled the volumes used for the assay and found more consistent results upon autoclaving at 100-110°C for periods of an hour or more. Using our Aminco Bowman spectrophotofluorometer and a 1P21 photomultiplier tube we obtained the same excitation maximum but the fluorescence maxima were at 410 nm and 505 nm. Our standard curves were established using the 505-nm peaks.

Urease Assay - A modification of the urease disc assay was employed for Task 3. The buffered substrate solution was actually pumped through the area containing immobilized urease at the rate of 300 ml/min. Hydrochloric acid (0.969 N) was added gradually to obtain a pH below 4.92. Each time the pH increased through 4.92, the time was noted and the volume of HCℓ acid neutralized was recorded.

Ion-Exchange Column Preparation - Two columns were prepared for Task 3 which contained Baker's ANGC-101 ion-exchange resin. Colume A was prepared for testing separated from the CST, and Column B was prepared for testing in conjunction with the second CST.

Column A: Fifty grams of the ion-exchange resin was placed in a column 25 x 400 mm. The system was washed with 2 ℓ of redistilled water and then 4.5 ℓ of 0.05 M phosphate buffer was circulated at 300 ml/min. through the system for 30 minutes. A sample of the phosphate buffer was taken before and after circulation through the column for atomic absorption measurement of the sodium-ion content. Ammonium chloride (ca. 2068 mg) was added to the circulating buffer and circulated overnight.

Column B: Into a 1-ℓ beaker was placed 150 g of ion-exchange resin. The resin was stirred twice with 0.5-ℓ portions of 0.05 M phosphate buffer. The suspension of resin was allowed to settle prior to decanting the supernatant. The same technique was used for rinsing with four 0.5-ℓ portions of redistilled water. The resin was transferred to a 42 x 450-mm column using redistilled

water as the transfer medium. Glass wool was used at both ends of the column for retaining the ion-exchange resin. The column was placed in the second CST for evaluation. The appropriate samples were taken for determining ammonium and sodium-ion content prior to and during column performance.

First Combined Systems Test

The first Combined Systems Test (CST) apparatus is shown in Figure 8. The 19 mg of NH_4Cl and 28 mg of urea (Table 1, Simulated Frog Excretia Calculations) were added daily to the CST along with sufficient sterile phosphate buffer and redistilled water to maintain the appropriate volume and concentration. Less than 1% of the total volume was removed daily for assay pruposes. The ammonium concentration was determined using the Orion 95-10 electrode before and after acid hydrolysis in order to determine the quantity of urea present. As reported earlier this was inadequate for urea determinations, but it did indicate that the Millipore membrane as shown in Figure 8 was not adequate for NH_3 removal. There was no significant change in pH during this 12-day CST (pH range 6.75 to 6.96). After making the decision to start the CST again, the immobilized urease from this CST (Immobilization #2) was assayed and found to have negligible activity at 18.5°C in 0.05 M phosphate buffer with 0.3 M substrate at pH 4.92 using the 60 rpm stirrer.

Second Combined Systems Test

The second CST apparatus is shown in Figure 9. Either arrangement A, B, or C was used throughout the 30-day period. Phosphate buffer (ca. 4.5 ℓ of 0.05 M) was heated to 95°C and circulated through the system (excluding enzyme preparations and ion-exchange column) for 2 hours. At this time the system was drained and 4.5 ℓ of sterile phosphate buffer was added. In addition, 0.012 ml of *Kantrex* was added and 1 ml of the circulating fluid was spread on a double strength trypticase soy agar plate for determining the microbial burden. The plates were incubated at 32°C for a minimum of 48 hours prior to counting. Information regarding *Kantrex* (1 g kanamycin/3 ml) additions, microbial burden and heating cycles is shown in Table 7.

Figure 8 First CST Apparatus

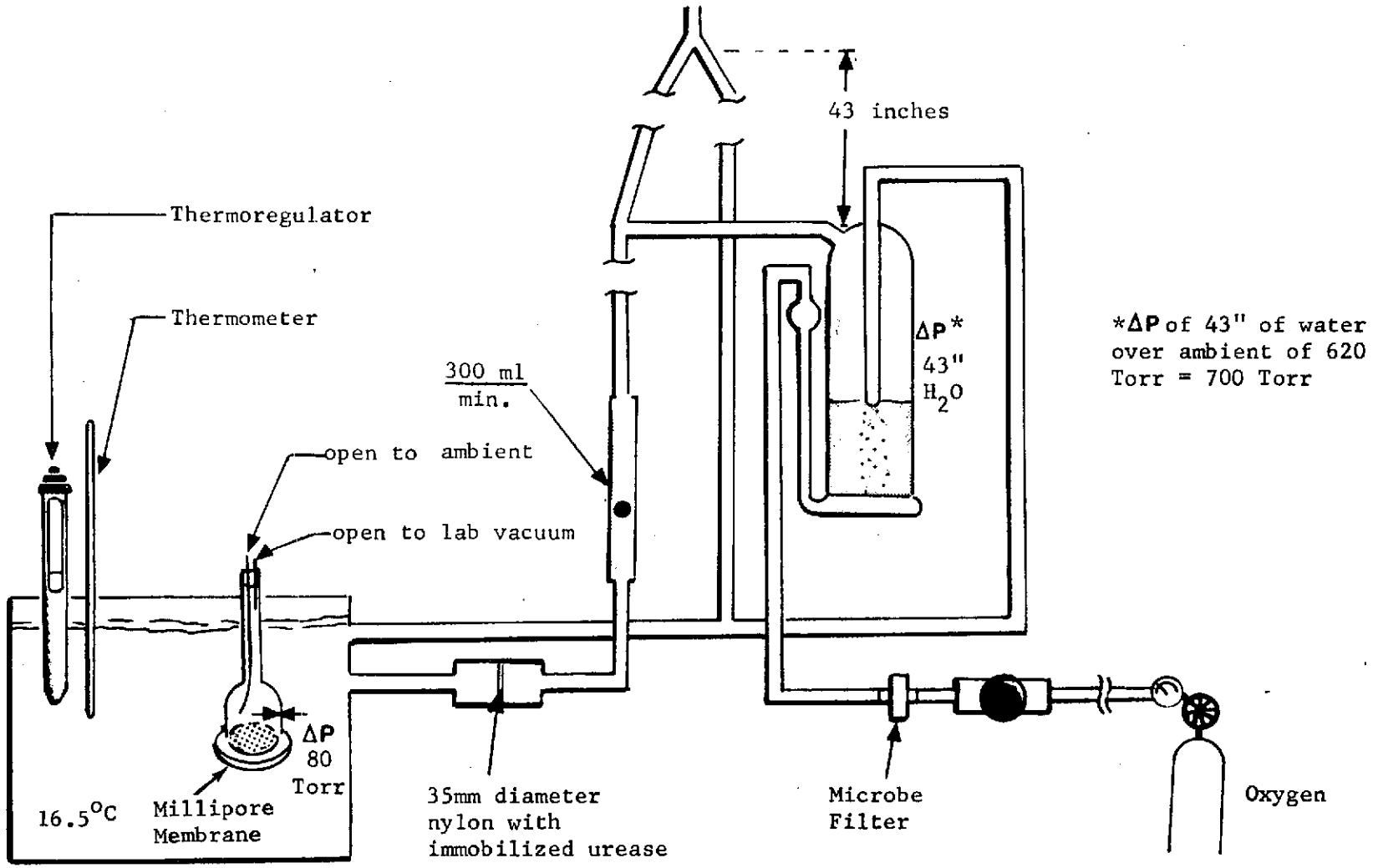


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Figure 9 Second CST Apparatus

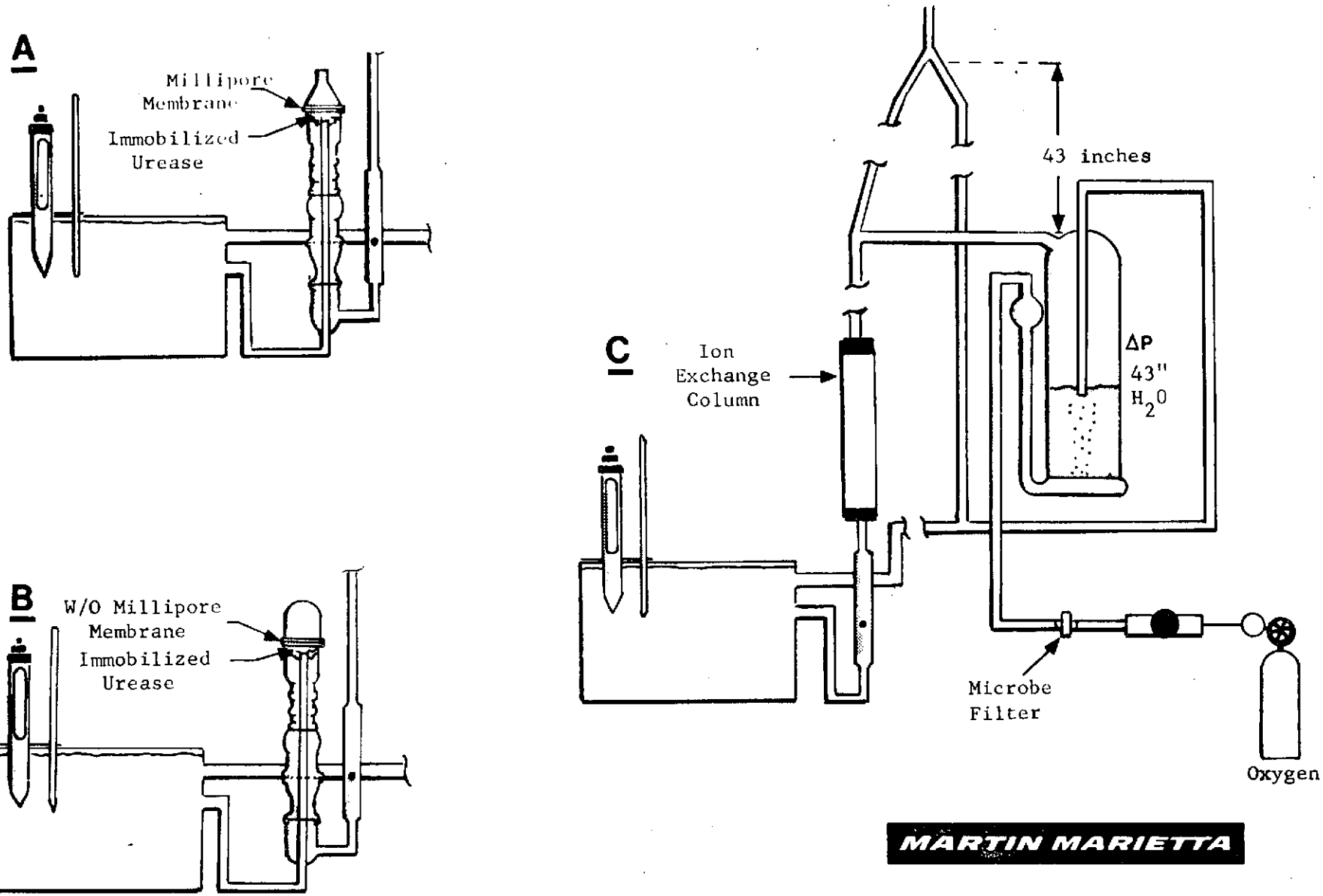


Table 7 - Kanamycin Additions and Microbial Burden

Date	Kanamycin Added mg/ℓ	Microbial Burden per millimeter	Circulation in hours at 95°C
09-13-74	0.9	-	2
09-14-74	0.9	0	0
09-15-74	0	25	0
09-16-74	0.9	-	0
09-18-74	0.9	>3000	0
09-19-74	0.9	>3000	0
09-20-74	0.9	>3000	0
09-23-74	9	>3000	0
09-24-74	9	0	1
09-25-74	-	>3000	0
09-26-74	-	>3000	1

Arrangement A in Figure 8 was used for the first five days; however, the constant ΔP of 80 Torr caused wetting of the membrane which prevents its performance as a liquid-gas separator. Thus, arrangement B which eliminated the Millipore membrane was used for days 6 through 10. On day 11, arrangement B was altered by removing the immobilized enzyme container and the second CST was continued through day 28 in that form. Free jack bean urease (ca. 50 mg) was added on days 11, 18 and 25. On days 9 and 11, the CST excluding urease (free or immobilized) was circulated at 95°C for one hour. The heating cycles were used to aid in microbial burden control. On day 29, arrangement C was employed in order to determine the efficacy of using the ion-exchange resin for ammonium control.

Results and Discussion

First CST - This 12-day test resulted in the adoption of a new technique for monitoring the presence of urea (fluorescence assay) and provided experience for use of the ammonium assay. The need for monitoring urease activity was established by the

fact that no urease activity was present at the end of the first CST. The decision was made to start the CST again in order to obtain better data for the feasibility study.

Second CST - The results and discussion of this 30-day test are covered under the heading ammonia or urea. This information provides the major basis for the conclusions of this feasibility study.

Ammonia: The information to the left of the day column in Table 8 shows the record of $NH_4C\ell$ additions, the ammonium available from $NH_4C\ell$ and hydrolysis of urea, and the measured ammonium concentration. Figure 10 is a graphic presentation of these ammonium concentrations. Initially, the available molarity of ammonium lags by a day the ammonium chloride molarity because the measurements were made prior to the ammonium chloride additions on any given day. Later as urea was hydrolyzed, it also contributed to the available ammonium concentration. On days 2, 3, 4, 8, 10, and 12, the measured ammonium exceeded the available ammonium for reasons unknown. On day 10, the available ammonium was adjusted to the measured quantity on day 9 plus the ammonium chloride added on day 9. This was done because of the heating cycle on day 9, which drove off some of the available ammonia. There was no apparent loss of ammonia resulting from the use of the Millipore membrane during days 1 through 5. On day 12, the same adjustment was made with regard to ammonium chloride, but on day 11 free urease was added which hydrolyzed the available urea (68.4 mg/ℓ) providing additional ammonium ions.

$$\frac{68.4\ \text{mg}}{\ell} \times \frac{2\ \text{moles}\ NH_4^+}{60{,}000\ \text{mg}} + 1.6 \times 10^{-4}\ \text{M} + \frac{18.8\ \text{mg}}{4.5\ \ell} \times \frac{\text{mole}}{53{,}500\ \text{mg}} = 25.2 \times 10^{-4}\ \text{M}$$

On days 19 and 26 the same adjustments were made for available ammonium resulting from the hydrolysis of urea using the difference between available and measured urea as the amount of urea hydrolyzed.

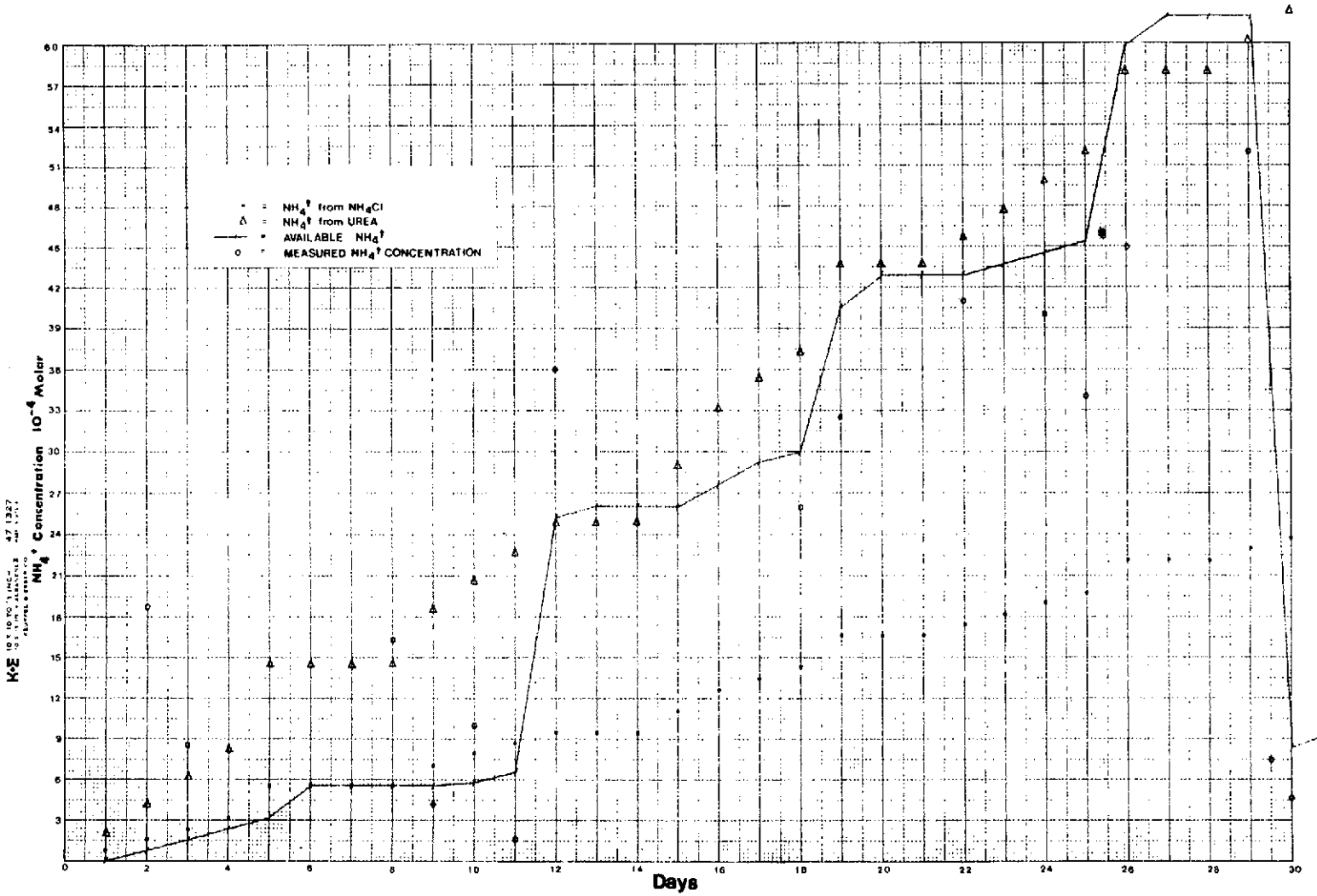
During performance of the second CST, Column A, described earlier, was tested for its ability to trap ammonia contained in phosphate buffer in a test separate from the CST. The 50 g of ion-exchange resin trapped 1081 mg of the 2068-mg challenge of ammonium chloride. The 2068 mg of ammonium chloride provides the same quantity of ammonium that a 30-day OFO experiment could provide.

Table 8 - Data from Second CST

mg NH_4Cl Cumulative	Available $[NH_4^+] \times 10^{-4}$	Measured $[NH_4^+] \times 10^{-4}$	$[NH_4Cl] \times 10^{-4}$	mg NH_4Cl Added	Day	Date	mg Urea Added	Urea mg/	Urea mg/ Measured	Urea mg/ Available	mg Urea Cumulative	Urease Assay Urea μmoles/min.
19			0.789	19	1	09-16-74	28	6.2		0	28	34.2[3]
37.8	0.789	18.8	1.57	18.8	2	09-17-74	27.9	12.4	12	6.2	55.9	19.5[3]
56.6	1.57	8.5	2.35	18.8	3	09-18-74	27.9	18.6	17	12.4	83.8	9.69[3]
75.5	2.35	8.0	3.14	18.9	4	09-19-74	27.8	24.8	15.7	18.6	111.6	4.65[3]
132.6	3.14		5.51	57.1	5	09-20-74	84	43.5		24.8	195.6	
132.6	5.51		5.51		6			43.5		43.5	195.6	
132.6	5.51		5.51		7			43.5		43.5	195.6	
132.6	5.51	16.3	5.51		8	09-23-74		43.5	65.5	43.5	195.6	25.5[4]
170.6	5.51	4.2[1]	7.09	38	9	09-24-74	56	55.9	56	43.5	251.6	6.14[4]
189.8	5.78	10.0	7.88	19.2	10	09-25-74	27.9	62.1	62	55.9	279.5	3.08[4]
208.6	6.38	1.6[1]	8.86	18.8	11	09-26-74	28.1	68.4	90	62.1	307.6	1.45[5]
227.7	25.2	36.0	9.46	19.1	12	09-27-74	28	74.6	<1	68.4	335.6	0.0[6]
227.7	26.0		9.46		13			74.6		6.2	335.6	
227.7	26.0		9.46		14			74.6		6.2	335.6	
265.7	26.0		11.0	38	15	09-30-74	56	87.0	9.5	6.2	391.6	
303.9	27.6		12.6	38.2	16	10-01-74	56.1	99.5		18.6	447.7	
323.1	29.2		13.4	19.2	17	10-02-74	28.2	106		31.1	475.9	
342.2	30.0	26.0	14.2	19.1	18	10-03-74	28.3	112	36.5	37.4	504.2	1.14[7]
399.4	40.5	32.5	16.6	57.2	19	10-04-74	83.9	131	14.5	43.7	588.1	0.112[7]
399.4	42.9		16.6		20			131		33.1	588.1	
399.4	42.9		16.6		21			131		33.1	588.1	
418.4	42.9	41.0	17.3	19	22	10-07-74	28.1	137	28	33.1	616.2	
437.4	43.7		18.2	19	23	10-08-74	27.9	143		39.4	644.1	
456.5	44.5	40.0	19.0	19.1	24	10-09-74	28.3	149	38.2	45.6	672.4	
475.5	45.3	34.0	19.8	19.0	25	10-10-74	28.0	156	47	51.9	700.4	
532.8	59.7	45.0	22.1	57.3	26	10-11-74	83.9	174	17	58.1	784.3	
532.8	62.1		22.1		27	-		174		35.6	784.3	
532.8	62.1		22.1		28			174		35.6	784.3	
551.9	62.1	52.0[2] 7.5[2]	22.9	19.1	29	10-14-74	28.1	181	21	35.6	812.4	
571.1	8.29	4.6[2]	23.7	19.2	30	10-15-74	28.1	187	26	41.9	840.5	
571.1	9.09	4.1[2]	23.7		31	10-16-74		187	28	48.1	840.5	

[1] The system was circulated at 95°C for 1 hour prior to measurement.
[2] The solution had been circulating through an ion-exchange column.
[3] This is the activity of immobilization #4 under standard assay conditions after use in the CST.
[4] This is the activity of immobilization #3 under standard assay conditions after use in the CST.
[5] This is the activity of Enzite urease under standard assay conditions after use in the CST.
[6] This is the activity of 51 mg of free urease (Beckman)/ 4.5% under standard assay conditions after overnight in the CST.
[7] This is the activity of 50-mg portions of free urease (Worthington)/ 4.5% under standard assay conditions after use in the CST.

Figure 10 - Record of Ammonium Concentrations



$$\left[\frac{28 \text{ mg urea}}{\text{day}} \times \frac{2 \text{ mmole N}}{60 \text{ mg urea}} \times \frac{53.5 \text{ mg } NH_4C\ell}{\text{mmole N}} + \frac{19 \text{ mg } NH_4C\ell}{\text{day}}\right] \times 30 \text{ days} = 2068 \text{ mg } NH_4C\ell$$

The 1081 mg of $NH_4C\ell$ corresponds to the trapping of 5.7 mg N/g of ion-exchange resin.

$$\frac{1081 \text{ mg}}{50 \text{ g}} \times \frac{14 \text{ mg N}}{53.5 \text{ mg } NH_4C\ell} = 5.7 \text{ mg N/g}$$

The concentration of sodium ions in 0.05 M phosphate buffer at pH 7.0 is:

$$[2(0.0306) + 0.0194]M \times \frac{23g}{\ell\ M} \times \frac{\ell}{1000 \text{ g}} = 1854 \text{ ppm}$$

For the atomic absorption measurement of sodium the samples were diluted 1 to 20. The sodium content prior to circulation through the column was 93 ppm and afterwards was 85 ppm which corresponds to an 8.6% loss of sodium.

$$\frac{93 - 85}{93} = 8.6\%.$$

It was felt that this sodium loss was acceptable, so Column B with a greater capacity for ammonia nitrogen was prepared for use in the second CST.

On day 29, Column B was placed in the second CST as indicated in arrangement C of Figure 9. The concentrations of ammonium before and after insertion of Column B were measured and are shown in Table 8 and Figure 10 on days 29 through 31. The corresponding measurements of sodium are shown in Table 9.

During the time that 11% of the Na^+ was absorbed (100% - 89%), 93.6% of the available NH_4^+ was absorbed.

$$1 - \frac{4.1 \times 10^{-4} \text{ M}}{\frac{19.1 + 19.2 \text{ mg}}{4.5\ \ell} \times \frac{\text{mole}}{53{,}500 \text{ mg}} + 62.1 \times 10^{-4} \text{ M}} = 93.6\%$$

Table 9 - Sodium Ion Concentrations

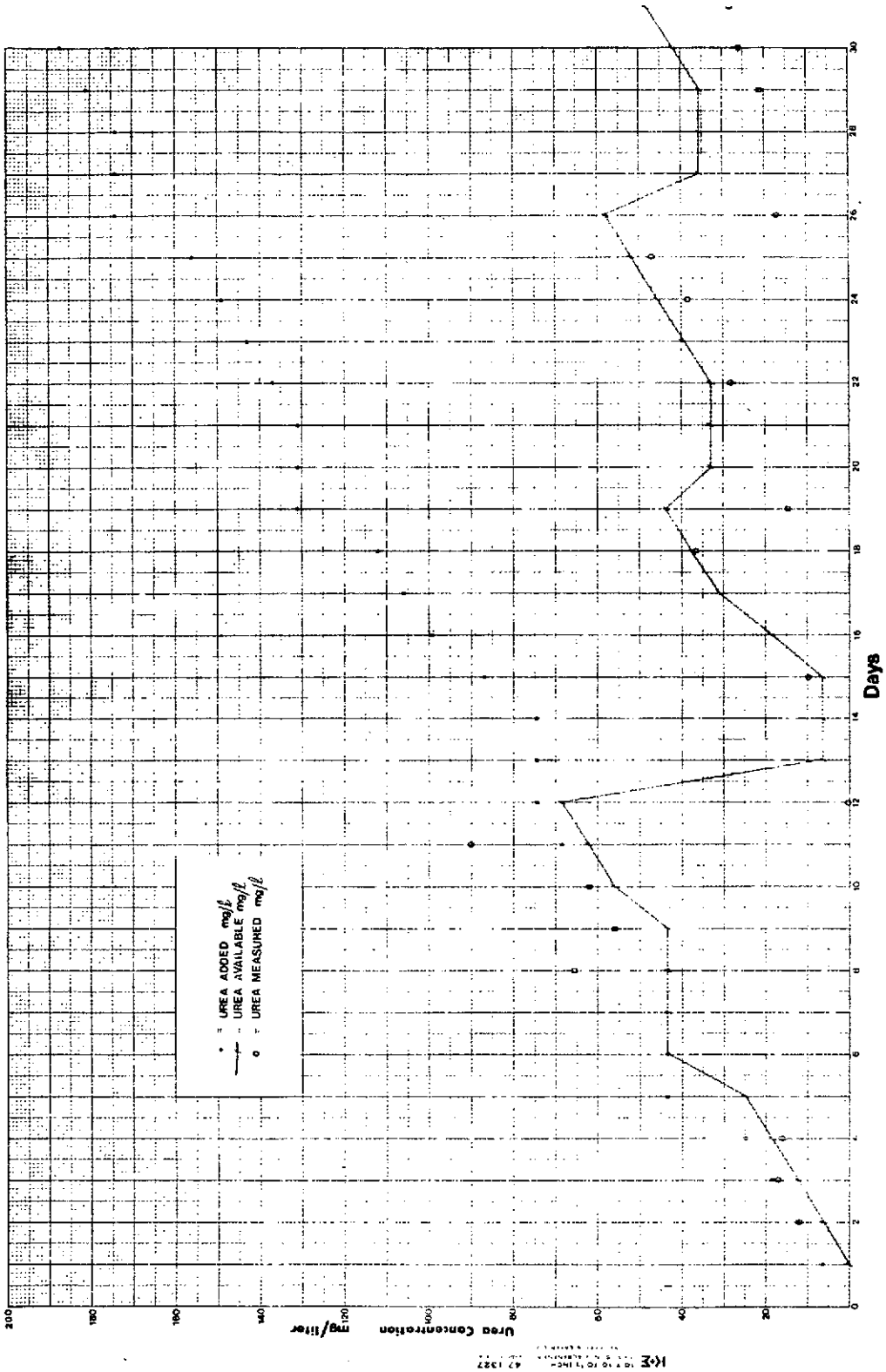
Day	Measured Na^+ ppm	$[Na^+]$	% Na^+ Remaining in Circulation
29 before	1680	0.073	100
29 after 2 hrs	1500	0.065	89
30	1520	0.066	91
31	1500	0.065	89

This suggests that the ANGC-101 ion-exchange resin could prove useful in controlling the concentration of ammonia in the presence of other required cations.

Urea: The information to the right of the date column in Table 8 shows the record of urea additions, measured urea, available urea and urease activities. Figure 11 is a graphic presentation of the urea concentrations. The available urea lags by a day the urea concentration, because the measurements were made prior to the urea additions on any given day. During the first 10 days while immobilized urease from either immobilizations #3 or #4 was employed in the CST, there was no significant hydrolysis of CST urea taking place and as was pointed out separate urease assays of these immobilizations showed significant losses of urease activity upon exposure to the OFO system (second CST). On day 10 Enzite urease (100 mg) was placed between two fritted glass discs and placed in the second CST. Its urease activity was 1.62 μmoles/min. prior to inclusion in the CST, and it was 1.45 μmoles/min. after 12 hours in the CST. There was no apparent hydrolysis of urea during its inclusion in the CST. During the first 11 days of this CST excluding day 4, the measured urea exceeded the available urea for reasons unknown. Because immobilized urease had no apparent ability to hydrolyze urea in the CST and was inactivated by exposure to the CST, the use of immobilized urease was abandoned in this CST.

On day 11, fifty-one mg of Beckman's jack bean urease (not immobilized) was added directly to the constant temperature bath of the CST. On day 12 the measured urea was less than 1 mg/ℓ and there was a corresponding increase in the measured ammonium ion. Fifty ml of the circulating solution was removed from the CST and found to have no urease activity at a urea concentration of 0.3 M.

Figure 11 - Record of Urea Concentrations



On day eighteen, 51.7 mg of Worthington's urease (URC) was added to the CST and 10 minutes later 100 ml was removed for assay. At a urea concentration of 0.3 M the urea hydrolysis rate was 1.14 μmoles/min. This free urease activity after 1300 minutes in the CST (day 19) was 0.112 μmoles/min. The measured urea concentration dropped from 36.5 to 14.5 mg/ℓ from day 18 to day 19 and there was a corresponding increase in the ammonium-ion concentration.

On day twenty-five, 50 mg of Worthington's urease (URC) was added to the CST. No urease activity measurements were made for this addition. The urea concentration decreased from 47 to 17 mg/ℓ and resulted in a corresponding increase in the ammonium-ion concentration.

The results of the addition of free urease to the circulating system suggest that this would be a plausible method warranting further investigation for use in controlling the OFO urea toxicity problem.

The results of the additions of free ureases to the circulating system suggest a difference in the free urease activities between the Beckman (reported 14 μg N/mg min) and Worthington (reported 1.1 mg N/mg min) preparations. Although the addition of Beckman urease showed no urease activity the following day, the one datum point suggests that it was more effective in reducing the presence of urea than were the additions of Worthington urease. However, the results of the addition of either of the free urease preparations to the circulating system suggest that periodic additions of free urease would be a plausible method warranting further investigation for use in controlling the OFO urea toxicity problem.

CONCLUSIONS

The objective of this program was to determine the feasibility of using immobilized urease for urea removal from waste water for space system applications, specifically the elimination of the urea toxicity problem in a 30-day Orbiting Frog Otolith (OFO) flight experiment. Because urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, control of their concentrations within nontoxic limits was also considered. Because the OFO A experiment had methods for controlling the CO_2 levels, only the control of NH_3 levels was addressed in this technical effort.

Task 1 - Immobilized Urease Technology Analysis

During performance of Task 1 the performance of various immobilized urease preparations was established. The nitrocellulose entrapment preparation provided by Worthington Biochemical Corporation showed loss of enzyme activity from one assay solution to the next caused by inadequate binding of the enzyme. Our acrylamide gel entrapment preparations on nylon showed greater stability from assay to assay and the loss of urease activity was less than 15% in Tris buffer at 4°C for 20 days. When the urease assays were conducted under the conditions of the OFO experiment, the resulting activities were greatly reduced. The differing conditions are shown in Table 10. Our results for activity reductions related to reduced assay temperature suggest that for a reduction of 6.5°C the activity would be reduced to 77%. For an increase of pH from 4.9 to 7.0, a reduction in activity of almost 50% was shown and for a reduction in substrate concentration to 0.3%, a reduction in activity to 1.5% was demonstrated. Because of these expected reductions in activity, higher initial activity preparations were attempted using acrylamide gel entrapment on glass wool. These preparations had a higher initial activity but this initial high activity was lost upon overnight cold storage indicating incomplete immobilization of the enzyme. Use of these immobilized enzymes in an OFO-type system resulted in significant losses of urease activity under the standard assay conditions and showed no urea hydrolysis in the combined system tests.

Table 10 - Urease Assay Conditions

Condition	Standard Assay	OFO Assay
Temperature	23°C	16.5°C
pH	4.92	7.0
Substrate Molarity	0.3	8.3×10^{-4}

Task 2 - Ammonia Removal Analysis

Initially the ANGC-101 ion-exchange resin (Baker) was selected for ammonia removal; however, during the oral review of Tasks 1 and 2 appreciable concern was expressed that the resin might reduce other cations below their required concentrations. Therefore, the hydrophobic membrane used for the ammonia-gas electrode was selected for testing as a means for ammonia removal.

The ultimate technique for NH_3 analysis was the use of the Orion 95-10 ammonia-gas electrode at a pH $\geq$11 on samples of solutions and comparison of their millivolt readings with a standard curve.

Task 3 - Combined Urea/Ammonia Removal Systems Test

The results of the first Combined Systems Test (CST) showed an inadequacy of the monitoring techniques for urea and the complete loss of urease activity for the acrylamide gel entrapment on nylon at the end of the 12-day test. Therefore, for the second CST, the determination of urea concentration by measuring the difference of ammonia concentration before and after acid hydrolysis was replaced by a method reported by Joon H. Rho [6], "Direct Fluorometric Determination of Urea in Urine" and urease activity was measured on a daily basis using a modified Worthington Disc Assay Procedure.

The second CST was started and run for 31 days. This test demonstrated the inadequacy of the hydrophobic membrane for ammonia removal under the CST conditions and showed wetting of the membrane preventing its function as a liquid-gas separator. As discussed in Task 2 under Results and Discussion, the use of this membrane or some other means of liquid-gas separation would probably prove inadequate for ammonia removal, because of the relatively low level of ammonia species present compared to their solubility under the OFO conditions.

The immobilized ureases in the CST had no apparent effect on the hydrolysis of urea and continually showed losses of activity following exposure to the CST. Although immobilized urease was ineffective under the OFO CST conditions, its use for reduction of urea concentration under more favorable conditions of pH, substrate concentration and temperature might be applicable.

When free urease was added to the second CST, there was a significant decrease in the measured urea and a concomitant increase in the measured ammonium-ion concentration. This indicated that free urease was effective in hydrolyzing urea under conditions where approximately three times as much immobilized urease was ineffective. There are no doubt numerous plausible explanations for this occurance and there is probably an obvious explanation to the alert enzymologist, but this author chooses not to speculate based on his limited knowledge in this area.

Based on the failure of the hydrophobic membrane in controlling ammonia concentration and the apparent preference of the ANGC-101 ion-exchange resin for ammonium over sodium cations, the resin was employed for reduction of the ammonium-ion concentration during the last two days of the second CST. This resulted in a reduction of the ammonium-ion concentration by 93.6% while reducing the sodium-ion concentration by only 9 to 11%.

Further investigation of the usefulness of the free urease and ion-exchange resin is warranted for establishing the feasibility of their use in controlling urea and ammonia concentrations in an OFO experiment. Specific questions which need to be addressed include:

1. Is free urease a detriment to the frog or vice versa?

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

3. Can the ANGC-101 be preloaded with required cations and remain active for ammonium ions in order to maintain the required cationic balance?

REFERENCES

1. G. G. Guilbault and J. Montalvo, *J. Amer. Chem. Soc.*, 92, 2533 (1970).

2. Catherine C. Johnson, letter to R. R. Husted, dated April 19, 1974.

3. Darius S. Rogers and Karl H. Pool, *Analytical Letters*, 6, 801 (1973).

4. *Handbook of Chemistry and Physics*, Forty-first Edition, Chemical Rubber Publishing Co., 1960, pages 1708 and 1743.

5. J. S. Fruton and S. Simmonds, *General Biochemistry*, Second Edition, published by John Wiley and Sons, Inc., New York, 1958, pages 87-88.

6. Joon H. Rho, *Clinical Chemistry*, 18, 476 (1972).