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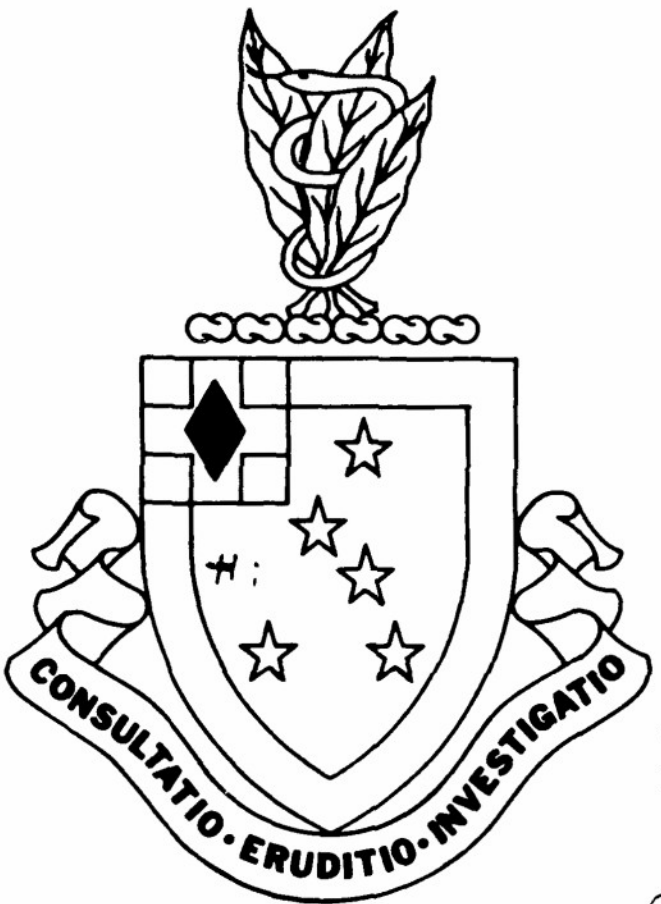
# ARMED FORCES INSTITUTE OF PATHOLOGY








## 1 JULY 1974 – 30 JUNE 1975

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## U.S.ARMY
## MEDICAL RESEARCH AND DEVELOPMENT REPORT
## RCS MEDDH-288 (RI)

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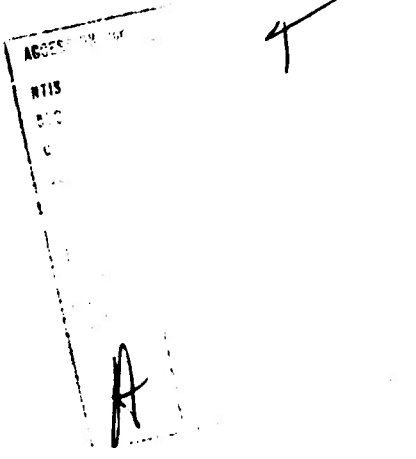

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Project: Army Aviation Medicine (3A762758A819)
Project: Military Animal Resources Development (3A762760A837)
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19.

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Exsheathment
Food
Infectious disease
In vitro survival and development
Laboratory animals
Military working dogs
Mycobacteria
Mycobacterium ulcerans
Occupant kinematics
Quantitative cytomorphology
Radiation
Sterilization
Toxin
Tropical disease

U.S. ARMY RESEARCH AND DEVELOPMENT

TECHNICAL REPORT

RCS-MEDDH-288 (R1)

ARMED FORCES INSTITUTE OF PATHOLOGY

WASHINGTON, D. C. 20306

ANNUAL PROGRESS REPORT

1 JULY 1974 - 30 JUNE 1975

TABLE OF CONTENTS

RESEARCH SUPPORTED BY:

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WASHINGTON, D. C.

| RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY | | | | 1. AGENCY ACCESSION* DA 0B 6606 | 2. DATE OF SUMMARY* 75 07 01 | | REPORT CONTROL SYMBOL DD-DR&E(AR)636 |
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11. TITLE (Precede with Security Classification Code)*
(U) Experimental Filariasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

| 13. START DATE | 14. ESTIMATED COMPLETION DATE | 15. FUNDING AGENCY | 16. PERFORMANCE METHOD |
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NAME: Garrigues, R. M., LTSG, MSC, USN

21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Experimental Filariasis, (U) Brugia pahangi, (U) Exsheathment (U) In vitro survival and development

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To initiate exsheathment and maintain viability of Brugia pahangi microfilariae with enzymes or balanced salt solutions; develop an in vitro system of tissue culture (TC) and/or media that would permit survival and development of B. pahangi from the $L_1$ to $L_3$ stages. Filariasis poses a potential threat if significant numbers of military personnel are stationed in one or more of the wide-spread endemic areas throughout the world.

24. (U) Microfilariae of B. pahangi were exposed to enzymes and balanced salt solutions to determine those parameters which govern the induction of exsheathment. Those larvae which exsheathed were subsequently incubated in selected mosquito cell TC and/or media for in vitro development.

25. (U) 7407-7506. In vitro microfilarial exsheathment has been accomplished to varying degrees with enzymes and modifications of Earle's balanced salt solutions. Completion of testing of the spectrum of selected exsheathment solutions has shown a wide range of stimulation. However, some of the active enzymes resulted in an 80% exsheathment rate with no detrimental effect on the exsheathed microfilariae. Of the development media tested, medium H-199 with serum, at 26C, provided the best conditions for survival after exsheathment and prior to introduction of the larvae into mosquito cell TC. Mosquito cell TC of Aedes aegypti Singh or Peleg cells augmented with serum and erythrocytes, at 26C, promoted moulting and development to the $L_2$ stage larvae between days 4 and 5. Subsequent development and survival continued for more than 21 days. A significantly greater per cent of larvae developed to the "sausage stage" in Peleg TC than in Singh TC.



*Available to contractors upon originator's approval

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3A161102B71Q Title: Experimental Filariasis

Task No. 00

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Medical Parasitology Branch

Period Covered by Report: 1 July 1974 - 30 June 1975

Professional Authors: Wayne T. Hockmeyer, CPT, MSC, USA
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Report Control Symbol: RCS-MEDDH-288(R1)

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BODY OF REPORT

Project No. 3A161102B71Q

Title: Development of Larval Forms of *Brugia pahangi in vitro*

Task No. 00

This work involved study of *in vitro* development of filarial parasites as a means to facilitate further study of the immunologic, physiologic and biochemical aspects of filarial infections. An aspect of this study involved determination of the triggering mechanism(s) of exsheathment prior to *in vitro* development of the larval stages.

## I. Experimental Exsheathment of Brugia pahangi Microfilariae

Development of the filarial parasite, *Brugia pahangi*, is complex and requires both an invertebrate and vertebrate host. Microfilariae undergo a complex developmental process in the mosquito vector resulting in the infective $L_3$ stage larvae. The developmental process initially requires the microfilariae to shed (exsheath) their cuticle. Normally this procedure occurs in the gut of the mosquito vector following ingestion of an infective blood meal.

The first step in establishing an *in vitro* maintenance system is to duplicate the exsheathing mechanism as it may occur *in vivo*. Microfilariae, isolated from infected dog blood, were treated with various solutions and enzymes at varying concentrations and temperatures in an attempt to induce *in vitro* exsheathment.

### A. Balanced Salt Solutions

The first solution examined for exsheathment stimulus potential was Earle's Balanced Salt Solution (BSS). Testing included different concentrations of BSS as well as single component deletions. Of concentrations consisting of 0.5X, 1X, 2X, and 5X, it was found that the higher the concentration, the lower the per cent of microfilarial exsheathment.

The 1X BSS concentration at 26 C stimulated exsheathment up to 30%. This rate was similar to the 35% reported for a taxonomically unrelated nematode, *Haemonchus contortus*, reported by Ozerol and Silverman (1969) although their exposure treatment was continuous for 24 hours at 37 C. The percentage similarity, despite the treatment temperature variance, is interesting in light of the variant ambient body temperatures of the hosts of the two parasitic nematodes involved (e.g., mosquitoes vs. sheep, etc.).

Since 1X BSS resulted in the highest per cent exsheathment this concentration was further analyzed to determine which of its chemical components were in fact contributing physiologic factors for the exsheathment

process. The components included NaCl, KCl, $CaCl_2$, $MgSO_4.7H_2O$, $NaH_2PO_4.H_2O$ and glucose. Deletion of NaCl or $CaCl_2$ significantly inhibited exsheathment by stimulating only 4 and 5% respectively of the microfilariae to react at 48 hours post exposure. Deletion of glucose or $NaH_2PO_4.H_2O$ were similarly inhibitory over a 72 hour test period. When $MGSO_4.7H_2O$ was deleted there was an insignificant reduction in the exsheathment rate. This was interesting because of its supposed conflict with the report by Rogers (1965) that Mg++ ions were required as cofactors for exsheathment activity. Our data suggests that the microfilariae, once placed in H-199 + 10% FeCASΔ which contains MG++ ions, were able to recover the Mg++ ions and maintain their requirement. It would seem that most of the ions contained in BSS, in addition to the Mg++ , are required for exsheatment and that glucose serves as an energy source.

B. Enzymes and Streptolysin-O

Enzymes with potlential exsheathment activity were examined singly and in selected combination at optimum pH and temperature.These included acid and alkaline phosphatase, collagenase, leucine aminopeptidase, saponin and trypsin. Exsheathment stimulation by acid phosphatase was highest (25%) at 0.1% concentration at 26 C. At 37 C it was 47% at 0.25% concentration. Total mortality prevented data collection after 5 hours post treatment.

The exsheathment activity associated with alkaline phosphatase appeared to be temperature dependent. Exsheathment was best (66%) at 0.5% concentration at 26 C, and at 37 C it was 58% at 0.1% concentration. Both readings were taken at 72 hours post treatment. That this effect was due to the alkaline phosphatase was confirmed utilizing the inhibition NaF. In this combination, only 6% of the microfilariae exsheathed.

Collagenase, leucine aminopeptidase, and saponin at both 26 and 37 C, and at all concentrations were ineffective. Maximum exsheathment with trypsin (0.5%) was 87% at 26 C and 77% at 37 C. Total mortality occurred by 24 hours post treatment. Streptolysin-O alone was ineffective as a stimulator.

In sequential combination, treatment with saponin, streptolysin-O and trypsin at 37 C resulted in a 52% microfilarial exsheathment rate. Effects of the different solutions on the viability of the microfilariae ranged from lethal to none.

II. In vitro Development of Brugia pahangi Microfilariae

Since no media or cell cultures have been reported as promoting development of B. pahangi to the "sausage stage," it was necessary to establish a system that would permit this development. Also, it was considered important to determine whether the per cent of exsheathed microfilariae would increase when they were placed in the development system.

Title: Development of Larval Forms of Brugia pahangi in vitro (page 3)

A. Exsheathment Rates Associated with the Development System

Exsheathment rates of the microfilariae treated with 2% alkaline phosphatase or 1X BSS at 26 C, held 24 hours in H-199 + 10% FeCasΔ, and subsequently introduced into cell cultures containing mouse L-929 or Aedes aegypti Singh or Peleg cells were 33% and 20% respectively for the mosquito cell cultures.

B. Larval Development

No larval development occurred in the cell cultures of the mouse L-929. In the A. aegypti Singh and Peleg cell cultures augmented with 5% erythrocytes and 10 and 15% FeCasΔ respectively, microfilariae developed and moulted to second state ($L_2$) larvae between days 4 and 5.

Development measurements at the "head" and anal pore of the microfilariae increased from 5.4 and 5.9μ respectively and length 280μ at 0 hours, to 12.6 and 15.3μ for the first two measurements and ± 200μ in length by day 5. Larval development data varied according to the type of exsheathment stimulation (e.g., 2% alkaline phosphatase vs. 1X BSS) and the mosquito cell culture type they were eventually placed on. Through day 25, there was no further significant increase in the width of the larvae at the "head" or anal pore. It was expected that the length would increase following the initial shortening associated with "sausage stage" development, however, the length continued to decrease through final measurements on day 25. Length measurements for those larvae exsheathed with 2% alkaline phosphatase and transferred to Singh or Peleg cell cultures were 177 and 193μ respectively. Larvae treated with 1X BSS and transferred to Singh or Peleg cells decreased in length to 153 and 144μ respectively. The difference in larval lengths for the two exsheathment treatments by day 25 suggests that an as yet unidentified development mechanism is deleteriously affected by 1X BSS during the initial exsheathment exposure.

Ten per cent of the larvae developed to the "sausage stage" in Singh cell cultures while only 5% of the larvae in Peleg cell cultures reached the same stage of development.

Results to date indicate that we can initiate rapid in vitro exsheathment - the first step in the developmental process of B. pahangi larvae. Once exsheathed, the larvae proceed to develop to the $L_2$ stage in A. aegypti Singh and Peleg cell cultures.

Further studies will determine those biochemical or physiological requirements necessary to sustain the larvae in development through the $L_2$ larval stage to infective $L_3$ larvae.

Literature Cited:

Ozerol, N. H. and P. H. Silverman. 1969. Partial characterization of Haemonchus contortus exsheathing fluid. J. Parasitol., 55 (1): 79-87.

Rogers, W. P. 1965. The role of leucine aminopeptidase in the moulting of nematode parasites. Comp. Biochem. Physiol., 14(2):311-321.

Following is a list of Publications during the last fiscal year:

Hockmeyer, W. T., B. A. Schinter, B. R. Redington, and B. F. Eldridge. 1975. Brugia pahangi: Effect on the flight capability of Aedes aegypti. Exp. Parasit. 38: 1-5.

Garrigues, R. M., W. T. Hockmeyer, and J. C. Balinas. 1975. Development of larval forms of Brugia pahangi in vitro. J. Parasitol., 61(3,supl.): IN PRESS.

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| 3. DATE PREV SUMRY 74 07 01 | 4. KIND OF SUMMARY H.Termination | 5. SUMMARY SCTY* U | 6. WORK SECURITY* U | 7. REGRADING* | 8. DISP'N INSTR'N NL | 8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO | 9. LEVEL OF SUM A. WORK UNIT |

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11. TITLE (Precede with Security Classification Code)*
(U) Isolation and Characterization of a Toxin Produced by Mycobacterium ulcerans

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

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19. RESPONSIBLE DOD ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
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TELEPHONE 202-576-2993
SOCIAL SECURITY ACCOUNT NUMBER [redacted]

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ASSOCIATE INVESTIGATORS
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NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Infectious Disease, (U) Tropical Disease, (U) Mycobacteria, (U) Mycobacterium ulcerans, (U) Toxin, (U) Armadillo

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop an effective treatment for Mycobacterium ulcerans infection by purifying and characterizing the toxin produced by M. ulcerans; developing an anti-toxin; testing hyperbaric oxygenation, as a potential therapeutic procedure, and by further evaluating the armadillo as a suitable experimental model. The impact of this disease on troops deployed in an endemic area is indicated by the fact that 9% of a migrant population became infected.

24. (U) Mycobacterium ulcerans cultures are grown in vitro and fractionated. The toxin is purified by potassium bromide isodensity ultracentrifugation and column chromatography. The toxicity is tested by inoculation of tissue culture cells and guinea pigs. Characterization of the toxin includes determination of molecular weight, chemical composition, heat and pH stability and enzyme sensitivity. Antitoxin is prepared by inoculating purified toxin into rabbits and isolating the serum fraction. The serum is used for therapeutic treatment of infections and prophylaxis in guinea pigs and armadillos. Mice are treated by hyperbaric oxygenation and the degree of infection in the treated mice is compared to positive controls (infected, not treated). Studies with the armadillo include determining the effect of route of inoculating dose, different strains of M. ulcerans, source of inoculum, age of the animal and environmental temperature.

25. (U) 7407-7506. Mycobacterium ulcerans produces a toxin which causes inflammation and destruction of guinea pig skin, cytopathic effects in mouse fibroblast cells, and is an important factor in the pathogenesis of the human infection. Hyperbaric

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oxygenation has a beneficial effect in mice infected with M. ulcerans. The treatment does not cure the disease but does delay the onset of symptoms and decrease the number of amputations and deaths. The infection in the armadillo is very similar in extent and appearance to naturally occurring human infections and the armadillo appears to be the best experimental model for studying the pathogenesis of this infection.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3AT61102B71Q Title: Isolation and Characterization of a Toxin Produced by Mycobacterium ulcerans

Task No. 00

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D.C. 20306

Name of Department: Microbiology Branch

Period Covered by Report: 1 July 1974 - 30 June 1975

Professional Author: Richard E. Krieg, Jr., Capt, USAF, BSC

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BODY OF REPORT

Project No. 3A161102B71Q Title: Isolation and Characterization of a Toxin Produced by Mycobacterium ulcerans

Task No. 00

Infections of humans by Mycobacterium ulcerans cause extensive necrosis of skin and subcutaneous tissue. Although the mechanism of this necrosis is not known, M. ulcerans in vitro produces a toxin which causes inflammation and necrosis of guinea pig skin and cytopathic effects (rounding of cells, enucleation) in mouse fibroblast cells. The organism was grown in Dubos broth and the culture was fractionated into culture filtrate, cytoplasmic fluid, particulate fraction, and cell wall. The changes in the guinea pig skin produced by intradermal injection of toxin fractions resemble the early changes in naturally occurring human infections. In both there is edema, calcification, loss of somatic cell nuclei, ulceration, reepithelization of the margin and a spreading contiguous necrosis involving full thickness of skin. When viable organisms are inoculated, a similar response is produced, although the infection is self-limiting. Fractions and viable cells of 15 other species of mycobacteria did not cause the same effects in guinea pig skin and did not produce cytopathic effects in mouse fibroblast cells.

The toxin is present in the culture filtrate, cytoplasmic fluid and the particulate fraction. It was retained by an ultrafiltration membrane with a molecular weight retention of 50,000 (XM 50). The toxin was separated from proteins present in the culture filtrate by preparative ultracentrifugation on a potassium bromide isodensity gradient. The top fraction from this ultracentrifugal run was toxic; the bottom fraction was not. The top fraction contains lipoprotein, lipids, carbohydrate, or carbohydrate containing material. The toxin was moderately acid labile but not affected by alkali; its activity was destroyed by pronase but resistant to trypsin and it is heat stable. The material is susceptible to reduction with 2-mercaptoethanol but not periodatcn. The results indicate that the toxin is a lipoprotein.

In addition to the guinea pig; mice, rats and mystromys have been inoculated with viable organisms in attempt to find a suitable experimental model for the infection. The infections in these other rodents follow essentially the same course. After inoculation in the footpad, it becomes swollen and red, the leg swells, hair falls out and there is diffuse crusting and weeping. Eventually the entire leg may slought or autoamputate. Histologically the infection in the rodents is characterized by severe edema, an acute inflammatory reaction and necrosis. The characteristics of the infections in these rodents and the guinea pig are distinctly different from the human infection and thus they are not ideal models for comparison with human infections.

The nine banded armadillo is susceptible to infection with Mycobacterium ulcerans. Two of the first four animals inoculated on the medial side of both thighs developed ulcers at the site of inoculation. The ulcers were very similar in extent and appearance to naturally occurring human infections. In both there is extensive necrosis, undermining of the margins of the lesions and a spreading contiguous necrosis involving full thickness of skin. Thirty-four additional armadillos have been inoculated to determine the effect of route of inoculation (intravenous, intraperitoneal, intradermal), dose, different strains of M. ulcerans, source of inoculum (isolated from infected mice footpads and grown in Dubos media), age of the animal, and environmental temperature. Animals inoculated by routes other than intradermal do not develop the typical ulcerated lesion. Five months after inoculation the animals died and were edematous with evidence of hemorrhage.

With high doses of bacteria ($10^{9}$-$10^{11}$) inoculated intradermally, small preulcerative nodules developed, receded, erythematous areas developed on the thighs, animals became edematous and died. The source of inoculum, strains, age of animals and environmental temperatures had no effect on the course of infection.

Chemotherapy has not been effective in the treatment of M. ulcerans lesions and extensive debridement followed by skin grafting is the treatment of choice. This often results in disfigurement, contraction deformity and amputation. There are other procedures which have promoted healing; heat treatment, rifampicin; but have not been completely evaluated. Hyperbaric oxygenation (HBO), an effective treatment for many bacterial diseases, including some mycobacterial infections, was used to treat mice with M. ulcerans infected footpads. Three groups (40 mice/group) were treated daily with 100% oxygen by three different protocols: 2 1/2 atmospheres pressure (2 1/2 Ata) for 2 hours; 2 1/2 Ata for 1 1/4 hours, twice a day; and 2 Ata for 3 1/2 hours. The degree of infection in the treated mice was compared weekly with 40 positive controls (infected, not treated). The HBO therapy was most effective in the group treated at 2 1/2 Ata for 1 1/4 hours, twice a day. After 20 weeks, no feet autoamputated and only 5 mice died as compared to 15 feet amputated and 18 deaths in the control group. Hyperbaric oxygenation has a beneficial effect and if used in conjunction with other therapeutic procedures, it may be an effective means of treating M. ulcerans infections.

Current studies include purification of the toxin, additional biochemical and biophysical characterization, elucidation of its antigenicity and development of an antitoxin. The armadillo will be further evaluated as a suitable experimental model. These studies will include determining the effect of inoculation of purified toxin, determining the armadillos response to infection, and testing the effect of hyperbaric oxygenation on infected animals following a more thorough analysis of this treatment with infected mice. Mice will be treated by hyperbaric oxygenation, heat, and rifampicin

alone and in all possible combinations. The successful completion of these studies will lead to a more thorough understanding of the disease, better control and preventive measures and earlier and more effective treatment to prevent the late sequalae of contraction deformity, lymphadema and amputation.

Following is a list of publications and/or presentations during this reporting period:


Hockmeyer, W. T., R. E. Krieg, R. D. Johnson, and R. Attanasio. 1975 Characterization of the Toxin of Mycobacterium ulcerans. Bacteriological Procedings, 75:21

Krieg, R. E., W. T. Hockmeyer, and D. H. Connor. 1974. The Toxin of Mycobacterium ulcerans (Production and effects in guinea pig skin). Archives of Dermatology, 110:783-788.

Krieg, R. E., G. P. Walsh, E. E. Storrs, H. P. Burchfield, and J. P. Heggers. 1975. Experimental Infection of Armadillo with Mycobacterium ulcerans. Bacteriological Proceedings, 75:24

Krieg, R. E., J. H. Wolcott, and R. D. Baker. 1975. Hyperbaric oxygenation of Mycobacterium ulcerans infection. Bacteriological Proceedings, 75:4

| RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY | 1. AGENCY ACCESSION | 2. DATE OF SUMMARY | REPORT CONTROL SYMBOL |
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| 10. NO./CODES: | PROGRAM ELEMENT | PROJECT NUMBER | TASK AREA NUMBER | WORK UNIT NUMBER |
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| a. PRIMARY | 6.27.20.A | 3A762720A835 | 00 | 150 |
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11. TITLE (Precede with Security Classification Code)

(U) Analysis of Cytotoxic Reactions Produced by MUST-Water Constituents

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

| 18. RESOURCES ESTIMATE | | a. PROFESSIONAL MAN YRS | b. FUNDS (In thousands) |
|---|---|---|---|
| FISCAL YEAR | PRECEDING 75 | 1.2 | 42 |
| | CURRENT 76 | 1.2 | 11 |

19. RESPONSIBLE DOD ORGANIZATION

NAME: Armed Forces Institute of Pathology

ADDRESS: Washington, D. C. 20306

RESPONSIBLE INDIVIDUAL

NAME: Hansen, J. L., COL, MC, USA

TELEPHONE: 202-576-2905

21. GENERAL USE

20. PERFORMING ORGANIZATION

NAME: Armed Forces Institute of Pathology

ADDRESS: Washington, D. C. 20306

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Bahr, G. F., M.D.

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Boccia, J. A., LTC, MC, USA

NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Cytologic Water Purity Assay, (U) Quantitative Cytomorphology, (U) Analysis of Cytologic Images, (U) Cytologic Pattern Recognition

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Develop cytoassay for detection of MUST-water impurities through extracting quantitative morphologic descriptors from digital images of cells cultured in waters tested. Method should extend to general water testing. At close of CY76, U. S. Army to possess mini-computer system and protocols for water toxicity cytoassay. Implication: reduce need for animal toxicologic testing. Desired result: higher and more quantitative data rates; lower costs; simpler laboratory logistics. Application to MUST re-use water primary; extension to water testing where military presence is possible, probable, or actual within applicability. Military-industrial effluent cytoassay an important consideration.

24. (U) Purchase upgrade for mini-computer hardware and software. Upgrade image digitizing light microscope subsystem. Design and fabricate (D&F) electronics for interfacing/control of scanner to/by mini-computer. Generate application specific programs. Standardize culture and staining procedures. Run feasibility experiments with raw water and water with known toxic additives; technical problems; standardize culture in presence of toxicants/raw water; theoretically develop and implement quantitative estimator of staining variability in contrasts and intensity; pre-analysis validation of image data; extract cell image descriptors of cytopathologic value which sensitive to exposure time and toxicant concentration; production of adequate experimental throughput on modest mini-computer system. Decelerating factors: procurement delays and defects in new equipment and software. Accelerating factor: addition of 1 man-year/year of professional effort, starting FY75.



*Available to contractors upon originator's approval

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY (DD Form 1498) (Page 2)

Title: (U) Analysis of Cytotoxic Reactions Produced By MUST-Water Constituents

Project No. 3A762720A835

25. (U) 7407-7406. Upgrade of equipment completed (7 mos). Design of electronics completed (4 mos) and fabrication presently 75% complete (3 mos). D&F instruments enabling virtual coordinate imposition and microscope slides completed (6 mos). Generation of program subsystem for building cell image date files and validation complete (2 mos). Initial selection of culture and staining technique complete (9 mos). In progress: final electronics fabrication; further culture standardization; continued software generation. Expected date of system completion and initiation of systematic feasibility testing is 1 October 1975.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3A762720A835 Title: Analysis of Cytotoxic Reactions Produced by MUST-Water Constituents

Task No. 00

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Cellular Pathology

Period Covered by Report: 30 September 1974 - 30 September 1975

Professional Authors: Gunter F. Bahr, M.D.
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Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 3A762720A835 Title: Analysis of Cytotoxic Reactions Produced by MUST-Water Constituents

Task No. 00

A. TECHNICAL OBJECTIVES

1. The development of a cytoassay for toxicity in MUST-Water. This assay is to be based upon the measurement of changing morphologic parameters in cells cultured using media diluted with the waters to be tested. Only quantifiable geometric, textural, or spectral descriptors of cytopathologic value are to be employed. The principal criteria of merit to be used in the analysis of feasibility are: sensitivity to concentration and type of toxicant, time-rate of morphologic response of cells to the presence of toxicants in culture media, and reproducibility (precision) of test results for any given water sample.

2. The development of procedural protocols for the laboratory assessment of cytotoxicity which are extendable to water testing in general.

3. By the close of CY-76, the U.S. Army be in possession of a research laboratory, minicomputer based system for the cytoassay of water toxicity. This system is to include instrumentation, minicomputer hardware, software, and laboratory protocols for the culturing, staining, and microscopy of cell types used in testing. Such a system is to be applicable to MUST-Water, but should extend to water testing in general. No dependence upon the source of water samples will be designed into this pilot system.

4. It is the feasibility as measured by such principal criteria as sensitivity, rapidity of cellular response, and reproducibility which are the objectives of this project. Further, a broad spectrum screening test is being sought, not one which is either quantitatively or qualitatively specific for a particular molecular species of toxicant. The potential application of a successful cytoassay is in situations where no a-priori knowledge of water contaminants may be presumed.

5. While chemical specificity and quantitation is beyond the scope of the proposed methodology, a correlation between the degree of quantitatively measured morphologic alteration in test cells and the concentration of cytotoxic water contaminants is sought. Such a correlation would open the possibility of setting level-of-use (e.g., potable, external use only, unusable, etc.) to a given water supply. Further, the response of a water supply to purification procedures might be monitored.

6. Chemical analysis and whole animal toxicity testing are not among the technical objectives of this project. The design of a field deployable system for water testing is beyond the scope of this project.

## B. HYPOTHESIS

It is possible to detect prenecrotic, quantitative morphologic alterations in cultured cells exposed to noxious water contaminants. This response is graded and proportional to the concentration of toxicants present in the sample. It is possible that cellular response is reversible by removal or dilution of contaminated water in culture media.

The means by which toxicity detection may be carried out is digitization of cellular light microscopic images after they have been suitably and reproductibly fixed and stained. Quantitative morphologic measurements are made on each digital image, and the values for cells exposed to clean water controls (distilled water) compared to those for cells exposed to various dilutions of the test sample. Upon comparison with controls, the values for morphologic descriptors will exhibit significant differences and trends which correlate with the concentration of contaminants. These differences and trends provide a basis for the cytoassay of water toxicity.

As stated in the previous two paragraphs, both the ends and means of cytoassay for water toxicity are under scrutiny in this project. The feasibility of such testing is contingent both upon cells' ability to react and the testing system's ability to detect any reaction by cells.

## C. MILITARY SIGNIFICANCE

The immediate significance is support of USAMRDC Environmental Quality Research pertaining to the medical aspects of water supply, waste disposal, sanitation, and occupational health for an army in the field. Particular application to MUST-Water is an objective.

A successful cytoassay for water toxicity has application to any water sample. Water quality of military significance includes: military-industrial wastes, water shed quality in geographic areas of potential or actual military presence.

A cytoassay based upon quantitative morphologic alteration would not yield chemically specific results. However, chemical specificity is not the intention in its design. Proper application of a cytoassay would be in circumstances where independence of the specific chemical entities which might be contaminants is either desirable or necessary. Such a circumstance would arise when the spectrum of possible water contaminants is unknown and/or time does not permit its determination. Another is when the biological effect of a water sample, toxicants known or unknown, is of primary importance. A chemical assay alone provides no prognostication on

biological effect. Correlative studies in a biologic system using an identified substance must be performed. Whole animal toxicity studies are relatively cumbersome, expensive, time consuming, slow, and yield fewer data than a successful cytoassay method.

## D. RESEARCH AND DEVELOPMENT PLAN

The basic experimental approach is to expose test cells in culture to graded dilutions of water samples for increasing periods of time. A group of cells cultured against a given dilution of water sample for a particular time interval will be termed a cell population. When a cell population is removed from culture it is immediately fixed and stained. After mounting with coverslip on an ordinary glass light microscope slide, a subpopulation of cells is selected and subjected to scanning microdensitometry.

Scanning microdensitometry performed on a cell yields a rectangular array of optical density values. Each value represents the density of a minute portion of the cell and the points at which these measurements are taken are equally spaced with high precision. The entire array of values is called a raster and is comprised of M rows and N columns of values, where M and N are large enough to encompass the entire cell with a small rim of slide background. Each point in the raster is called a pixel which is a contraction for "picture element". The distance between pixels is the interpixel distance (IPD). Another term for this raster of density values, especially when it was obtained by scanning an object of interest, is digital image.

Such images are termed digital because they are in two ways quantitized versions of the object they depict. First, there is spatial quantitization, since what appears as a spatially continuous image is sampled for optical density at discrete points. Secondly, the range of obtainable density values is quantitized to a suitably fine, but none the less finite, number of admissible values. Digital images are appropriate for transmission to, storage on, and analysis by digital computers and their common peripheral devices such as magnetic tape and disc units.

Digital images may be analysed by computer for size, shape, and textural properties of structures they contain. These properties may also be analyzed as a function of the spectral bandwidth of the illumination used in the scanning process.

Since digital image analysis relies more upon the relationship between pixel values and their spatial distribution than upon the absolute magnitude, the use of terms like density or transmission when referring to pixel values is needlessly specific. Any measure of pixel optical weight which varies in a determined manner with the scene brightness at each pixel location may be used to create a digital image. However, since optical density value is the measure of brightness used in this project, continued use of the term will be made in the sequel. More generally applicable terminology is grey level or grey value.

Scanning microdensitometry will be used to produce digital images from selected members of culture cell population. The spatial quantitization will be at an interpixel distance (IPD) of 0.5μ and density quantitization will be to 186 parts (optical density range of 0.00 -1.85).

A battery of numerical morphologic descriptors is then computed from the image of each cell scanned. These descriptors are called features. Some features are quantitative analogues of traditional cytopathologic indicators of cell injury. Others have no humanly observable equivalent. In the former category are cell area, nuclear area, cell perimeter edge length, nuclear perimeter edge length, granularity of cytoplasm, granularity of nuclear chromatin, total cytoplasmic density, total nuclear density, regularity of cell shape, regularity of nuclear shape, cell convex hull, and nuclear convex hull.

Features which have not been traditionally accessible by unaided human microscopic examination are frequency distribution of cell, cytoplasmic, and nuclear pixel densities, geometric center of cell, geometric center of nucleus, center of optical gravity (using density as measure of optical mass) for both cell and nucleus, density transiton probability matrix. The last is a complex feature and constitutes the empirically estimated conditional probability of transition from one density class interval to another by pixels spatially separated a designated number of positions.

Features enumerated in the previous two paragraphs do not exhaust the possibilities. They are given as an indication of the range of morphologic properties which may be extracted from each cell. In case a feature has an unaided, humanly observable analogue, the difference is this methodology makes a quantitative assessment of the morphologic parameter. Therefore, comparisons among cells for each such parameter can be made objectively. The same is true for features with no human analogue, except that here no assessment or comparison can be performed by unaided human observation.

The act of computing features from the digital image is called feature extraction. Features will be extracted from each cell selected from a population and the data is recorded. It should be added that the digital images as well as feature data shall be permanently recorded on magnetic tape.

In each experiment, the feature data computed will be compared among the various cell populations which differ in the dilution of water sample to which the members have been exposed and/or the time of exposure in culture. The experimental design in each case is a two factor treatment by level of dilution and exposure time. After an appropriate two-way analysis of variance is performed, dose and time response curves are plotted.

For each feature extracted, a cell population's response to water sample dilution and time of exposure in culture can be assessed by the frequency of outlyers from an N% condifence interval for the feature value in the

corresponding control population. The higher the frequency, the earlier it is manifested, and the higher the dilution of water sample at which it is manifested-the more sensitive is the feature as an indicator of cytotoxicity. An appropriate value for the level of confidence, N, is the lowest value which will not generate frequencies of outlying the confidence interval for controls resulting in an unacceptably high risk of false positivity for cytotoxicity based on use of the single feature. Too high a value for N would result in a loss of sensitivity for the feature. Generally, a trade-off between sensitivity and false positives must be made. This trade-off is made in the selection of N for each feature.

From the set of all features computed is to be selected a subset of those showing greatest sensitivity at a cost of least false positivity. This evaluation is based upon dose and time response data to be developed. A single composit descriptor of cytotoxic effect may be created by forming a linear combination of the features in this most sensitive subset of those originally evaluated. This linear combination is written:

$$F(c,t) = w_1(c,t)f_1(c,t)+\ldots+w_K(c,t)f_K(c,t),$$

where $f_k(c,t)$ for $k = 1,\ldots K$ are the members of the most sensitive subset of features and evaluated at water sample dilution c with exposure for time t in culture. The values of the $f_k(c,t)$ represent the frequency (expressed fractionally) with which the particular feature lay outside the $N_k(0,t)$% confidence interval for the corresponding control cell population exposed to a 0:1 dilution of the water sample for time t in culture.

The $w_k(c,t)$ are weights assigned to each feature at dilution c and time t in culture. The $w_k(c,t)$ will be assigned values in the interval $[0,1]$ in accordance with a strategy which increases weight directly with sensitivity of the feature and inversely with its propensity to yield false positives for cytotoxicity. Sensitivity and false positivity for each feature can be assessed by trial against water samples containing known cytotoxicants at well defined concentrations.

It should be noted that, since $0 \leq f_k(c,t) \leq 1$ and $0 \leq w_k(c,t) \leq 1$ for $k = 1,\ldots K$ where K is number of features in the selected subset of all features available,

$$0 \leq F(c,t) \leq K.$$

If this cytoassay methodology is successful, it will result in a scale for the measure of cytotoxicity in the range of $[0,1]$. With sufficiently standardized procedures, not only detection of cytotoxicity in individual water samples would be possible, but also the monitoring of water supplies and effluents over time. A case in point would be comparison for cytotoxicity before and after the application of purification procedures to a given water supply.

To enable the experimentation and analysis necessary to these objectives the following capabilities must be developed.

1. Test cell(s) must be selected for use in tissue culture and a culturing technique must be settled upon which will yield certain results. The test cell(s) must react sensitively to the presence of toxicants in the culture. It must be of a size amenable to rapid image digitization with reasonable storage space requirements in a digital minicomputer system of modest size. This applies to storage requirements for both main memory and auxilliary memory containment of the digital images. A cell not larger than 50μ in maximum diameter is ideal. The cell and culture technique used should permit growth in a monolayer short of confluence for a period of four days from initiation. Removal from culture of a cell population must be easily accomplished. It is desirable that cell morphology manifest measurable changes within one day or less. Cellular morphologic response to toxicants should be graded and not all-or-none in nature. There must be sufficient cell growth, though in a possibly injured state, to permit efficient location of cells for observation and image digitization.

2. A standard fixation and staining technique must be used. This technique must be capable of rendering a reproducible staining effect in terms of staining intensity contrast between slide backround vs. cytoplasm, and contrast between cytoplasm vs. nucleus. A measure of staining effect should be developed which is accessible by the same microscope densitometer which digitizes the cell images. This check should be run on the on-board control populations included in every experimental run. This assures comparability of results from different experiments in which the cells are not stained together-or even with the same batch of reagents and dyes.

3. A self-contained, in-laboratory, minicomputer based image acquisition and analysis system must be developed by upgrading a previously existing, off-line, non-self contained one. This system should have the capability to acquire and analyse the images of from 1200-2500 test cells per experiment in a period of approximately six weeks. This is a proposed research rate-not a production capacity. The scanning microdensitometric subsystem must be fast and reliable enough to meet these requirements. Data should be transmissible directly to computer storage devices, whence it may be fetched for analysis. The system must have the capability for display of images and associated data-both intermediate and final. The mincomputer subsystem, hardware and software supplied by its manufacturer, must efficiently support the very development of which it is a part.

4. Every successful bench-top procedure, hardware development, software technique, and experimental run must be adequately documented to assure, not only a demonstration of feasibility (or lack of it), but also transferability of a successful outcome to other Army activities.

## E. PROGRESS

At this writing, the test cells selected for use are the Mouse L-cell line and the WI-38 human fibroblast. The former cell line had been in use by the Kettering Laboratory, University of Cincinnati Medical Center, at the time this project was initiated in October 1974. The WI-38 human fibroblast was selected for additional trial in this laboratory because it is diploid and of human origin. Both species are relatively easily grown.

Only the Mouse L-cell can be digitized within a raster of 100 x 100 dimensions. (The WI-38 human fibroblast varies between 75-100μ in maximum diameter. This would require 150 x 150 pixels per digital image.) This corresponds to a maximum diameter of 50μ and makes storage on and manipulation by the laboratory minicomputer subsystem feasible. The microscope scanner's measuring rate is 60 pixels/second at an IPD of 0.5μ. An upper bound estimate of scanning time for a raster of these dimensions is therefore 2.8 minutes. This does not include search time to locate a cell or to perform various editing operations described subsequently.

### 1. IMPROVED STEPPING STAGE

This scanning rate constitutes an improvement in speed of 400% over what existed previously. The obsolete stepping stage with which the scanning microscope was equipped stepped at 30 0.5μ displacements per second. Further, the design of processing software on the AFIP IBM 360/30 computer was such that the images which were submitted in local batch mode (the AFIP 360/30 is exclusively batch oriented) must have each scan row in left to right order. Since the digital image was recorded directly on magnetic tape, with no intervening preprocessing, the order of pixels in a scan row was the order in which they were measured. Consequently, a comb scan pattern was utilized in which each scan row was measured at 30 steps/second, then backstepped over. Upon return to the left hand margin of scan, the specimen was stepped to the next row and the process repeated. This means that each pixel location is passed over twice-once to measure and once upon return. The effective scanning rate is therefore not even 30 steps/second, but in reality 15 steps per second.

On-line data acquisition with the upgraded system permits a meander pattern of scan. No motion is wasted. Further, a newly acquired stepping stage with maximum speed of 200 0.5μ steps per second will allow the specimen to be moved at a rate commensurate with the microdensitometer's speed-which has always been 60 measurements per second.

### 2. FURTHER IMPROVEMENT OF SCANNING RATE AND QUALITY BY JOYSTICK PRE-EDITING

Additional low cost speed is purchased by stepping at full throttle with the new stage (Carl Zeiss, Inc.) over pixels in the rectangular raster where there is no cell of interest. These points will be traversed at the rate of 200 steps per second. Because data acquisition is being brought on-line, the

scanning operator may indicate to the scanning program in the minicomputer a scan boundary within which the cell lies. This boundary is then framed by a tight rectangle which dictates the gross dimensions of scan. Only pixels interior to both the circumscribed rectangle and the scan boundary are measured at the rate of 60 per second. All other points are traversed at 200 steps per second and assigned the value of zero.

Note, this procedure also eliminates data from near-lying cells which formerly and unavoidably found its way into the image of a cell being digitized. A similar statement is true for near-lying debris on the slide. Effectively, these unwanted data are pre-edited from the digital image. This constitutes a major departure from procedures commonly used on many current image analysis systems-even those which are on-line. The usual procedure is to post-edit. That is, the image is digitized uncleaned. Then, usually using a cathode ray tube terminal (CRT), the digital array of values or a graphic representation is cleaned interactively by light pen, data tablet, or some other equivalent graphic device. The disadvantages of this technique are: 1) the system must bear the cost of a CRT and supporting software; 2) cleaning and scanning occupy separate computer sessions (frequently at separate computer installations)-this is time consuming and decreases throughput; 3) the image facsimile being cleaned is usually a course approximation to the cell as seen through the miscroscope-cleaning must be accomplished with the aid of photographs or other visual aids and frequently results in editing uncertainties.

The pre-editing capability being built into the system for this project permits cleaning of the digital image before it is taken. This is accomplished by use of a Pulsar-639 (Measurement Systems, Inc., Norwalk, Conn.) isometric, digital output joystick. The joystick is used to move the eyepiece crosshair on the microscope over trajectories determining scan, cell, and nuclear boundaries. These are stored in computer memory.

The running program reads the joystick activity at approximately 5 millisecond intervals. Directional information is thereby transmitted to the computer which then moves the microscope stage in the direction indicated. In the process, the co-ordinates of each position traversed are stored in memory for use during scanning.

With these factors considered, a scanning time of 2.8 minutes per cell is deceptively conservative. By moving at 200 steps per second over pixels not overlying the cell of interest, an entire raster of approximately 100 x 100 pixels need not be traversed at 60 steps per second-which is the rate that yields a scanning time of 2.8 minutes. Depending upon how many points are outside the scan boundary, this time will be reduced conceivably as low as 2.0 minutes per scan, but this will vary from cell to cell. To these considerations must be added the weeks it takes to post-edit images numbering even several hundred when time must be awaited for use of a batch

computer. The time savings here dwarf those of increased scanning speed. Lastly, the quality of editing should be improved due to having a better "picture" to work with.

## 3. IMPROVEMENT OF SCANNER-HARDWARE AND SOFTWARE

To enable the procedures discussed above, a 200 step/second (0.5μ) motorized precision stage (Carl Zeiss, Inc.), a model 639 Pulsar digital output, isometric joystick (Measurement Systems, Inc.), and the microdensitometer have been interfaced to a model 70 Interdata, 16-bit word minicomputer. The interfacing was performed using Interdata's Universal Logic Interface (ULI) card. The design and fabrication has been completed entirely within this laboratory and at this writing is undergoing checkout. Wiring diagrams, logic schematics, and cabling layouts have been produced as documentation for the maintenance and reproduction of this system.

All application software for this system runs in an operating system environment. A small Disc Operating System (Interdata DOS) was purchased which is source code sysgenable. Via system generation, 5 peripheral device drivers have been written and included in DOS. Specifically these are drivers for: the ULI interface itself (no equipment connected), the Zeiss 200Hz stepping stage, the Pulsar 639 isometric joystick, the microscope densitometer, and two Kennedy Model 9000 magnetic tape drives on a Xebec Systems model 300 controller.

The drivers for the ULI and Zeiss stepping stage are completely debugged and both the ULI interface and Zeiss stepping stage are fully operative. The respective drivers for the joystick and microdensitometer are being debugged along with hardware checkout at this writing.

The magnetic tape driver had to be written even though the magnetic tape drives pre-existed, because of the acquisition of a new operating system (DOS). This software module drives up to four magnetic tape decks (45 IPS, 800 BPI, 9 Track, IBM compatible, NRZI) and includes features especially useful in the processing of magnetic tape files of digital images. These features are not available in the magnetic tape system available from Interdata, Inc. The magnetic tape software is completely debugged, documented, and the tape subsystem completely functional.

Four diagnostic application programs have been written for the purpose of initial checkout and future maintenance of the ULI, stepping stage, joystick, and microdensitometer. The diagnostic for the ULI enables checkout of ULI electronic function without any equipment being connected. This is why a separate driver for it is included in DOS. Given a functioning ULI, the stage diagnostic allows propulsion through an arbitrary stepping pattern. The joystick diagnostic presumes a functional ULI and stage. The joystick is checked for its ability to direct movement

of the stage. The microdensitometer diagnostic presumes the function of only the ULI. It checks measuring and data transmission of the microdensitometer. These programs run in an operating system environment and require the driver software described previously. They are fully documented.

The stage, at this writing functional, has been tested for the meeting of specifications (both as required and advertised by its manufacturer). Since there are two stage stepping motors which may be activated independently in either of two directions, steps may be taken in any one of eight directions. Each motor's axis of motion is perpendicular to the other and termed the X and Y axis respectively. As an operator faces the stage +X is rightward, -X leftward, +Y forward, and -Y backward. For purposes of notation, 0X and 0Y shall mean no movement on the corresponding axis. The eight possible directions a step may take are coded with the integers modulo-8, 0,...,7. The special symbol 8 indicates 0X and 0Y motion (no step). Each of the codes 0,...,8 is called a link and a concatenation of links a chain. This coding has become common in computer graphics applications and is used throughout the system.

The following table gives the relation between link codes and physical direction. The column labled "motors" gives the activity on each motor in terms of previously described symbolism.

| LINK CODE | MOTORS |
|---|---|
| 0 | +X,0Y |
| 1 | +X,+Y |
| 2 | 0X,+Y |
| 3 | -X,+Y |
| 4 | -X,0Y |
| 5 | -X,-Y |
| 6 | 0X,-Y |
| 7 | +X,-Y |
| 8 | 0X,0Y |

Each link direction determines a vector in 2-space making an angle with the positive X-axis which is a multiple of 45 degrees. That angle is calculated by multiplying the link code by 45 degrees. Eg., 4 x 45 = 180 degrees. Consultation of the above table reveals that this is indeed motion purely in the negative X axis direction.

Calibration of stage speed and precision has been carried out using the diagnostic software described previously. Positioning accuracy was checked with a microscope stage micrometer calibrated in 10μ graduations for 1mm. Test trajectories with a distance between start and stopping point equal to a multiple of 10μ were devised. These trajectories involved individual links which ranged through the entire sequence 0,...,8 and not

necessarily in order. The actual distance between start and stop points was measured with the stage micrometer and eyepiece crosshair. Results indicate the stepping distance is 0.5μ for each motor regardless of stepping direction. These trajectories range from 4000-120000 steps in length, require single and simultaneous motor activation, and utilize both directions of motor propulsion. If at any point in the 200Hz stepping trajectory one or more motors failed to respond accurately, the distance from start to stop point would not likely measure equal to the value calculated geometrically. It does.

Stage speed was checked by commanding steps at 200Hz. An octagonal trajectory was used, starting with motion in link = 0 direction for 500 steps, and continuing for 500 steps in each of the link directions 1,...,7. This amounts to 4000 steps per traversal of the octagonal trajectory. Thirty cycles around the trajectory were performed in each experiment. The number of steps per experiment is therefore 4000 x 30 = 120000. The stage would start and come to a stop after 30 cycles at a point on the stage micrometer in alignment with the eyepiece crosshairs. If any of the 120000 steps was not accurately executed, it is not likely that the start and stop points would be the same. As nearly as could be measured with the stage micrometer, they were identical. During this cycling time was kept by electronically operated timer. Stepping speed was calculated at 201.79 Hz.

It should be mentioned that the stepping stage need not be moved at full throttle of 200Hz. The software stage driver included in the operating system has the facility of modulating the rate of stepping. This modulation is accomplished totally by software. The stage hardware permits pulsing the stage motors, separately or together, at a maximum rate of 200Hz (manufacturer's specification). The pulsewidth (300μsec), duration between pulses (minimum 5msec from leading to leading edge), and direction (TTL 5 to 0 volts negative going) are shaped in software. Pulse rate and duration are controlled by programmed timing loops.

The stage drive can modulate stage speed to 15 levels. It does this by entering a 5 msec timing loop from 1 to 15 times after giving pulse commands to the stage. A program running under DOS containing this driver calls for motion of the stage by specifying link direction codes associated with a speed factor of from 1 to 15. The stepping rates corresponding to each factor are given in the table below:

| SPEED FACTOR | STEPPING RATE (Hz) |
|---|---|
| 1 | 202 |
| 2 | 104 |
| 3 | 71 |
| 4 | 53 |
| 5 | 43 |
| 6 | 34 |
| 7 | 30 |

| SPEED FACTOR | STEPPING RATE (Hz) |
|---|---|
| 8 | 27 |
| 9 | 24 |
| 10 | 20 |
| 11 | 19 |
| 12 | 18 |
| 13 | 16 |
| 14 | 13 |
| 15 | 10 |

The stepping rates entered in this table were measured during actual function of the stage and are not merely nominal calculations. The usefully distinct speeds are those with factors of 1,2,3,4,7,10,15. Fifteen levels were programmed for because timing by software is constrained by the execution times of instructions in the repertoire of the CPU. Until actual measurements could be made, a degree of uncertainty existed as to just which speed factors would be most useful. Consequently, a sufficient range of speeds was programmed for to provide among them rates which are useful.

To facilitate the movement of the stage in any trajectory required by a running program and at any of the possible stepping rates, a special data structure called a link list has been created. A link list consists of one or more, consecutive, 16-bit words, the last of which contains the value zero. Each word in the list is called a list element. A list element contains three fields, labeled RPT, SP, and LNK respectively. The LNK field is 4 bits wide and contains a link code from 0,...,8. The RPT field is 8 bits wide and contains a non-negative integer in the range 0-255. The SP field is 4 bits wide and contains a speed factor from 1,...,15. The number of elements which may be included in a single link list is limited only by available memory.

Another data structure maintained by all programs which cause stage motion is the location counter. This structure is composed of two consecutive 16 bit words. The data field comprised by the first word is labeled XLOC and that for the second YLOC. Both fields contain a signed integer in the range -32,767 to +32,767. The meaning of the XLOC field is the number of X-motor steps from a reference position. A similar interpretation holds for the YLOC field.

When a running program requires stage motion, it executes a call for service from DOS containing the stage driver. In making its request, the program passes the address in main memory of both a link list it has composed and the location counter it is keeping. CPU control then passes to DOS and the stage driver.

Upon gaining CPU control along with the addresses of link list and location counter, the stage driver consecutively executes each element in the link list. For each element, the stage is stepped in the direction indicated in the link field, LNK. This step is repeated for a number of

times as indicated in the RPT field. The stepping is carried out at a rate specified in the speed factor field, SP. After completion of the stepping directions contained in one link list element, the next is fetched and executed as described. The process is terminated when a zero link list element is encountered.

The advantages of this approach are several. Complex, irregular trajectories for stage motion may be accomplished. Such trajectories will result from the tracing of boundaries by the Pulsar 639 joystick. These trajectories can be traversed at variable speeds. Slower speeds are extremely useful when "playing back" previously traced boundaries which pass close between wanted and unwanted detail in the microscopic field. Playback consists of moving the stage through a trajectory stored in chain code fashion during the scanning operators tracing with the Pulsar joystick. The link list provides compactification in the case of highly regular stepping patterns such as those for scanning and searching. When multiple repetitions of the same link code occur, the repeat count greatly reduces the amount of memory needed to contain the link specifications. For one hundred steps in the link 0 direction, for example, the minimum storage required for the links specified separately is 25 words-4 bits per link. The repeat count permits this motion to be specified in one word with a speed specification included. Lastly, the link list permits an entire stepping pattern to be specified at once, with only one passage of control to the operating system and one return to the running program. This greatly reduces CPU operating system overhead time and thereby improves efficiency.

After every step taken in the execution of a link list, the stage driver updates the location counter by the proper X and Y co-ordinate components for the direction stepped. These components are always valued -1, 0, or + 1 for both the XLOC and YLOC fields. The particular values depend upon the link stepped. For example, a link 5 step will result in an update of XLOC = XLOC - 1 and YLOC = YLOC - 1. When control is returned to the program, the location counter correctly indicates the position of the stage in terms of steps from reference marks. These calculations need not be performed by every program, since they are performed by stage driver. This simplifies one aspect of writing application programs which move the stage.

The interfacing for the microdensitometer and its driver software are completely installed and at this writing are being debugged. The electronics are placed on the same ULI card (INTERDATA) as are the electronics for stage and joystick. Input of digital image data from the microdensitometer occurs on an interrupt driven basis. Data is transmitted to the computer as 4 ASCII coded characters per pixel measurement. Sixty measurements per second are possible.

The microdensitometer is equipped with its own analogue to digital conversion equipment. During a measurement period of 16.67 msecs, four consecutive 8-bit ASCII characters are placed on the data input lines to the computer. The transmission is 8-bit parallel. The availability of each data character to the computer is signaled by a data available pulse which is used by the interface to generate an interrupt.

After responding to the first character's interrupt, the stage is moved by the microdensitometer driver to the next pixel position. Then, in response to the remaining three interrupts, the last three characters for the measurement are read into the computer. The stage may be moved upon receipt of the first interrupt, because the A/D conversion of analogue photomultiplier tube output is complete at that time. Only the transmission of 4 data characters needs be accomplished. Since movement to the next pixel position (one 0.5μ step) takes only 5 msec and there are approximately 12 msecs more required to read the remaining 3 characters into the computer, the stage is actually in position long before the microdensitometer is ready to make and transmit another measurement.

In order not to waste time just waiting for character transmission, a useful, memory saving task is performed during the 16.67 msec measurement period. The 4 data characters, representing a decimal number in the format n.nnn (the decimal point is not transmitted as a character), are converted to a binary integer and stored in a main memory buffer. Since each character requires 8 bits of storage and the binary number they are converted to requires 8 bits, this constitutes a 75% compression of data buffer storage requirements. The advantages are that the main memory available for data buffering can either be smaller or more data can be accommodated in a buffer using binary number representation than can be using character data. Since data buffers, when full, have to be written to magnetic disc storage to capture the complete digital image, the number of disc accesses for this purpose is reduced to 25% of what it would be if character data were maintained.

A program requiring scanning calls upon DOS containing the microdensitometer driver much as for stage movement. Here too there is a link list, location counter. In addition, there must be a data buffer into which measurements will be placed by the microdensitometer driver. The link list is in most respects identical to that for stage movement alone. The exception is the speed field is replaced with a field which specifies the number of separate measurements to be made at each pixel position. This field is labeled NM and is 4 bits wide. The value in NM may be 0-15. The LNK and RPT fields are as for stage movement without measurement.

In calling for scanning, the running program passes the addresses of the link list, the location counter, and the data buffer to DOS and the driver. Upon gaining control of the CPU, the microdensitometer driver executes each link list element consecutively until a zero element is encountered. For each element, a number of measurements are made at the present stage position. When the first character interrupt of the last measurement arrives, the stage is moved in the direction specified by the LNK field. The number of measurements performed at the pixel is specified in the NM field. Since a value of zero is permitted, no measurements need be made at a particular point. This feature makes possible the performing of image scans which are coarser than 0.5μ IPD. This process is repeated the number of times specified in the RPT field. When the RPT field count is exhausted, the next link element is fetched and executed as described.

During each 16.67 msec measurement period, four characters are read, the stage may be moved to the next position directed by the link list, the characters are converted from 4 characters of 8 bits each to one 8 bit binary number, the binary value is stored in the running program's data buffer in a position immediately after the previous measurement, XLOC and YLOC in the location counter of the program are updated in accordance with the LNK field code. In addition, data is checked for range validity in the process. If a value which cannot be stored in 8 bits is detected, and appropriate error status is returned to the running program and the scanning process is immediately terminated. Control passes back to the program.

When CPU control is restored, the program checks the error status for a possible data check error. If one occurred it may repeat the call for scanning or generate an appropriate error message to the scanning operator at the scanning teletype. If no error occurred, the program is in possession of a data buffer filled with measurements in accordance with the link list directions and an updated location counter indicating stage position. From the implicit order of increasing 8 bit memory locations and comparison with the directions given in the link list, the program may process the scanning data in the buffer as is appropriate.

Note, since the data is transmitted as four characters, nnnn, where n = 0,1,...,9, the range of microdensitometer values is 0.000 - 1.850, the decimal point is implicitly taken to be placed after the most significant digit, and the least significant digit is always 0, the following transformation is a part of the conversion to binary. The character stream nnnn is converted in 16 bits to a binary number. Then it is divided by ten. This results in a binary number identical to what would of been the case if the original data were three characters in length, nnn. The largest decimal number in character form received from the microdensitometer is 1.850. Ignoring the decimal point and treating this value as an integer yields 1850. Dividing by ten gives 185. This can be stored in 8 bits. So can any non-negative integer bounded above by 185. Therefore, using this transformation of the character data, all measurements may be stored in 8 bits of memory.

When a running program wishes to read the Pulsar joystick, it passes only the address of a single computer word location. This word constitutes a single field of 16 bit width. When the joystick driver gains CPU control it reads the stick. Depending upon how hard (0 - 2 lbs force) the operator is pushing and in what direction, the driver converts digital information read from the joystick into a link code = 0,...,8. These have the same interpretation as discussed previously. The link code is returned to the running program at the location passed in the call for joystick information.

The harder the operator pushes on the joystick, the more often will the link returned to the program be one of 0,...,7 rather than 8. Recall, 8 means no movement. If the program reading the joystick is also moving the stepping stage in accordance with the link value returned from the joystick, the operator will observe stage motion corresponding to his directives from the stick. Programs which move the stage by joystick will repeatedly read the stick and move the stage at a rate of approximately 200 joystick readings per second. If most of the returned links are 8, motion will be slow, because a request for stage motion in link direction 8 produces no motion. If most of the links returned are 0,...,7, motion will be faster. In this manner the operator may throttle by the differential application of force to the stick.

The joystick has been chosen for positional accuracy. It is an isometric stick which does not displace under a force. The electronics in the device transduce force, not displacement, into direction and magnitude of force signals. The joystick driver returns non-zero motion links corresponding to the directional information read from the joystick in direct proportion to the magnitude of the force information supplied. The device acts both as a direction indicator and throttle. Response to manipulation of the joystick is rapid, because changes of force are sensed more rapidly than displacements. This positioning accuracy is a requirement for the editing to be done in conjunction with scanning.

Several notions should be emphasized at this point. First, only two driver modules alter a program's location counter after executing a link list. They are the stage and microdensitometer drivers. This is because only these modules actually move the stage. Second, the programming code for stage movement by the stage driver and that in the microdensitometer driver are distinct. Common code is not shared. Thirdly, no speed specification is given to the microdensitometer driver because stage motion is yoked to the speed of measurement by keying on the first character interrupt of the last measurement to be made at each pixel. Fourth, the joystick throttles the stage via differential force applied to the stick. The stage driver throttles stage speed by the specification of a speed factor. These mechanisms are distinct.

## 4. SLIDE MARKER AND HOLDER-VIRTUAL CO-ORDINATES

Individual cells in this proposed cytoassay method are analogous to the individual animals in traditional toxicological assays. The animal has its cage and quarters; the cell its co-ordinates and slide. Slides can be filed after labeling, but co-ordinates for the individual cells on a slide take a bit more doing.

A precision slide marker has been designed and fabricated in this laboratory. This device is completed, functional, and both mechanical and electronic drawings are available. The marker is for placing four reference marks on ordinary glass microscope slides. These marks are made cleanly and without fissuring of the glass and are microscopic. They are sighted upon for purposes of alignment with a reference position using the eyepiece crosshairs of the microscope.

A companion precision slide holder has also been completed with mechanical drawings available. The holder firmly grips glass slides on the microscope during scanning and searching. Further, and of extreme importance, it orients the glass slide with respect to the microscope's central optical axis. At four designated positions of the stage's X and Y verniers, the corresponding mark will appear in a 400X field. They are microscopic and refractile (it is not necessary to color them), but if one had to search for them, it could take many minutes.. This method assures they will be somewhere in the first field of view, if the stage is correctly positioned with its verniers. Positioning to vernier graduations is easily done using thumbwheels provided on the sides of the stepping motor housings.

The marks themselves are placed at the verticies of 1 x 2 cm rectangle. The sides are essentially perfectly parallel, angles exactly 90 degrees, and as close to 1 x 2 cm as precision machining equipment can make them.

Their shape is stellate, providing a small intersection for aligning with the microscope crosshairs. To afford accurate and reproducible alignment, they are made free of distortion and their impression into the glass slide does not cause fissuring.

The marker makes an indentation in the glass by camming down a doubly spring loaded shaft on which is mounted a diamond objective. The springs in the shaft chamber modulate the downward pressure once contact has been made with the slide and return the shaft to its apogee after a mark has been made. The cam is specially machined to provide smooth downward and upward motion.

The slide mounts on a motorized rotating table. The table is round and grooves are machined in the periphery at precise positions. A microswitch armature rolls along the edge as it is rotated by its motor and

eventually drops into the next groove (there are four). When the microswitch drops into a groove, table rotation immediately stops because power to its low-drift motor has been cut by the switch. Reciprocally, the motor for the cam is actuated and impression of a mark begins. When the shaft returns to its apogee, another microswitch is triggered to cut power to the cam motor and restore it to the table motor. The initial force of rotation kicks the microswitch armature out of the groove it's in and it rolls freely with table rotation until the next groove is encountered. Since there are four grooves, four marks are made. After the last mark is impressed, the entire process comes to an automatic halt. An electromechanical counter has been attached which is incremented by one for every four marks. Thus, a given number of marked slides may be produced without manually counting them. The process is started by merely mounting an unmarked slide and pushing a start button. A green light goes on and stays that way until the slide is completely marked. At this point it is extinguished and the newly marked slide can be removed.

The grooves in the edge of the rotating table and its orientation with respect to the central axis of the shaft are set so the table will cease rotation and the shaft impress its mark at the corners of the rectangle described. The slide holder for the microscope and that on the marker are mated in certain critical dimensions.

The microscope slide holder grips a glass slide the same way the marker does. When in the proper vernier positions and if the stepping stage could rotate about the optical axis, the four marks would come under the central optical axis of the microscope as they did the central axis of the marker shaft.

The microscope holder is also pre-aligned on the stepping stage so the sides of the virtual rectangle dilineated by the marks are in parallel with the X and Y axes of motion produced by the motors. As a result, if an operator has one mark centered, the others can be positioned to by use of one thumbwheel (X or Y, + or -) exclusively. The mark being positioned to will pass through the field of view at 400X. If opposite sides of the rectangle deviate too much from parallel or the angles from 90 degrees, one side could be aligned with one axis of stage motion. However, one or more of the other sides would be out of alignment. The result would be inability to find a mark 2cm or 1cm away by motion along one axis at 400X.

The marker and slide holder provide absolute reference marks for assigning cell co-ordinates. Each mark will be taken as the center of a "quadrant." A quadrant's dimensions are -32,767X to +32,767X and -32,767Y to +32,767Y steps about the mark as its center. The marks are numbered, from the upper left position in the rectangle and counting clockwise 1,2,3, and 4. This arrangement assigns 4 to the lower left hand mark.

Quadrants overlap each other and the long sides of the slide. The total area for which co-ordinates are assigned is bounded by a rectangle of dimensions 5.28 x 4.28 cm. Within this rectangle, any point may be assigned co-ordinates: q,x,y. Here q = 1,2,3, or 4; x and y are the respective number of steps from mark q and both range between -32,767 and +32,767. In areas where quadrants overlap, the mark used for reference, i.e., the value for q, is chosen for convenience or arbitrarily.

A cell may be designated as occupying any co-ordinate it overlays. Boundaries may be specified with one position designated by co-ordinates and a chain code. In this way not only the shape and size of the boundary can be recorded, but also where it is on the slide. It can be refound and played back.

Both the slide marker and holder are adaptable to performing all that has been described with previously coverslipped specimens. In this case a precision "L" shaped abutment for the slide's edge has one limb exchanged for a tunneled version that permits the slide to pass but not the coverslip. In this case, the specimen is held in abutment against the L's limbs along one edge of the coverslip and one edge of the slide. Having this capability permits the scanning of specimens fixed and stained elsewhere.

## 5. SELECTION OF TEST CELLS AND CULTURE TECHNIQUE

The Kettering Laboratory has forwarded to the AFIP fixed Mouse-L cells from a variety of experiments. These experiments involved culturing the cells against control water, 2,4-DNPH, or Reverse Osmosis Concentrate (RO). In all cases the cells were cultured for 1,2,3, or 4 days. The following table summarizes the Kettering Laboratory experiments for which cultured cells were received.

| Experiment | Cell Type | Summary of Culture Conditions |
|---|---|---|
| MUSTEP-1 | Mouse L | Cells cultured in control water only. Three initial concentrations of cells (cells/ml) were used. These were: 100,000, 150,000, and 200,000 respectively. Medium used was Minimal Essential Medium (MEM) with 1% fetal calf serum (FCS). Exposure was for 1,2,3, and 4 days and fixation was performed with ETOH. Coverslips were shipped both dry and immersed in 50% ETOH. Further reference to this protocol will be: MUSTEP-1. |
| MUSTEP-6 | Mouse L | Cells cultured against varying concentrations of 2,4-DNPH. Concentation range: 0.0 - 10.0 mg/L. Initial concentration of cells: 37,500 cells/ml. Medium: MEM with 1% FCS. Exposure times: 1,2,3, and 4 days. Fixative: ETOH. Medium renewed after 2nd and 3rd days. Shipped both dry and immersed in ETOH. Fixative for SEM: Glutaraldehyde. |

| Experiment | Cell Type | Summary of Culture Conditions |
|---|---|---|
| MUSTEP-7 | Mouse L | Protocol used is identical to that for MUSTEP-6, with the following exceptions: concentration of 10.0 mg/L 2,4-DNPH deleted; concentration of 0.1 mg/L 2,4-DNPH added to experimental matrix. Although the protocol received from the Kettering Laboratory indicated the exceptions listed, the actual coverslips were an exact replication of MUSTEP-6. |
| MUSTEP-8 | Mouse L | Cells cultured against varying concentrations of synthetic hospital waste water (MUST waste). Original waste subjected to ultrafiltration and reverse osmosis, then concentrated to 1.84% of initial volume. From this RO concentrate MEM cultures were run containing concentrations, under conditions otherwise identical to those in MUSTEP-6, in the range of 0.0 -18% of the RO concentrate. Exposure time: 1,2,3, and 4 days. Fixative: ETOH. Coverslips shipped both dry and immersed in ETOH. |
| MUSTEP-9 | Mouse L | Protocol used is identical to MUSTEP-8, with the following exeption: range of RO concentrate dilution: 0.0 -10.0%. |

Coverslips shipped to the AFIP in a dry condition are used for light microscopy. Those shipped under ETOH are used for scanning electron microscopy (SEM).

MUSTEP-1 was an exploratory experiment performed for the purpose of determining the concentration of cells (cells/ml) to be used in initiating cultures, staining techniques, scanning time required per cell, the amount of editing time required to rid each digital image of extraneous content, the amount of computer memory (main and auxilliary) needed for capture and storage of digital cell images, and an estimate of the range of optical density values (grey values) resulting from digitization of cell images stained by a particular methodology. For the last stated purpose, 800 cells stained with the Papanicolau (400 cells) and Feulgen (400 cells) techniques were scanned using the laboratory's pre-existing, off-line scanning system.

Examination of cell populations produced in MUSTEP-1 revealed a sparsity of cell growth at all initial concentrations of Mouse L cells (100,000, 150,000, and 200,000 cells/ml). Coverslips shipped under ETOH for SEM were observed to contain cells inadequately preserved for these studies. The SEM slips also contained excessive, artifactious debris which frequently obscured cell surface detail.

Cell cultures using medium 199 (HANKS' salts) were performed at the AFIP. Ten per cent fetal bovine serum, 0.1g streptomycin/ml, and 100,000 units penicillin/ml were added. Initial cell concentrations of 100,000 cells/ml were employed. Under atmospheric conditions of 3% CO2 and 37 degrees centigrade, the cells grew vigorously. Sampling after 1,2,3 and 4 days produced even monolayers and the 4 day populations had not yet reached confluence.

From the SEM results obtained, it was concluded that the fixative for SEM coverslips was changed to 2% glutaraldehyde to correct for poor preservation of cell surface features.

Recommendations based on these findings were forwarded to the Kettering Laboratory and reported to USAMRDC. For further details consult the report entitled: "Analysis of Cytotoxic Reactions Produced by MUST-water Constituents, Renewal Proposal and Progress Report," dated 1 February 1975.

Based in part on these recommendations, adjustments in the culture protocol used by the Kettering Laboratory have resulted in subsequent coverslips containing adequate numbers of well preserved cells-both wet and dry shipped. Success has been achieved with continued use of MEM and the initial cell concentration of 37,500 cells/ml appears adequate to assure enough cells on each slip. Confluence is not reached, even in the four day populations. SEM coverslips are now adequate for study of surface properties of cells cultured in the presence of contaminants.

Cell populations from MUSTEP-1 were stained with the Papanicolau (MUSTEP-3), Feulgen (MUSTEP-2), and Geimsa (MUSTEP-4) techniques. Geimsa staining of Mouse L cells was judged least efficient in the production of nucleo-cytoplasmic and slide backround - cytoplasmic contrast. Since these properties are of great value in subsequent image analysis, it was decided to confine further initial study to the Papanicolau and Feulgen techniques.

Four hundred cells each from populations stained by Feulgen and Papanicolau techniques were scanned. The digital images contained optical densities in the range of 0.00 - 1.20 optical density units (ODU). Since the microdensitometer being employed in this project has an analogue-to-digital conversion dynamic range of 0.00 - 1.86, these results indicate that 8 bits of grey resolution is easily sufficient for the project's needs. Generally, nuclear pixels in regions of chromatin concentration range from 0.90 - 1.20 ODU. Cytoplasmic density rranges from 0.80 - 0.90 ODU. This is for Papanicolau stain only, since Feulgen stained populations were not counterstained for cytoplasm. Hence, they are useful for assessment of nuclear staining properties only. Slide backround in the vicinity of stained cells ranged between 0.04 - 0.10 ODU. The cleaner slide backround can be kept, the more useful are density threshold techniques in distinguishing between "naked slide" and the cell. Thus the cell border is more easily discernable. A similar statement may be made for the contrast in density between cytoplasm and nucleus.

A raster of pixels measuring 50 x 50μ appears sufficient for the overwhelming number of Mouse L cells observed and scanned. This includes only the mononucleate members of each population. The project will be confined to this type of culture cell. A raster of these linear dimensions contains at most 100 x 100 = 10,000 pixels. Each pixel will be stored in one 8 bit byte of data storage space. Therefore, digital images of Mouse L cells will require (if no further compression technique is employed) approximately 10,000 bytes of storage per cell.

Given the expected scanning rate of the improved system, the time required to scan cells of this size should not exceed 3.0 minutes. This is the elapsed time the scanner will be automatically taking data without operator intervention. Not included is search and pre-editing time. For reasons given in an earlier section of this report, scanning time will probably average closer to 2.0 minutes per cell.

The fact that cells stained with the Papanicolau and Feulgen techniques were selected for the most intense initial study should not be construed as an indication that the Geimsa technique has no value. Not only might it be superior with other test cells, but further study with the Mouse L cell may reveal advantages with respect to the measurement of specific features. The Papanicolau and Feulgen stains have been initially used because they show greatest promise in qualitative determinations. Ultimately, when scanning can be accomplished by the improved system at faster rates, a final determination of best stain will be quantitatively established.

Aside from production of adequate contrast among intracellular areas of interest in the assessment of cytotoxicity, a good stain must render a reproducible effect on control cell populations (cells cultured in the absence of any contaminant). The effect desired is stability of optical density distributions for pixels overlying important cell infrastructure. Examples of such infrastructures are heterochromatin, euchromatin, cytoplasm, nuclear border, and cell border.

Since the cells used in a particular test are all of the same line, the overall distribution of pixel densities (relative frequency distribution of densities constructed using only pixels overlying the cell proper) should be relatively stable in both shape (moments, modes, scew ) and absolute value of class interval limits. If the densities measured for each cell in a control population are pooled, a "typical" distribution for the control population can be constructed. This should be invariant under mathematical transformations in 2-space which are pure magnifications or contractions of the cell image resulting in independence of size and shape provided the areal relationships among infrastructures are preserved.

The variations in areal proprotions among major cell infrastructures are reflected in the "typical" relative frequency distribution of densities obtained by pooling the values of all cells digitized from the population.

Thus, though nucleo-cytoplasmic ratio varies from cell to cell in a population of controls, it should range over certain values with predictable frequency for the test cell line under constant culture conditions.

The strategy used to produce relatively uniform staining effects is to define and compute a function of this "typical" relative frequency distribution. The function should be sensitive to changes in shape and class interval limits of the distribution. A candidate for this function has been decided upon, but is not yet implemented in software. Testing the effectiveness of this strategy requires completion of improvements to the scanning system which are in progress.

For every batch of stain prepared, a control cell population will be stained and a subpopulation scanned. From the relative frequency distribution of pooled densities for each subpopulation the above mentioned function is computed. Based upon the resulting values, the staining protocol is adjusted to produce an end effect, as measured by the functional value, which is not significantly different from that produced by previous usage of the staining technique with different batches of stain.

The major advantage of this approach is ability to compare results obtained from the cytoassay of a water source at different times (e.g., periodic monitoring, before and after purifciation procedures). The in-built controls for each cytoassay are sufficient to distinguish significant cytomorphologic alterations within a given experimental run. However, for comparison of one run with another standardization of staining effect is required.

The useful life of a given batch of stain may also be monitored. Generally, a stain batch may be used as long as it produces a staining effect which is not significantly different from the established standard. Thus, this technique permits the development of data concerning the shelf life of stock solutions used in a given cytoassay.

The control cell populations contained in each experimental run may be used to check the actual staining effect for the run. The control cells used to standardize a staining protocol for a new batch of stain need not be part of an actual test. However, the controls which are included in each test may be checked against the standards expected for the protocol. The analytic results of cytoassay are relied upon only in the presence of a positive demonstration of standard staining effect.

SEM and light microscopic examination after Geimsa staining has been performed on the cell populations received from the Kettering Laboratory. The coverslips involved are those produced by the experiment referenced as MUSTEP-6. SEM examination of cell surface morphology reveals cytopathologic effects in all populations exposed to 2,4-DNPH. The effects were observed at all concentrations and times of exposure. Even after 24 hours of exposure at 0.1 mg/l considerable numbers of cells possessed an altered morphology compared to controls.

The principle cytopathic effects observed were: loss of normal angularity in cell outline with rounding of cell configuration; apparent loosening of attachment to the coverslip; development of outward blebbing of the cell membrane; Light microscopic examination after Geimsa staining confirmed the loss of cell angularity with rounding. Also present was nuclear hyperchromatism with coarser clumping of the chromatinic material. The light microscopic effects were observed in all populations.

These effects appear more commonly in populations exposed for longer periods of time and/or to higher concentrations of 2,4-DNPH. While the abnormalities in populations with short exposure (1 day) to the lowest concentration (0.1 mg/L) are not uncommonly found, they are common in populations exposed the longest (4 days) to the highest concentrations (10.0 mg/L).

SEM examination has been initiated on populations from the experiment referenced as MUSTEP-8. These are cells exposed to various dilutions of RO concentrate. At this writing, only the cells exposed to 18.0% for 4 days have been examined. The effects are similar to those described for 2,4-DNPH except they are more frequent in the population than in the 10.0 mg/L, 4 day population for 2,4-DNPH. Further, many cells are represented merely by a "ghost" membrane which is smoothed, sometimes fragmented, and typical of no physiologically significant activity.

These studies are being conducted concurrently with development of an improved scanning system to provide insight to the quantitative features most likely to yield a sensitive quantitative assay. Further, they may be used to provide a qualitative index of suspicion concerning the feasibility of the cytoassay method. The quantitative analogue of these studies, under light microscopy, will be performed upon completion of system improvement. Replicate coverslips for the various populations in each experiment are being stored in the meantime.

The conclusions drawn so far are several. First, observable cytopathic effects are obtained even after relatively short exposure to a cytotoxicant. The effects are apparently a measure of cell damage and not specific to the insulting agent. Response of a population is graded-proportional to both time of exposure and concentration of toxicant. It is probably possible to conduct a cytoassay using less than 24 hours of exposure and much lower concentrations of toxicants. The effects of purification procedures might be checked by renewing the medium using purified water after 1 or 2 days, since the culture system is well behaved for four.

## 6. ACQUISITION AND INSTALLATION OF NEW EQUIPMENT

Installation of additional memory, a small magnetic disc, and a printer/plotter for the minicomputer subsystem has been accomplished. Some items of documentation for the printer/plotter are yet to be delivered from its manufacturer. At this writing, the new equipment has been available for integrated use for approximately 6 months.

Considerable time and energy was required in the acquisition order to comply with Army regulations relating to the purchase of automated data processing equipment and purchasing and contracting procedures. Vendor delays have also occurred-particularly with respect to the printer/plotter. Lastly, not all equipment delivered functioned properly from the start. The experience has been, for essentially all hardware acquired (ADPE or otherwise), at least one month of trouble-free function is required to assure continued reliable performance. After initial installation, multiple re-visits from vendor installation personnel have been required before one month of trouble-free function is achieved. All equipment purchased for this project has now reached this level of performance.

F. PROJECTED SCHEDULE OF WORK

The scanning subsystem should be completed between 1 October '75 and 15 December '75. At that time scanning with the improved system will commence on populations from previously received experimental runs. Concurrent with scanning, development will continue of software for feature extraction, analysis, and display of results. Continued heavy use of the electronics design and maintenance laboratory is anticipated. Similarly, heavy use of the precision instrumentation machine shop will continue. The latter facility with its associated E-6 precision machinist is critical for the success of this project.

Completion of the investigation for feasibility of a cytoassay for water toxicity is anticipated by 31 December 1976. Upon full implementation of the improved system, approximately one experiment per six weeks can be performed. This includes the scanning of 1200 - 2500 cells per run, analysis, and computerized report generation. This rate is not a limitation of the methodology. Equipment is commercially available which would increase the production rate of completed analyses manyfold. Since the purchase of such equipment involves expenditures considerably beyond those allocated for this project, feasibility ought be demonstrated first. What is being sought in the design of the present system is an acceptable experimental rate and research flexibility. . . .

G. ANTICIPATED MANPOWER ALLOCATION BASED ON CURRENT STRENGTH

Tablulated below are the estimates of manhours which are or will be dedicated to this project:

| Category & Description | Number of Aggregate Hrs/Wk | Individuals Performing |
|---|---|---|
| Professional: Cytopathology, cell biology, cell culture, system design, data analysis, software development | 90 | Gunter F. Bahr, M.D., Chairman; Joseph A. Boccia, M.D., LTC, MC, USA: Robert H. Shoemaker, Ph.D., CPT, MSC, USA |
| Technical (biologic): Cell culture, staining, SEM, scanning | 40 | 3 Cytotechnologists; 1 Cytotechnology research assistant (supervisor) |

| Category & Description | Number of Aggregate Hrs/Wk | Individuals Performing |
|---|---|---|
| Technical (electronics): Implementation of electronic design, maintenance | 30 | 1 electronics technician |
| Technical (instrumentation design, fabrication, and mainenance-mechanical) | 30 | 1 master machinist with drafting capability and instrumentation experience |

# RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

| 1. AGENCY ACCESSION* | 2. DATE OF SUMMARY* | REPORT CONTROL SYMBOL |
|---|---|---|
| DA OB 6608 | 75 07 01 | DD-DR&E(AR)636 |

| 3. DATE PREV SUM'RY | 4. KIND OF SUMMARY | 5. SUMMARY SCTY* | 6. WORK SECURITY* | 7. REGRADING* | 8a. DISB'N INSTR'N | 8b. SPECIFIC DATA-CONTRACTOR ACCESS | 9. LEVEL OF SUM |
|---|---|---|---|---|---|---|---|
| 74 07 01 | D. Change | U | U | | NL | ☒ YES ☐ NO | A. WORK UNIT |

| 10. NO./CODES:* | PROGRAM ELEMENT | PROJECT NUMBER | TASK AREA NUMBER | WORK UNIT NUMBER |
|---|---|---|---|---|
| a. PRIMARY | 6.27.58.A | 3A762758A819 | 00 | 061 |
| b. CONTRIBUTING | | | | |
| c. CONTRIBUTING | CARDS 114F | | | |

11. TITLE (Precede with Security Classification Code)*

(U) Dynamics of Aircraft Accident Victims: Computer Simulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

| 13. START DATE | 14. ESTIMATED COMPLETION DATE | 15. FUNDING AGENCY | 16. PERFORMANCE METHOD |
|---|---|---|---|
| 72 07 01 | CONT | DA | C. In-House |

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

| 18. RESOURCES ESTIMATE | a. PROFESSIONAL MAN YRS | b. FUNDS (In thousands) |
|---|---|---|
| FISCAL YEAR PRECEDING 75 | | 22 |
| FISCAL YEAR CURRENT 76 | | 25 |

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Armed Forces Institute of Pathology

ADDRESS:* Washington, D. C. 20306

RESPONSIBLE INDIVIDUAL

NAME: Hansen, J. L., COL, MC, USA

TELEPHONE: 202-576-2905

21. GENERAL USE

20. PERFORMING ORGANIZATION

NAME:* Armed Forces Institute of Pathology

ADDRESS:* Washington, D. C. 20306

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* McMeekin, R. R., LTC, MC/FS, USA

TELEPHONE: 202-576-3232

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Ballo, J. M., MAJ, MC, USA

NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Computer Simulation, (U) Aircraft Accidents, (U) Occupant kinematics, (U) CALSPAN three dimensional program model

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text with each with Security Classification Code.)

23. (U) Computer simulation of the kinematics of occupants in selected aircraft accidents in order to recreate the sequences of injuries and to determine their specific cause(s). This will lead to an increased understanding of the pathogenesis of such injuries and lead to more effective means of preventing them. The results of this project will enable the Army to better understand aircraft injuries and allow it to better cope with same.

24. (U) Operate the CALSPAN three-dimensional program as a model using selected Aerospace Pathology Division cases as sources of input data. Correlate the actual injuries observed at autopsy with the injuries predicted from the occupant kinematics of the computer simulation.

25. (U) 7407-7506. The new version of the program, as received from CALSPAN, has been validated and run. Actual field measurements have led to improved vehicle data for the UH1H/D, AH1G and OH58. The quality of data being collected by field investigators has improved. After review, 10 cases involving UH1H/D accidents are being presently simulated.



*Available to contractors upon originator's approval.

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ANNUAL PROFRESS REPORT

TITLE PAGE

Project No. 3A762758A819

Title: Dynamics of Aircraft Accident Victims: Computer Simulation

Task No. 00

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Aerospace Pathology Division

Period Covered by Report: 1 July 1974 - 30 June 1975

Professional Author: R. R. McMeekin, LTC, MC, USA

Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 3A762758A819

Title: Dynamics of Aircraft Accident Victims: Computer Simulation

Task No. 00

Since the last report, dated 1 July 1974, an updated version of the CALSPAN three-dimensional program model has been obtained. This new version has been validated and is now operational. A significant improvement in the program is a "restart" feature which allows input parameters to be changed at will at selected moments during the course of the simulation. Thus multiple values for an input parameter may be modeled without the entire program being re-run. This results in a significant saving in both time and costs.

Actual field visits to Ft. Rucker, Alabama, have resulted in improved and more accurate input data for simulation of accidents involving the UH-1D/H and OH-58 helicopters. Correspondence with the technical staff of Bell Aircraft Co., has resulted in the capability of simulating accidents involving the AH1G attack helicopter.

The project has received updated accident data from the U. S. Army Agency for Aviation Safety (USAAAVS). Correlation of this data, with material accessioned by the Aerospace Pathology Division, has led to the selection of 10-12 cases which are suitable for simulation. The quality of data collected in the field continues, in many cases, to be insufficient for accurate calculation of the forces involved at impact. It is expected that actual on-site investigation of selected accidents by the Aerospace Pathology Division will lead to a core of cases suitable for accurate simulation as well as leading to the creation of a check list or form, filled out by the contributor, which will allow accurate simulation.

The ban on expenditure of travel funds (from 3 January 1975 to 7 February 1975), as well as our inability to hire a secretary for the project, until recently, has unavoidably slowed expected progress.

| RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY | | | | 1. AGENCY ACCESSION* | 3. DATE OF SUMMARY* | REPORT CONTROL SYMBOL |
|---|---|---|---|---|---|---|
| | | | | DA OB 6607 | 75 07 01 | DD-DR&E(AR)636 |

| 3. DATE PREV SUM'RY | 4. KIND OF SUMMARY | 5. SUMMARY SCTY* | 6. WORK SECURITY* | 7. REGRADING* | 8A. DISP'N INSTR'N | 8B. SPECIFIC DATA - CONTRACTOR ACCESS | 9. LEVEL OF SUM |
|---|---|---|---|---|---|---|---|
| 74 07 01 | K.Completion | U | U | | NL | ☒ YES ☐ NO | A. WORK UNIT |

| 10. NO./CODES:* | PROGRAM ELEMENT | PROJECT NUMBER | TASK AREA NUMBER | WORK UNIT NUMBER |
|---|---|---|---|---|
| a. PRIMARY | 6.27.60.A | 3A762760A837 | 00 | 076 |
| b. CONTRIBUTING | 6.21.10.A | 3A162110A830 | | |
| c. ~~CONTRIBUTING~~ | CARDS 114F | | | |

11. TITLE (Procede with Security Classification Code)*

(U) Spontaneous Lesions in the Ears and Noses of Military Working Dogs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

| 13. START DATE | 14. ESTIMATED COMPLETION DATE | 15. FUNDING AGENCY | 16. PERFORMANCE METHOD |
|---|---|---|---|
| 72 04 13 | | DA | C. In-House |

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

| 18. RESOURCES ESTIMATE | a. PROFESSIONAL MAN YRS | b. FUNDS (In thousands) |
|---|---|---|
| FISCAL YEAR PRECEDING 75 | | 13 |
| FISCAL YEAR CURRENT | | |

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Armed Forces Institute of Pathology

ADDRESS:* Washington, D. C. 20306

RESPONSIBLE INDIVIDUAL

NAME: Hansen, J. L., COL, MC, USA

TELEPHONE: 202-576-2905

20. PERFORMING ORGANIZATION

NAME:* Armed Forces Institute of Pathology

ADDRESS:* Washington, D. C. 20306

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Casey, H. W., Lt Col, USAF, VC

TELEPHONE: 202-576-2601

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Biosensor systems, (U) Military working dogs, (U) Auditory and olfactory ability

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Study will determine on a morphologic basis the type and extent of lesions that may have contributed to an auditory and/or olfactory sensory loss in military working dogs. Such studies are vitally important in maintaining maximum effectiveness of military sentry and security dogs.

24. (U) Noses and temporal bones will be examined grossly and histologically.

25. (U) 7407-7506. Gross and microscopic examinations have been completed on the 100 noses and 50 ears of military working dogs. No lesions that were considered to be functionally significant were detected in any of the noses although minor lesions of a chronic inflammatory nature were observed in 14 dogs. In 3 of the 14 dogs, the inflammatory lesions were associated with nasal mites, Pneumonyssus caninum. Of the 50 ears examined, only two animals exhibited lesions. One dog had an area of atrophy in the cochlear nerve and ganglion in the basal cochlear turn with loss of hair cells in the organ of Corti. These lesions are consistent with an aging change. A cholesteatoma, associated with bilateral otitis media, was located in the right temporal bone and extended into the middle ear in one dog. No other significant lesions were observed in the ears. A brief scientific article reporting the findings is being prepared by F. M. Robinson, USAF (Ret.) former principal investigator, and V. J. Hyams, CAPT, MC, USN. These studies indicate that the incidence and severity of morphologic lesions in the nose and ears of military working dogs would not contribute to an auditory and/or olfactory sensory decrement in a significant number of military working dogs.



*Available to contractors upon originator's approval

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3A762760A837

Task No. 00

Title: Spontaneous Lesions in the Ears and Noses of Military Working Dogs

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Veterinary Pathology Division*
ENT Pathology Branch**

Period Covered by Report: 1 July 1974 - 30 June 1975

Professional Authors: H. W. Casey, Lt. Col., USAF, VC*
V. J. Hyams, CAPT, MC, USN**

Report Control Symbol: RCS MEDDH-288 (R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 3A162110A820

Task No. 00

Title: Spontaneous Lesions in the Ears and Noses of Military Working Dogs

The usefulness and efficiency of the military working dog is directly related to their ability to sense by sight, smell and hearing an enemy, intruder or explosive device. The eyes of these dogs have been studied routinely, but the ears and noses have not because of expense and time involved. Continued studies will determine on a morphologic basis the type and extent of lesions that may have contributed to an auditory and/or olfactory sensory loss. By defining these lesions possible medical and surgical treatments may be devised to extend the useful life of these valuable dogs and also provide basic information useful in the selection of candidate military working dogs.

The objective of the research is to determine on a morphologic basis the cause of the loss of auditory and olfactory sensory ability in the military working dog.

Gross and microscopic examinations have been completed on the 100 noses and 50 ears of military working dogs. No lesions that were considered to be functionally significant were detected in any of the noses although minor lesions of a chronic inflammatory nature were observed in 14 dogs. In three of the 14 dogs the inflammatory lesions were associated with nasal mites, Pneumonyssus caninum. Of the 50 ears examined, only 2 animals exhibited lesions. One dog had an area of atrophy in the cochlear nerve and ganglion in the basal cochlear turn with loss of hair cells in the organ of Corti. These lesions are consistent with an aging change. A cholesteatoma, associated with bilateral otitis media, was located in the right temporal bone and extended into the middle ear in one dog. No other significant lesions were observed in the ears. A brief scientific article reporting the findings is being prepared by F. M. Robinson, USAF (Ret.) former principal investigator, and V. J. Hyams, CAPT, MC, USN. These studies indicate that the incidence and severity of morphologic lesions in the nose and ears of military working dogs would not contribute to an auditory and/or olfactory sensory decrement in a significant number of military working dogs.

| RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY | | | | 1. AGENCY ACCESSION* DA OE 6605 | 2. DATE OF SUMMARY* 75 07 01 | REPORT CONTROL SYMBOL DD DR&E(AR)636 |
|---|---|---|---|---|---|---|
| 3. DATE PREV SUMRY 74 07 01 | 4. KIND OF SUMMARY D. Change | 5. SUMMARY SCTY* U | 6. WORK SECURITY* U | 7. REGRADING* | 8a. CHG IN INSTR'N NL | 8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO | 9. LEVEL OF SUM A. Work Unit |

| 10. NO./CODES:* | PROGRAM ELEMENT | PROJECT NUMBER | TASK AREA NUMBER | WORK UNIT NUMBER |
|---|---|---|---|---|
| a. PRIMARY | 6.47.13.A | 1J664713D47 | 00 | 001 |
| b. CONTRIBUTING | | | | |
| c. CONTRIBUTING | CARDS | | | |

11. TITLE (Precede with Security Classification Code)*

(U) Lesions in Animals Fed Enzyme Inactivated Frozen and Irradiated Beef

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

006500 Food; 014100 Radiobiology

| 13. START DATE | 14. ESTIMATED COMPLETION DATE | 15. FUNDING AGENCY | 16. PERFORMANCE METHOD |
|---|---|---|---|
| 71 07 01 | CONT | DA | C. In-House |

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

| 18. RESOURCES ESTIMATE | a. PROFESSIONAL MAN YRS | b. FUNDS (In Thousands) |
|---|---|---|
| FISCAL YEAR PRECEDING 75 | | 32 |
| FISCAL YEAR CURRENT 76 | | 35 |

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Armed Forces Institute of Pathology
Washington, D. C. 20306

ADDRESS:*

RESPONSIBLE INDIVIDUAL

NAME: Hansen, J. L., COL, MC, USA

TELEPHONE: 202-576-2905

20. PERFORMING ORGANIZATION

NAME:* Armed Forces Institute of Pathology
Washington, D. C. 20306

ADDRESS:*

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Casey, H. W., Lt Col, USAF, VC

TELEPHONE: 202-576-2601

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

21. GENERAL USE

ASSOCIATE INVESTIGATORS

NAME: Stedham, M. A., LTC, VC, USA

NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Food, (U) Radiation, (U) Sterilization, (U) Beef, (U) Experimental, (U) Laboratory Animals

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To determine the wholesomeness for human consumption of radiation sterilized beef by studying the pathologic effects, if any, of feeding dogs, rats and mice irradiated beef. Preservation of food is vital to military operations.

24. (U) The U.S. Army is conducting contractual studies on the wholesomeness for human consumption of radiation sterilized beef. Pathological results obtained from experimental animals will be statistically analyzed and submitted together with other experimental data to the FDA and USDA to establish a regulation permitting unlimited consumption of radiation sterilized beef. The AFIP serves as monitor and reviewer of the pathologic findings in the contractor's experimental and control animals.

25. (U) 7407-7506. During FY 1975 six site visits were made to Industrial Bio-Test Laboratories. Three of these visits were made to coincide with periodic sacrifices of the $F_0$ generation rats on study for 12, 18, and 24 months. Two visits were made specifically to deal with a disease problem which was a potential threat to outcome of the mouse study. One visit was made to brief the new AFIP monitor. Tissue, blocks and protocols from 404 periodic sacrifices and spontaneous deaths in rats, mice and dogs have been received, processed and accessioned by the AFIP. Microscopic diagnoses have been made on 368 animals and filed in the Registry of Veterinary Pathology. To date the AFIP pathologists have been in essential agreement with diagnoses submitted by Industrial Bio-Test Laboratories.



*Available to contractors upon originator's approval

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 1J664713DL47

Task No. 00

Title: Lesions in Animals Fed Enzyme Inactivated Frozen and Irradiated Beef

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Division of Veterinary Pathology

Period Covered by Report: 1 July 1974 - 30 June 1975

Professional Author: H. W. Casey, Lt. Col., USAF, VC

Report Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 1J664713DL47

Task No. 00

Title: Lesions in Animals Fed Enzyme Inactivated Frozen and Irradiated Beef

The need for fresh meat to meet the dietary requirements of large numbers of troops in isolated areas where logistics do not permit the use of refrigeration requires the development of a new preservation and sterilization method for meat products. Preservation by radiation offers a practical means of overcoming these logistical problems while maintaining the wholesome and organoleptic properties of fresh meat.

The AFIP serves as monitor and reviewer for the U. S. Army of pathologic findings in experimental and control animals utilized in the present study now under contract with the Industrial Bio-Test Laboratories, Northbrook, Illinois.

Consultation with the contractor's pathologists has been very active during FY 1975. Two site visits were made to deal solely with a potentially serious disease problem among male mice with urethral obstruction. Appropriate laboratory tests were done but the causative agent or agents were not identified. Fortunately, the disease has run its course and no pathology related problems are currently present in the mouse study. Examination of tissue sections from all studies by Bio-Test pathologists as well as those monitored by the AFIP are proceeding on a reasonable schedule.

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