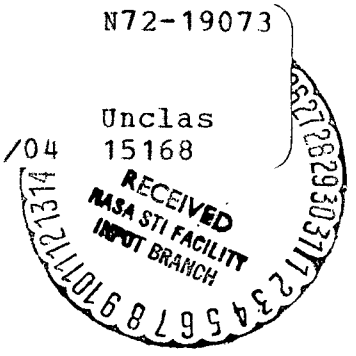
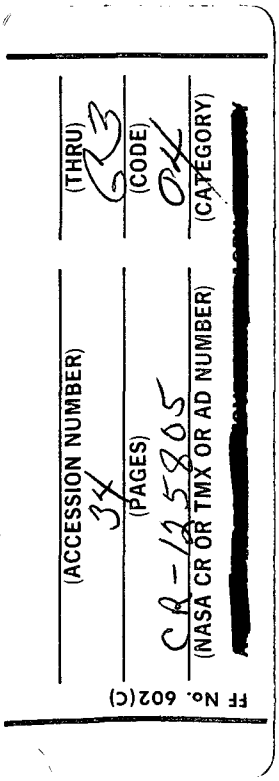
FINAL REPORT

Contract No. NAS 9-7951

"THE EFFECTS OF SPACECRAFT ENVIRONMENTS ON SOME HYDROLYTIC ENZYME PATTERNS IN BACTERIA"





Prepared by

J. M. Prescott and B. G. Foster
Department of Biochemistry and Biophysics
Texas A&M University
College Station, Texas 77843

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INTRODUCTION AND SPECIFIC AIMS

A number of microorganisms not generally considered to be pathogenic, but which are known to produce proteolytic enzymes, are frequently found as normal flora of man. Although most bacterial proteinases from aerobic or facultatively aerobic species appear not to attack the skin and epithelial tissues of man or to act as toxins, some of them possess the ability to attack living tissues. For example, the crude enzyme system of *Aeromonas proteolytica* has been found to cause pronounced necrosis when injected into experimental animals (1) as is shown in Figure 1. The possibility must be considered, therefore, that non-pathogenic species of bacteria might produce sufficient levels of proteolytic enzymes to constitute a health hazard to space crew members, especially since the ability of the latter to control bacterial growth through normal hygenic procedures is limited and the organisms might gain entrance through abrasions or cuts.

Furthermore, it is well established that the growth of microorganisms and the production of their extracellular enzymes are profoundly influenced by such cultural conditions as medium, temperature and degree of aeration. The relatively oxygen-rich atmosphere of a spacecraft cabin might thus be expected to promote growth and proteinase production by certain microorganisms, and it is possible that the artificial environment of the cabin might also produce alterations in the ratios of production of individual proteinases by the same organism. The spacecraft environment might also cause a decrease

in the population of certain species or eliminate organisms which are not adaptable to these environments. A reduction in the microbial spectrum would serve to reduce natural competition and permit overgrowth of those organisms that are suited to life in the artificial environment.

If any of the conditions in the spacecraft cabin should result in greatly increased proteolytic enzyme production or severely altered ratios of the different proteinases, a potential hazard to man might well exist. Because of these unknown factors, it was the objective of this research to investigate the effects of space flight on the production and characteristics of proteolytic enzymes of a number of bacterial species isolated from crew members and spacecraft. The research covered in this report fell under two general categories, as follows:

I. Determination of the enzymatic make-up and cultural characteristics of bacteria isolated from spacecraft crew members.

II. Studying the organism *Aeromonas proteolytica* and the proteolytic enzymes which it produces as models for use in future spacecraft experiments.

GENERAL METHODS

Microbiological procedures. Cultures were isolated from the spacecraft and space crew members by personnel at NASA Manned Spacecraft Center and forwarded to Texas A&M University. All cultures were maintained by accepted microbiological techniques, and a number of them were screened for confirmation of their taxonomic classification. These experiments were done by the procedures outlined in *Mannual of Microbiological Methods* (2), and by those described by Bailey and Scott (3). Details of the individual media and growth conditions used in the various experiments are described in the Experimental and Results Section.

Enzyme assays. Qualitative screening for gross proteolytic activity was done by placing a drop of culture filtrate from the organism on the emulsion side of a sheet of exposed but undeveloped photographic film. After a suitable incubation period, the film was washed in cold tap water and examined for a zone of clearing that resulted from hydrolysis of the gelatin in the emulsion. Quantitative assays for proteolytic enzymes were performed by slight modifications of the procedure of Anson (4), using either the denatured hemoglobin substrate that we previously described (5) or a 2% casein solution in phosphate buffer, pH 7.0. For the assay, 5 ml of substrate and 1 ml of enzyme solution were incubated at 37°C for the desired period of time (usually 5 to 30 minutes). Enzymatic action was halted and undigested protein precipitated by the addition of 10 ml of 5% trichloroacetic acid. The tubes were kept in the water bath to

permit complete coagulation and precipitation to occur (usually 30 minutes), then the suspensions were filtered through Whatman No. 3 paper. The extent of hydrolysis was assessed by reading the absorbance of the filtrate at 280 nm. One unit of enzyme activity toward hemoglobin was defined as the amount of enzyme that produced an absorbance of 1.0 in 5 min. at 37°C.

Aminopeptidase assays were run according to the general principles of the method of Goldbarg and Rutenburg (6), using a substrate *L*-leucyl-β-naphthylamide. The substrate was dissolved in 0.015 M Tricine buffer, pH 8.0, at a concentration of 0.2 mg per ml; 0.5 ml of substrate and 0.5 ml of enzyme were incubated at 37°C for time periods up to 20 minutes. The reaction was stopped by the addition of 1 ml of 40% trichloroacetic acid, and diazotization and color development were carried out as described by Goldbarg and Rutenburg (6).

Other procedures. Disc gel electrophoresis was performed by the procedure of Ornstein (7) and Davis (8), and the physical and enzymatic characterization of the aminopeptidase of *Aeromonas proteolytica* were performed according to procedures described previously (9-11).

EXPERIMENTAL AND RESULTS

The original investigation was undertaken to determine whether exposure to spacecraft environments alters the production and properties of proteolytic enzymes of bacteria isolated from crew and spacecraft. Various cultures examined were obtained from chamber flights and from Apollo flights 8, 9, 10, 11, and 12. Our earlier attempts to assess the effects of spacecraft environments, however, were hampered by the fact that we seldom received postflight bacterial species that corresponded to the preflight cultures. Thus, meaningful comparisons of proteolytic enzyme levels were not possible, and our work was largely directed toward screening the cultures for classification, searching for media that permitted maximum production of proteinases, and developing new methods for the assessment of the specific bonds cleaved by proteinases. Many of the earlier results were reported in a Final Report dated July 24, 1969.

With Apollo 12, however, excellent matching of preflight and postflight cultures was obtained for a number of *Bacillus* species. The sampling obtained appeared to be adequate to evaluate the relative proteolytic enzyme production of the species obtained preflight and those isolated postflight, inasmuch as six preflight cultures of *Bacillus* were received along with ten postflight *Bacillus* cultures.

Apollo 12 Experiments. Cultures in each experiment were grown on both rotary (a) and reciprocal (b) shakers in order to account for any differences in growth and enzyme production caused by variations in the degrees of aeration. Both incubators were maintained

at a temperature setting of 30 ± 1°C with a shaker rate of 130 rpm. The enclosed chamber of the reciprocal shaker was gassed with filtered compressed air through a gas port with a flow meter setting of 5 mm. Gassing of the rotary shaker was not necessarily due to free air exchange through a motor driven fan.

The medium used for this set of experiments was composed of 1% Bacto-peptone, 1% beef extract and 0.5% NaCl, distributed in aliquots of 100 ml/500ml erlenmeyer flask and autoclaved 12 minutes at 121°C.

Inoculum source was obtained from 18-24 hour nutrient broth tube cultures of the bacteria grown in a 37.5°C static incubator. All cultures were centrifuged in an International Clinical Centrifuge for 20 minutes, the cells were resuspended in sterile physiological saline and the turbidity at 660 nm was read on a Coleman Junior Spectrophotometer. The inoculum was adjusted so that approximately 1.0 ml of cell suspension showing a turbidity of 80% T at 660 nm was added to each flask. An equal aliquot of the cell suspension was added to each flask (a and b). Blanks consisting of uninoculated medium were subjected to the same conditions. Previous experiments indicated that optimal proteolytic enzyme activity was obtained from cultures grown for 40 hours or more; therefore all cultures were incubated for 46 ± 2 hours as designated in Table I.

Duplicate experiments were run with a one to two week interval between to account for differences in the age of the stock cultures; these experiments are designated as Harvest #1 and Harvest #2. Difficulty was encountered with two *Bacillus* cultures (#78 and #88)

due to clumping and in those cases a third harvest was made.

Termination of culture growth was designated as "Harvest Time"; at this time final turbidity of the culture was recorded and a Gram stain of each culture was made as a check for possible contamination. All cultures and blanks were centrifuged in a Sorvall RC2-B refrigerated centrifuge for 20 minutes at 12,000 x *g*. All cells were discarded and the experiment was completed using culture supernatants as the source of enzyme.

The pH values of blanks and culture supernatants were recorded using wide range pH paper. Proteolytic enzyme activity of supernatants toward gelatin was checked by the photographic emulsion technique described above, and those samples which showed activity were then assayed quantitatively by the hemoglobin procedure.

The results of the proteinase studies on the cultures from Apollo 12 are shown in Table I, which reveals that both growth and proteolytic enzyme production for the *Bacillus* species varied over a fairly wide range. Most of the strains were found to produce protease, however, and it is likely that activity could have been elicited from the other cultures by varying the media and other cultural conditions. Similarly, higher production of enzyme by the proteolytically active cultures could probably be optimized by similar variations. The average protease production of all postflight cultures was higher than the average production by the preflight cultures. Whereas these results in themselves are certainly not conclusive, they suggest the possibility that spacecraft environments may select for strains that are more proteolytic or that they may produce genetic alterations

which result in increased proteolysis.

Studies on Aeromonas proteolytica as a model system. The potential health hazard posed by increased protease production following exposure in a spacecraft cabin prompted an investigation of *Aeromonas proteolytica* as a model organism to be used in pursuing this line of research. This organism produces at least two proteolytic enzymes--an endopeptidase and an aminopeptidase--both of which we have previously isolated (9-11). Moreover, the unfractionated proteolytic enzymes of *A. proteolytica* have been shown (1) to attack the tissues of living animals (cf. Fig. 1) and its use in such studies therefore appeared especially appropriate. Our efforts were directed toward elucidation of the detailed molecular properties of these two proteolytic enzymes, in order that any changes induced by space flight or lunar surface exposure might be detected by a comparison of properties of the enzymes produced by the organism preflight with those produced post-flight. The studies were performed by standard methods of protein chemistry, descriptions of which are included in the reprint (11) attached hereto, and in the work of Prescott and Wilkes (9) and Griffin and Prescott (10).

A summary of the physical properties of the *Aeromonas* endopeptidase and aminopeptidase is shown in Table II, and in Table III the amino acid compositions of the two enzymes are shown. From the data in these tables, it is evident that a considerable amount of precise information is available concerning these two enzymes and that the data provides a baseline for comparisons of the properties of enzymes isolated from cultures of *A. proteolytica* exposed in space flight experiments.

Irrespective of the biochemical attributes of *A. proteolytica*, however, it is evident that its microbiological characteristics must be such as to render it suitable for the conditions to be encountered in the spacecraft experiments. Therefore, a study was inagurated to assess its suitability for such purposes.

Preliminary studies were made to determine the best medium to use for growth of the organism (in preparation for survival studies) and recovery of the organism following storage in a suitable holding medium.

The following media were compared:

1% peptone

1% peptone with 5% glucose

1% peptone with 0.005 g K_2HPO_4/liter

1% peptone with 5% glucose and 0.005 g K_2HPO_4/liter

1% proteose peptone No. 3

1% proteose peptone No. 3 with 5% glucose

1% proteose peptone No. 3 with 0.005 g K_2HPO_4/liter

1% proteose peptone No. 3 with 5% glucose and 0.005 g K_2HPO_4/liter

1% phytone

1% phytone with 5% glucose

1% phytone with 0.005 g K_2HPO_4/liter

1% phytone with 5% glucose and 0.005 g K_2HPO_4/liter

5% peptone

5% peptone with 1% glucose

1% peptone with 1% glucose

All of these were made in artificial sea water since *A. proteolytica* grows and survives best at relatively high ionic strength.

Turbidimetric assessment of growth as a function of time, along with determinations of the changes in pH led to the conclusion that the most suitable growth medium would be 1% peptone, which allowed steady log growth while maintaining a physiological pH value. The optimal recovery medium was found to be 5% peptone, which gave maximum recovery following prolonged storage in non-nutrient holding media.

The next series of studies included the development of a non- or low nutrient holding medium that would give maximum cell recovery after 20 days of storage, while maintaining a cell concentration between 90 and 100% transmittance at 253.7, 260, 280 and 300 nm.

The following holding media were tested:

distilled-deionized water

distilled water

sea water (artificial)

physiological saline

asparagine in distilled water

physiological saline with added K_2HPO_4

The study consisted of 5 day preliminary runs and 20 day experimental runs at 20°C. The first five day preliminary runs consisted of distilled-deionized water, distilled water, physiological saline solution and artificial sea water as a holding media. A 4.9 ml aliquot of each holding medium was inoculated with 0.1 ml of a twice-washed 18 hour culture of *A. proteolytica* (3.1×10^6 cells per ml). At day 1, 3 and 5 triplicate samples were processed and delivered into

recovery media. Growth was found at the end of each processing period in all media except distilled-deionized water, in which no growth was found, and in distilled water, where no growth was found after a three day holding period. A 20 day experimental run was then set up to test the use of distilled-deionized water over a longer time period. This test also showed no viable organisms to be present after twenty-four hours holding time.

A 20 day experimental run using artificial sea water was then set up. This consisted of a triplicate set of tubes (unsealed and sealed with sterile mineral oil) for each day of the 20 days in the run, and 20 samples (sealed and unsealed) for processing on the last day. Each screw cap tube contained 4.9 ml of holding medium to which was added 0.1 ml of a twice-washed 18 hour culture (6.8×10^6 organisms per ml). The optical density at 660 nm was recorded for each sample. Each day triplicate samples, sealed and unsealed, were processed and the viable cell count and percent survival calculated. On the twentieth day, an additional 20 sealed tubes and 20 unsealed tubes were processed, as well as the three sealed and unsealed tubes regularly scheduled for that day. The results are shown in Graph I, in which are plotted the average percentage survival of organisms for each of the 20 days. The additional 40 tubes (20 sealed; 20 unsealed) held throughout the experiment and processed on the twentieth day yielded a survival rate of 44% for sealed tubes and 42% for unsealed.

The next holding study was a 20 day experimental run with a medium consisting of 0.1, 0.05 and 0.01% L-asparagine in 66% artificial sea water. The holding medium was inoculated with 8.2×10^6 organisms per ml

(prepared as above) and processed at 5 and 20 days. The results indicated that due to growth of the cells by day 5 (4.7×10^7) which exceeded transmittance limits the medium was unacceptable for use.

Physiological saline (8.5 g NaCl per liter) solution with 0.05 g K_2HPO_4 per liter was tested as a holding medium in a 20 day experimental run at 20°C. Triplicate samples were inoculated with 7.4×10^6 organisms per ml and sealed with mineral oil. The samples were processed on the fifth, tenth, fifteenth and twentieth day when optical density, viable count and percent survival was determined. Results of this study are summarized in Graph II, which shows a 35% survival.

The absorbance at 253.7, 260, 280 and 300 nm was run on all of the holding media considered. All were found to be within acceptable limits (greater than 90% transmittance) except the physiological saline-phosphate. It appeared possible that the latter might be due to the difficulty in removing the mineral oil overlay which might interfere with the spectrophotometer readings. However, a second 20 day run made under the same conditions except that the mineral oil overlay was omitted, gave similar results as is shown in Table IV. On day one the percent transmittance readings for 253.7 and 260 nm were 91.0% and 91.04% respectively and each increased to below the acceptable level by the fifth day. A third 20 day run was then made using a lower cell count (4.4×10^6 organisms per ml). The results are shown in Table V. As indicated the percent transmittance readings for each wavelength after the twentieth holding day remained within acceptable limits. The pH of the holding medium varied only slightly during the

20 day period and the percent survival was 27%. Since all of the parameters for a holding medium had been met, it was decided that physiological saline solution (0.85%) with 0.05 g K_2HPO_4 per liter in demineralized water would be used.

Since at least one *Aeromonas* species (*A. liquifaciens*) is known to produce an extracellular toxin and a hemolysin, tests were initiated to determine whether these are present in cell free extracts of *A. proteolytica*. Initially, the organisms were grown using the cellophane plate technique. Sterile cellophane sheets were placed aseptically on plates containing nutrient agar made with artificial sea water and inoculated with broth cultures of *A. proteolytica* grown for 24 hours. After growth had occurred for five days, the plates were flooded with sterile saline solution and the cells were removed by centrifugation; the supernatant fluid served as the source of material for toxin and hemolysin studies. A 0.2 ml aliquot inoculated subcutaneously produced an erythema in rabbits within four hours. Several incubation times and temperatures were used to determine optimum conditions for the hemolysis reaction; sheep and human erythrocytes were also used as variables. Human erythrocytes at an incubation time of 24 hours at 37°C seemed optimal and were used throughout the remainder of the studies. The hemolysin titer was 1:32. Neither the cells or the cell-free extract was lethal to mice. The extract, when inoculated into guinea pigs, produced an intracutaneous hemorrhage almost immediately. Within 24 hours the lesion had formed a scab.

Similar experiments were repeated with organisms grown in 1% peptone (in sea water) at 37°C for 72 hours. The cells were removed

by centrifugation and filtration through a 0.45 μ pore membrane filter, and the filtrate was used for the study. Within 5 minutes after injection of a 0.2 ml dose, intracutaneous hemorrhage occurred in guinea pigs. Within 24 hours the lesions formed scabs and in some cases showed signs of necrosis. The hemolysin titer was 1:32. Perevaporation of the filtrate increased the intensity of the guinea pig reaction and elevated the hemolysis titer. The filtrate was stable at 70°C for thirty minutes; however, this did reduce the hemolysis titer. Higher temperatures were not tested. The filtrate still was not lethal to mice by intraperitoneal injection, but 10^9 organisms did kill mice.

From these experiments it was concluded that *A. proteolytica* elaborated a toxic substance which produced immediate intracutaneous hemorrhage followed by scabbing and sometimes necrosis, and which also caused hemolysis of human erythrocytes. These effects might, of course, have resulted from more than one substance.

Tests were begun to determine the effect of altering the time and temperature of growth on the production of the toxic substance and hemolysin. Several media of varying composition were also used, both as stationary cultures and on shakers. The following growth media were tested: nutrient broth, tryptic soy broth, 1% peptone and 5% peptone (each of which were made with artifical sea water). A semi-synthetic (SS) medium was also tested. It was composed of the following:

Peptone	10.0 g
NaCl	8.12g

$MgCl_2 \cdot 6H_2O$	4.22g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
TRIS*	1.0 g
$MgSO_4 \cdot 7H_2O$	0.04g
Trace Mineral solution+	5.0 ml

*Trishydroxymethylamino methane

+Components and concentration in grams per liter

(1) ethylenediaminetetracetic acid (tetrasodium salt)	1.0
(2) H_3BO_4	1.14
(3) $FeCl_3 \cdot 6H_2O$	0.049
(4) $FeSO_4 \cdot 7H_2O$	4.0
(5) $MnSO_4 \cdot H_2O$	3.08
(6) $ZnSO_4 \cdot 7H_2O$	0.022
(7) $CuSO_4 \cdot 5H_2O$	0.00016
(8) $CoSO_4 \cdot 7H_2O$	0.00048

The organisms were grown in each medium at 37°C and at room temperature for 24, 48, 72 and 120 hours either on a reciprocal shaker or stationary. Filtrates from each experiment were tested for hemorrhage and necrosis in guinea pigs using 0.5 ml aliquots and for hemolytic activity. Because of the similiarity of the toxic substrate to the endopeptidase isolated and characterized by Griffin and Prescott (10), a hemoglobin assay for endopeptidase activity was run on a sample from each test condition with results as

shown in Tables VI, VII, VIII, IX and X. As can be seen there is good correlation between the guinea pig reaction and endopeptidase activity and, therefore, indirect evidence that the reaction in guinea pigs is due to the endopeptidase. There seems to be no relation between endopeptidase and the hemolysis reaction.

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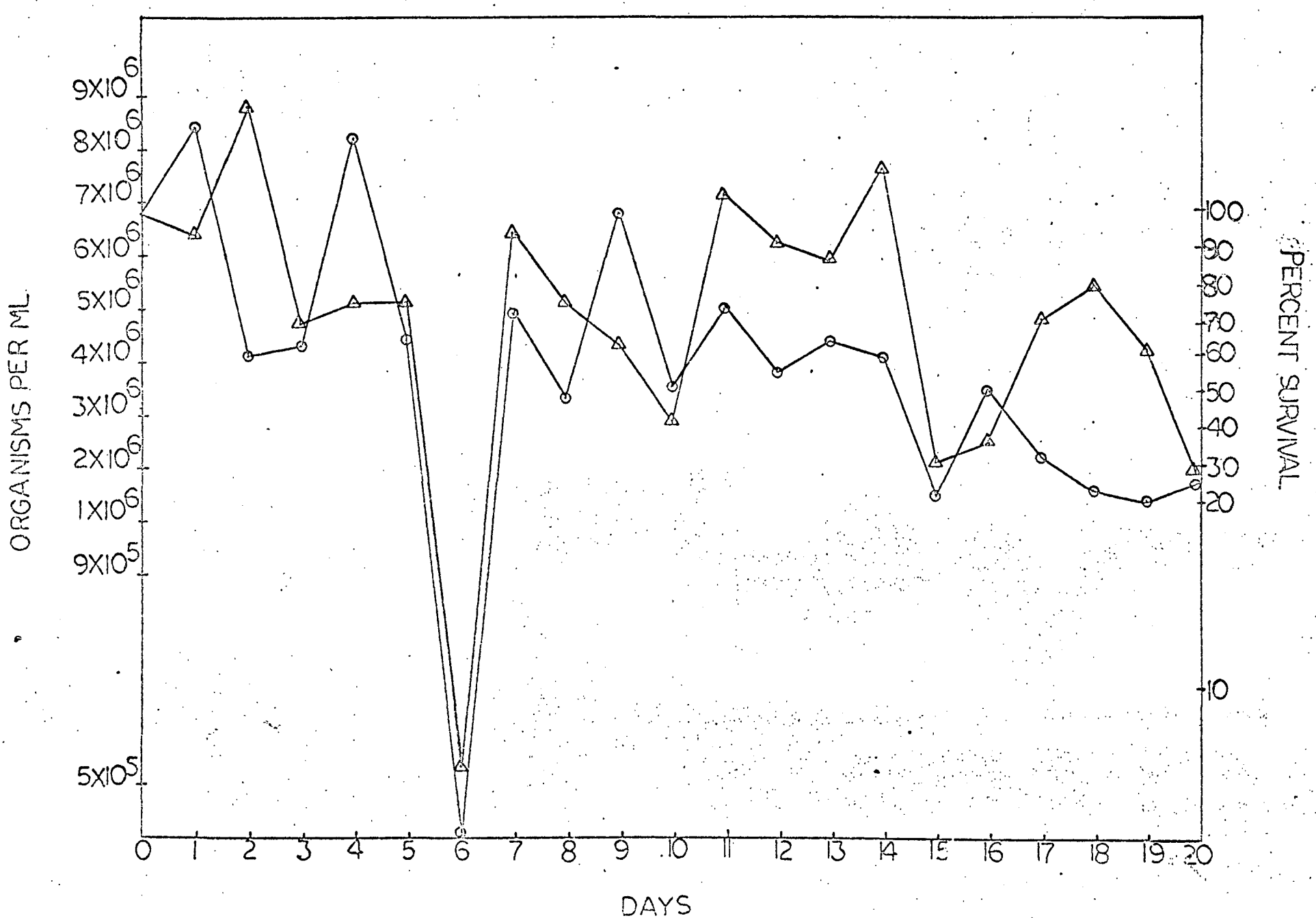
Fig. 1 Lesion produced in a rabbit that had been injected with *Aeromonas proteolytica* culture filtrate for the purpose of generating antibodies. The lesion subsequently healed. (Courtesy of Dr. Frank F. Hall.)

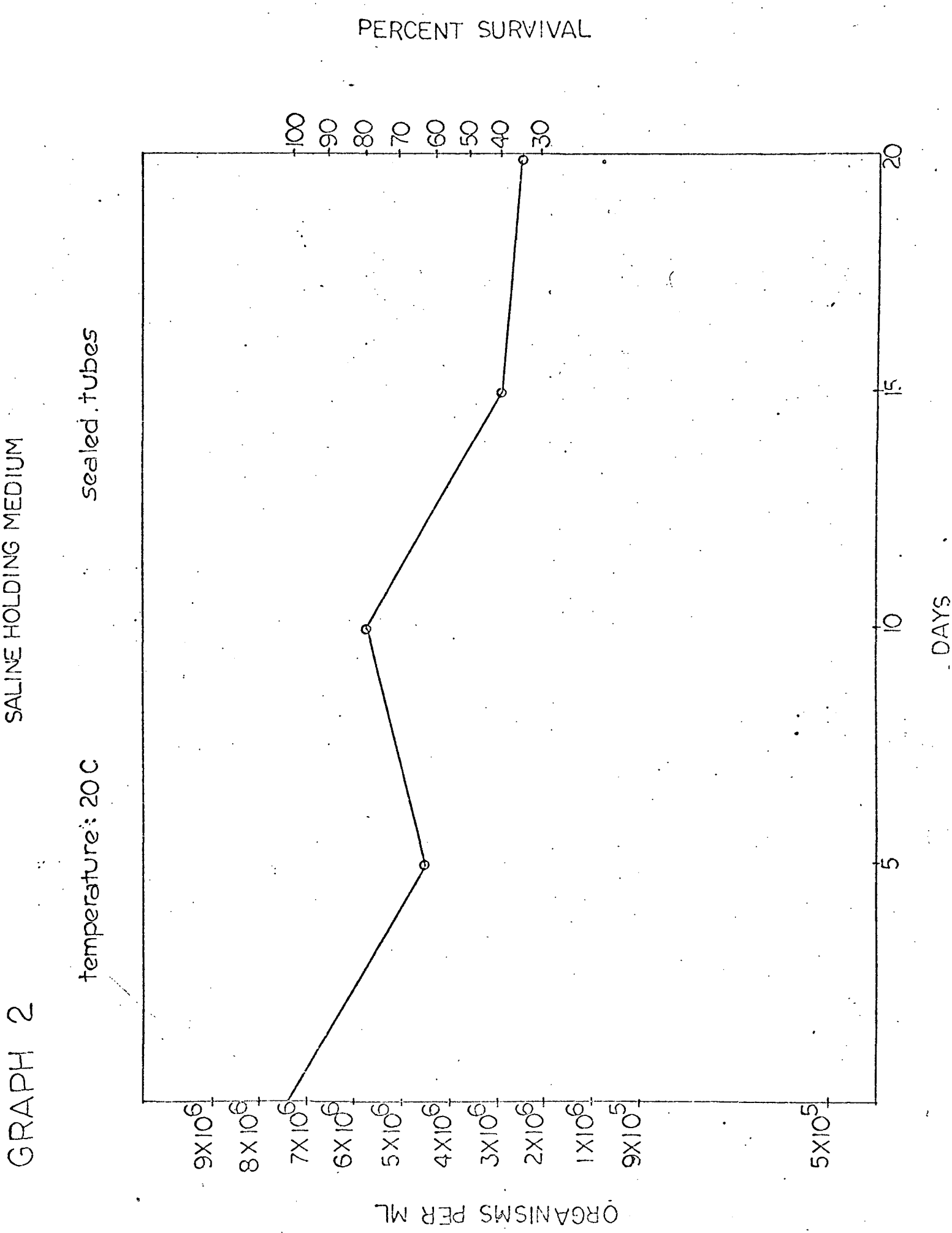
GRAPH 1'

SEA WATER HOLDING MEDIUM

temperature: 20 C

unsealed: -o-
sealed: -Δ-




PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
15
10
5
DAYS
ORGANISMS PER ML
9X10^6
8X10^6
7X10^6
6X10^6
5X10^6
4X10^6
3X10^6
2X10^6
1X10^6
9X10^5
5X10^5
SALINE HOLDING MEDIUM
sealed tubes
temperature: 20 C

GRAPH 2

TABLE I

Proteolytic Enzyme Production by Organisms Isolated From Apollo 12

Cultures marked a and b were grown in rotary and reciprocal shakers, respectively. Hemoglobin assays were not performed on cultures that gave a negative gelatin assay (≠). Growth was measured turbidimetrically and is expressed as absorbance at 660 nm.

Culture	Age of Culture at Harvest	Growth		Proteinase Units per ml		Proteinase Units per ml, average	
		a	b	a	b	a	b
Bacillus badius - EI_1b Pre-flight (#76) Harvest #1	46.0	0.52	0.51	Neg.	Neg.	Neg.	Neg.
Bacillus badius - EI_1b Pre-flight (#76) Harvest #2	46.0	0.57	0.48	Neg.	Neg.		
Bacillus sp. 1041 Pre-flight (#77) Harvest #1	46.0	0.69	0.41	0.07	0.02	0.08	0.03
Bacillus sp. 1041 Pre-flight (#77) Harvest #2	46.0	0.57	0.36	0.08	0.04		
Bacillus subtilis Pre-flight (#78) Harvest #1	46.0	0.98	0.49	0.08	0.30	0.07	0.3
Bacillus subtilis Pre-flight (#78) Harvest #2	48.0	1.11	0.96	0.07	0.1		
Bacillus subtilis Pre-flight (#78) Harvest #3	46.0	1.08	0.43	0.07	0.5		

Culture	Age of Culture at Harvest	Growth		Proteinase Units per ml		Proteinase Units per ml, average	
		a	b	a	b	a	b
Bacillus licheniformis -C12 Pre-flight (#79) Harvest #1	46.0	1.03	0.51	0.07	0.20	0.07	0.2
Bacillus licheniformis -C12 Pre-flight (#79) Harvest #2	48.0	1.11	0.61	0.07	0.19		
Bacillus unidentified -C9 Pre-flight (#80) Harvest #1	46.0	0.89	0.96	0.10	0.04	0.11	0.03
Bacillus unidentified -C9 Pre-flight (#80) Harvest #2	46.0	0.88	0.57	0.11	0.02		
Bacillus subtilis -C4 Pre-flight (#82) Harvest #1	46.0	0.89	0.74	0.07	0.18	0.07	0.17
Bacillus subtilis -C4 Pre-flight (#82) Harvest #2	48.0	0.88	0.48	0.07	0.15		
Bacillus cereus -36 Post-flight (#86) Harvest #1	46.0	1.08	0.81	Neg.	Neg.	Neg.	Neg.
Bacillus cereus -36 Post-flight (#86) Harvest #2	47.5	1.24	0.95	Neg.	Neg.		
Bacillus 1063 -28 Post-flight (#87) Harvest #1	46.0	0.46	0.30	1.14	1.24	1.12	1.16
Bacillus 1063 -28 Post-flight (#87) Harvest #2	47.5	0.52	0.27	1.09	1.07		

Culture	Age of Culture at Harvest	Growth		Proteinase Units per ml		Proteinase Units per ml, average	
		a	b	a	b	a	b
Bacillus subtilis -C8 Post-flight (#88) Harvest #1	46.0	0.68	0.44	0.18	0.28	0.16	0.25
Bacillus subtilis -C8 Post-flight (#88) Harvest #2	47.5	0.85	0.41	0.16	0.18		
Bacillus subtilis -C8 Post-flight (#88) Harvest #3	46.0	0.82	0.42	0.13	0.28		
Bacillus 1063 -34 Post-flight (#89) Harvest #1	47.5	0.64	0.34	0.36	0.17	0.37	0.17
Bacillus 1063 -34 Post-flight (#89) Harvest #2	47.5	0.67	0.30	0.38	0.17		
Bacillus 1063 -42 Post-flight (#90) Harvest #1	47.5	0.51	0.30	1.00	1.10	1.00	1.11
Bacillus 1063 -42 Post-flight (#90) Harvest #2	47.5	0.43	0.56	0.99	1.11		
Bacillus cereus -n27 Post-flight (#91) Harvest #1	47.5	1.16	0.89	≠	≠	--	--
Bacillus cereus -n27 Post-flight (#91) Harvest #2	47.5	1.17	0.86	≠	≠		
Bacillus 1041 -37 Post-flight (#92) Harvest #1	46.0	0.32	0.28	0.04	0.03	0.04	0.05
Bacillus 1041 -37 Post-flight (#92) Harvest #2	45.5	0.39	0.32	0.04	0.06		

Culture	Age of Culture at Harvest	Growth		Proteinase Units per ml		Proteinase Units per ml, average	
		a	b	a	b	a	b
Bacillus 1063 -46 Post-flight (#93) Harvest #1	46.0	0.61	0.35	1.12	1.14	1.13	1.17
Bacillus 1063 -46 Post-flight (#93) Harvest #2	46.0	0.58	0.41	1.14	1.20		
Bacillus 1030 -n26 Post-flight (#94) Harvest #1	46.0	0.65	0.50	Neg.	Neg.	Neg.	Neg.
Bacillus 1030 -n26 Post-flight (#94) Harvest #2	45.5	0.56	0.43	Neg.	Neg.		
Bacillus sp. unidentified Post-flight (#95) Harvest #1	46.0	0.77	0.50	0.14	0.05	0.14	0.05
Bacillus sp. unidentified Post-flight (#95) Harvest #2	45.5	0.70	0.58	0.13	0.05		

	a	b	All
Average all protease positive preflight (Hgb. units)	0.08	0.16	0.12
Average all protease positive postflight (Hgb. units)	0.54	0.54	0.54

TABLE II

Physical Properties of the Extracellular Endopeptidase and Aminopeptidase of *Aeromonas proteolytica*

Property	Endopeptidase[a]	Aminopeptidase[b]
Sedimentation coefficient ($s_{20,w}$)	3.50 S	3.12 S
Diffusion coefficient ($D_{20,w}$; $cm^2 sec^{-1} \times 10^7$)	8.52	9.29
Partial specific volume ($cm^3\ g^{-1}$)	0.709	0.724
Isoelectric pH	~ 3.5	~ 3.0
Molecular weight by:		
Approach to equilibrium	34,800	------
Equilibrium	------	28,480
Sedimentation velocity	34,800	29,500
Metal ion bound	Zn (1 g atom/mole)	Zn (2 g atom/mole)

[a] From Griffin and Prescott (10) and shown for sake of comparison.

[b] From Prescott *et al.* (11); supported in part by contract NASA - 7951 (reprint attached).

TABLE III

Amino Acid Composition of the Extracellular Endopeptidase and Aminopeptidase of *Aeromonas proteolytica*

Amino acid	No. of residues in	
	Endopeptidase	Aminopeptidase
Aspartic acid	52	30
Threonine	20	31
Serine	27	26
Glutamic acid	19	28
Proline	12	13
Glycine	34	19
Alanine	25	28
Valine	24	15
Half-cystine	4	2
Methionine	8	6
Isoleucine	6	12
Leucine	11	16
Tyrosine	22	12
Phenylalanine	18	9
Lysine	12	8
Histidine	6	6
Arginine	8	5
Tryptophan	8	5

TABLE IV

Twenty-Day Survival Study I

The cultures were held at 20°C in physiological saline solution (0.85% NaCl in demineralized water) to which 0.05 g K_2HPO_4 per liter had been added. The cell density of each culture was 7.5×10^6 organisms per milliliter; culture volume was 5 ml.

Day	% Transmittance at--				Organism Count	pH	Percent Survival
	253.7 nm	260 nm	280 nm	300 nm			
0	91.0	91.0	92.5	95.0	5.5×10^6	7.0	100
5	88.8	89.0	90.8	93.5	5.1×10^6	6.9	93
10	84.7	85.5	88.3	90.8	5.0×10^6	7.1	91
15	88.2	88.5	90.5	94.2	4.6×10^6	6.9	83
20	87.5	88.5	90.5	94.2	3.9×10^6	6.9	71

TABLE V

Twenty-Day Survival Study II

The cultures were held at 20°C in physiological saline solution (0.85% NaCl in demineralized water) to which had been added 0.05 g K_2HPO_4 per liter. Cell density, 4.4 x 10^6 organisms per milliliter; culture volume, 5 ml.

Day	% Transmittance at--				Organism Count	pH	Percent Survival
	253.7 nm	260 nm	280 nm	300 nm			
0	91.6	91.6	93.8	4.0	4.4 x 10^6	7.0	100
5	91.0	91.6	94.8	4.4	3.6 x 10^6	6.9	82
10	93.5	93.5	95.5	2.5	3.0 x 10^6	6.87	68
15	85.5*	89.0*	95.2	2.4	1.8 x 10^6	6.98	41
20	95.5	94.6	97.5	1.4	1.2 x 10^6	6.9	27

TABLE VI

Production of Toxic Substance and Hemolysin

by *Aeromonas proteolytica* on Nutrient Broth Medium[a]

Incubation Temperature	Incubation Condition	Incubation Time (Hr.)	Units of Endo-peptidase per ml	Guinea Pig Reaction[b]	Hemolysin Titer[c]
37°C	Stationary	24	0.00	Neg.	2
		48	0.00	2	2
		72	0.25	7	4
		120	0.35	10	4
	Shaker	24	0.48	8	2
		48	0.55	10	2
		72	0.55	8	2
		120	0.41	4	4
Room[d]	Stationary	24	0.27	Neg.	±[e]
		48	0.47	3	2
		72	1.11	8	±
		120	2.01	15	2
	Shaker	24	2.31	15	4
		48	1.68	8	4
		72	2.04	7	2
		120	2.18	7	2

[a] Made in 66% artificial sea water

[b] Hemorrhage measured in mm along greatest distance of scab

[c] Reciprocal of titer

[d] 25°C ± 2°C

[e] Equivocal in first tube

TABLE VII

Production of Toxic Substance and Hemolysin

by *Aeromonas proteolytica* on Trypticase Soy Medium[a]

Incubation Temperature	Incubation Condition	Incubation Time (Hr.)	Units of Endo-peptidase per ml	Guinea Pig Reaction[b]	Hemolysin Titer[c]
37°C	Stationary	24	0.31	Neg.	32
		48	1.52	4	8
		72	0.10	Neg.	32
		120	0.01	Neg.	16
	Shaker	24	0.32	Neg.	16
		48	0.13	Neg.	2
		72	0.96	Neg.	4
		120	1.25	9	2
Room[d]	Stationary	24	0.31	Neg.	16
		48	0.00	Neg.	64
		72	1.43	18	4
		120	2.84	18	8
	Shaker	24	1.99	15	4
		48	8.93	15	4
		72	8.89	15	4
		120	7.65	18	2

[a] Made in 66% artificial sea water

[b] Hemorrhage measured in mm along greatest distance of scab

[c] Reciprocal of titer

[d] 25°C ± 2°C

TABLE VIII

Production of Toxic Substance and Hemolysin

by *Aeromonas proteolytica* on 1% Peptone Medium[a]

Incubation Temperature	Incubation Condition	Incubation Time (Hr.)	Units of Endo-peptidase per ml	Guinea Pig Reaction[b]	Hemolysin Titer[c]
37°C	Stationary	24	.0.48	Neg.	2
		48	0.16	Neg.	2
		72	0.36	Neg.	2
		120	1.28	10	4
	Shaker	24	1.61	8	2
		48	2.87	13	2
		72	2.77	16	4
		120	4.00	14	8
Room[d]	Stationary	24	0.13	Neg.	Neg.
		48	1.82	10	2
		72	0.96	14	2
		120	2.20	8	2
	Shaker	24	3.65	20	8
		48	3.36	20	8
		72	3.18	20	8
		120	3.69	15	4

[a] Made in 66% artificial sea water

[b] Hemorrhage measured in mm along greatest distance of scab

[c] Reciprocal of titer

[d] 25°C ± 2°C

TABLE IX

Production of Toxic Substance and Hemolysin

by *Aeromonas proteolytica* on 5% Peptone Medium[a]

Incubation Temperature	Incubation Condition	Incubation Time (Hr.)	Units of Endo-peptidase per ml	Guinea Pig Reaction[b]	Hemolysin Titer[c]
37°C	Stationary	24	0.00	6	8
		48	0.78	7	2
		72	1.34	20	8
		120	1.24	12	4
	Shaker	24	4.18	15	±[e]
		48	7.50	25	±
		72	9.17	20	2
		120	6.68	15	2
Room[d]	Stationary	24	0.41	2	2
		48	1.83	20	2
		72	2.82	20	±
		120	2.02	15	±
	Shaker	24	4.60	50	8
		48	8.42	60	8
		72	19.21	70	8
		120	6.62	20	16

[a]Made in 66% artificial sea water

[b]Hemorrhage measured in mm along greatest distance of scab

[c]Reciprocal of titer

[d]25°C ± 2°C

[e]Equivocal in first tube

TABLE X

Production of Toxic Substance and Hemolysin

by *Aeromonas proteolytica* on Semisynthetic Medium[a]

Incubation Temperature	Incubation Condition	Incubation Time (Hr.)	Units of Endo-peptidase per ml	Guinea Pig Reaction[b]	Hemolysin Titer[c]
37°C	Stationary	24	0.00	Neg.	Neg.
		48	0.05	15	Neg.
		72	0.06	15	Neg.
		120	0.03	10	Neg.
	Shaker	24	1.16	Neg.	2
		48	0.87	2	Neg.
		72	1.02	2	±[e]
		120	0.36	Neg.	Neg.
Room[d]	Stationary	24	0.00	Neg.	Neg.
		48	0.00	Neg.	Neg.
		72	0.00	Neg.	±
		120	0.00	Neg.	2
	Shaker	24	0.86	8	2
		48	1.68	12	±
		72	1.59	10	±
		120	0.90	9	±

[a] Made in 66% artificial sea water

[b] Hemorrhage measured in mm along greatest distance of scab

[c] Reciprocal of titer

[d] 25°C ± 2°C

[e] Equivocal in first tube