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FINAL REPORT ON THE RESEARCH PROGRAM ON
BW DETECTION

SGC 382R-8

TASK II OF RESEARCH PROGRAM ON BW DETECTION
CONTRACT DA 18-064-AMC-137(A)

VOLUME I
TECHNICAL DISCUSSION



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Volume I
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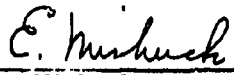
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Fort Detrick
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on
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Section 1

INTRODUCTION

This Final Report on the Research Program on New Concepts in BW Detection (Task II of the original two-part program*) is provided by Space-General Corporation in accordance with the requirements of Contract DA 18-064-AMC-137(A). The report is divided into two volumes: Volume I, Technical Discussion, and Volume II, Management and Financial Summary.

Volume I contains a detailed review of the work carried out in examination of the feasibility of novel concepts for solution of various aspects of the BW detection problem. It describes the research activities performed and the work accomplishments in the areas of detection, collection, separation and concentration research. In addition, work on the characterization of atmospheric aerosols is also reviewed. The conclusions presented in these areas are supported by experimental data, technical analyses, calculation, and illustrations. This report is arranged in four major parts:

1. Introduction
2. Program Definition
3. Summary
4. Technical Discussion

The Table of Contents presents a more detailed outline of the items reviewed in this report. Section 2, Program Definition, contains a concise discussion of the Objectives and Scope of the program, a review of the Technical Approach to the problem, and a description of the Program Management. A brief review of some of the technical highlights of the program is given in Section 3, the Summary.

The Technical Discussion, Section 4, briefly discusses the philosophy of the program and provides a detailed examination of the technical

* The Final Report on the Exploratory Development of the FAST Breadboard, Task I of the Research Program on BW Detection, was issued on 15 June 1964.

approaches which have been examined. It is the objective of this review to indicate the technical reasons for the eventual disposition of each study, together with conclusions and recommendations which may be pertinent on the basis of current information.

Financial and managerial aspects of the program are described in Volume II of this report.

Section 2
PROGRAM DEFINITION

2.1 OBJECTIVES AND SCOPE

2.1.1 OBJECTIVES

The primary objective of the Research Task of Contract DA 18-064-AMC-137(a) has been to provide new and more effective approaches to the problems associated with the detection of small numbers of aerosolized pathogenic micro-organisms among the normal larger numbers of airborne particulates of similar size.

Specific objectives have included (a) the ultimate development of a capability for detecting one pathogen-bearing particle per liter of air in less than five minutes, (b) evaluation of the effectiveness of recommended concepts for detection in terms of speed, sensitivity, specificity, and multi-agent capability, and (c) establishment of the engineering feasibility of attainable instrumentation in terms of simplicity, cost, development lead time, and logistic support requirements.

The immediate objectives of the work have been (a) to recommend new approaches to BW detection which are attainable on the basis of current technological information, (b) to recommend the most promising approaches for development in automated detection devices, (c) to design and fabricate bread-board models of components and first generation prototypes, and (d) to evaluate the performance of these new detection instruments. In addition, a constant effort has been expended to discover new phenomena which might lead to improved detection methods.

2.1.2 SCOPE

The effort, which was directed toward the objectives indicated above, has included the following specific work areas as required in the contract:

Conceive and perform a research program designed to focus on areas

which show maximum potential for leading to improved biological detection principles, utilizing new phenomena for improved collection, concentration, separation and detection of dilute microbiological aerosols, and to reliably and quantitatively evaluate the feasibility of biological agent detection by such methods.

2.2 TECHNICAL APPROACH

During the period May 1963 through November 1965, the technical approaches were subdivided into three basic categories:

Category A: Generation of New Ideas

Category B: Research on Known Concepts

Category C: Development of Breadboard Models

This effort was organized to give an integrated, well-balanced program which met all the objectives specifically mentioned or implied in the contract.

In Category A, Generation of New Ideas, a deliberate, organized effort was made to uncover phenomena of use for rapid, specific, and positive means of detection. Organized technical meetings, which brought together the most knowledgeable talents from multiple disciplines, were used to focus attention on areas such as (1) virus detection, (2) instrumentation, and (3) immunological approaches. In addition, experts have been consulted frequently and on an organized basis in each part of the program. Constant contact with academic progress in related fields was maintained by placement of research subcontracts within the academic community. Frequent, scheduled exchanges of ideas with industrial subcontractors also served as a source of input in devising new techniques and concepts. A continuing literature search was conducted by all personnel related to the technical effort. This served to keep the input to the continuous analysis of the state of the art in BW detection on a current basis.

Category B, Research on Known Concepts, comprised three tasks:

(a) reduction of a large number of generated ideas into a smaller practical number for further study, (2) definition of the nature of the technical effort on each of the acceptable ideas, and (3) conduct of a sound research program. In sifting of potentially good ideas, an analysis was usually constructed of how the

system might operate, what defects were inherent and how they might be overcome, what data would be needed to evaluate the suitability of the technique, the time required to carry out the necessary research to demonstrate feasibility, and finally an estimate of the eventual performance of the system. In general, the research effort was divided into consideration of the four essential component parts of any system: (1) collection, (2) concentration, (3) separation, and (4) detection. Section 4.1 of this volume reviews the detection concepts which were examined. Efforts on collection, concentration, and separation are reviewed in Section 4.2.

Development of Breadboards, Category C, was carried out in two parts. Task 1 implemented the decision to develop the Fluorescent Antibody Staining Technique (FAST). However, during the period 1 October 1964 - 1 November 1965, several other first-generation prototypes were constructed and tested. These devices were the direct results of research carried out in Categories A and B. Each had high promise with respect to development into practical devices. The Chemiluminescence, Bacteriophage, and Radioantibody Detection Systems, which resulted from earlier work on this program, serve as potent proofs that the methods applied in this integrated program yielded practical devices and results. In addition, a number of component parts were developed from the concept phase to operational utility. These include the porous electrode electrostatic precipitators and the liquid partition separator. All of these devices were evaluated during this program in terms of properties required to meet the ultimate requirements for BW aerosol detection.

2.3 PROGRAM MANAGEMENT

2.3.1 SPACE-GENERAL CORPORATION

This program at Space-General Corporation has been conducted in the Chemical and Biological Operations under Dr. E. Mishuck, Senior Division Manager. From its inception in May 1963 through September 1964, Dr. Mishuck served as Program Manager. From 1 October 1964, Program Management has been carried out by Dr. D. J. Sibbett. Figure 2-1 indicates the overall program organization under the Research Task of the contract. In addition, active participation by top

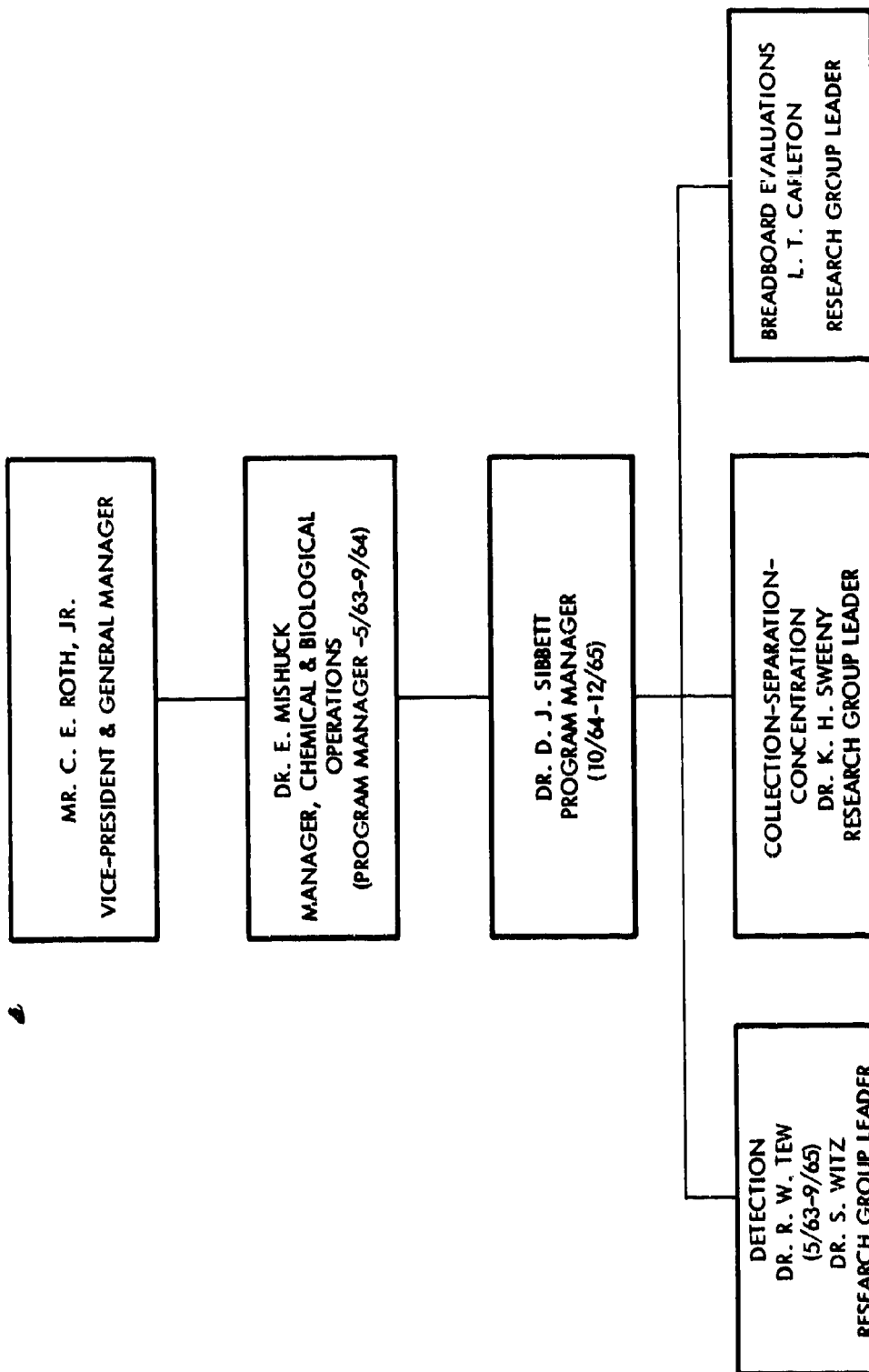


Figure 2-1. Program Organization

Space-General management has played an influential role throughout the program. Prominent among individuals involved were Dr. J. C. Fletcher, former Chairman of the Board of Directors of Space-General and current President of the University of Utah, Mr. F. W. Lehan, President of Space-General and Chairman of the BW Program Technical Advisory Board, Dr. J. E. Froehlich, Executive Vice-President of Space-General, and Mr. C. E. Roth, Jr., Vice-President and General Manager of Space-General.

At the program level, Mr. L. T. Carleton has been responsible for Breadboard Evaluation, Drs. S. Witz and R. W. Tew have been group leaders for Detection Research. Dr. K. H. Sweeny has supervised research on Collection, Separation, and Concentration methods.

2.3.2 SUPPORTING ORGANIZATIONS

A number of supporting organizations have participated in this effort, providing an integrated, balanced team. Their areas of responsibility and key personnel were as follows:

Beckman Instruments, under Dr. R. Gafford and Mr. A. Strickler, has been responsible for research in the areas of Fermentation Matrix Methods, Bioelectrochemical Methods (Magna), Zone and Thin-Film Electrophoresis, and Density Gradient Centrifugation.

Metronics Associates, headed by Dr. W. A. Perkins, President, has conducted research and development on collection methods including Porous Electrode Electrostatic Precipitators and Electrostatic Spray Collectors and has been responsible for research on Characterization of Atmospheric Background Properties, Electrostatic Separation Methods, Concentration in Foams, the Vertical Filter-Concentrator, and the Double Plate Impeller Separator.

Varian Associates conducted research on EPR detection methods.

Hyland Laboratories, under the direction of Dr. T. Asher and Dr. E. Shanbrum, provided the high-titer, highly-tagged fluorescent antisera and carried out research on Fluorescein Isothiocyanate Tagging of Antisera.

The University of California at Los Angeles conducted research on Virus Detection and Purification Methods under the direction of Prof. M. J. Pickett.

Professors D. W. Hill and L. P. Gebhardt at the University of Utah studied methods for Rapid Detection of Group A Arboviruses.

In addition, Bio-Science Laboratories, the International Chemical and Nuclear Corporation, and the Magna Corporation have also contributed, although in relatively limited capacities, to the team effort.

2.3.3 SCHEDULES AND MILESTONES

The overall program plan and schedule as it was carried out since May 1963 is depicted in Figure 2-2. This plan clearly illustrates the inherent flexibility in program direction which resulted from the close liaison between U.S. Army Biological Laboratories at Fort Detrick and Space-General. This liaison allowed rapid adjustments in the technical directions of the various program tasks as changes became desirable on the basis of technical developments.

A detailed breakdown of the manpower loading utilized to fulfill these efforts is presented in Volume II of this report.

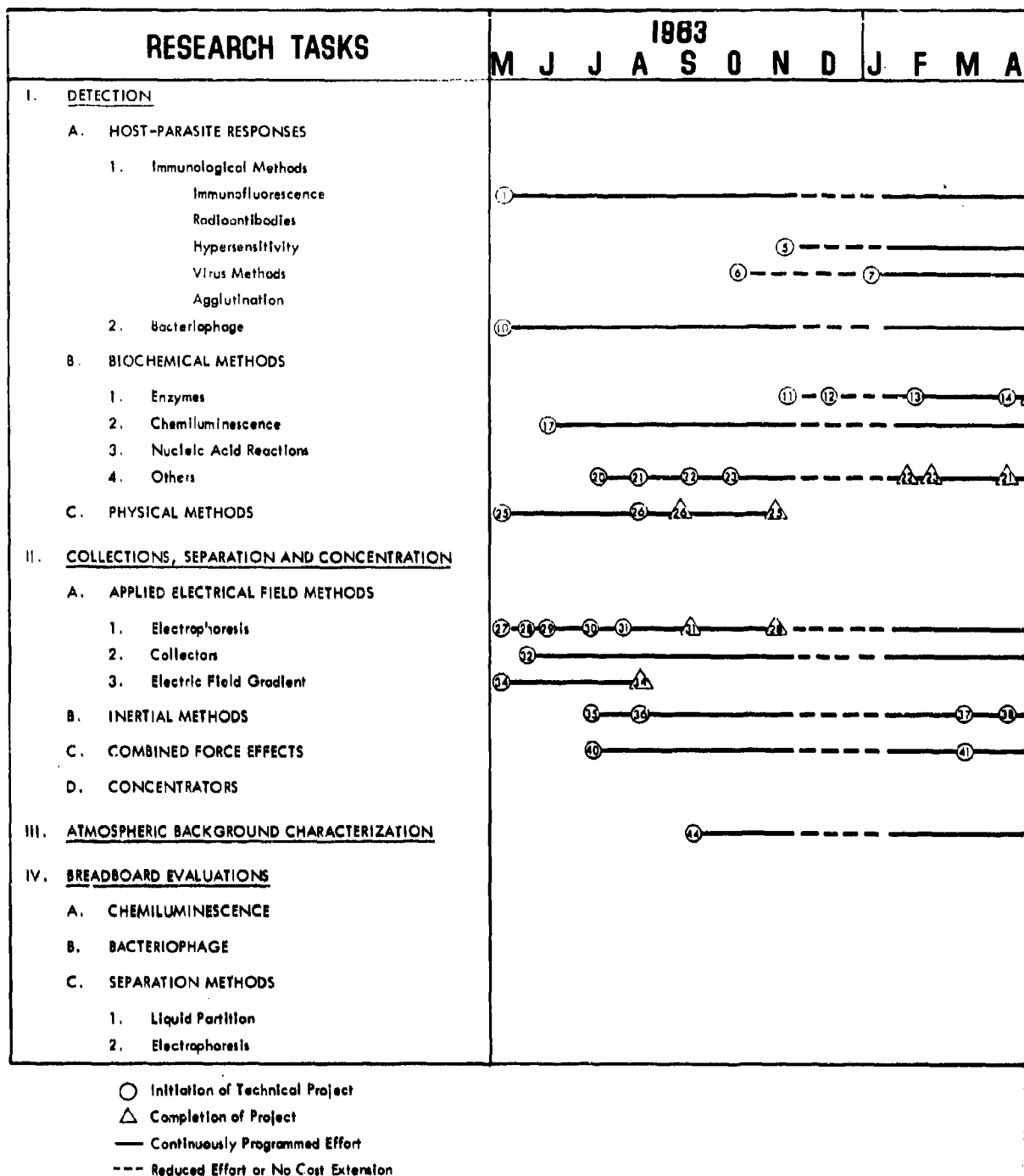
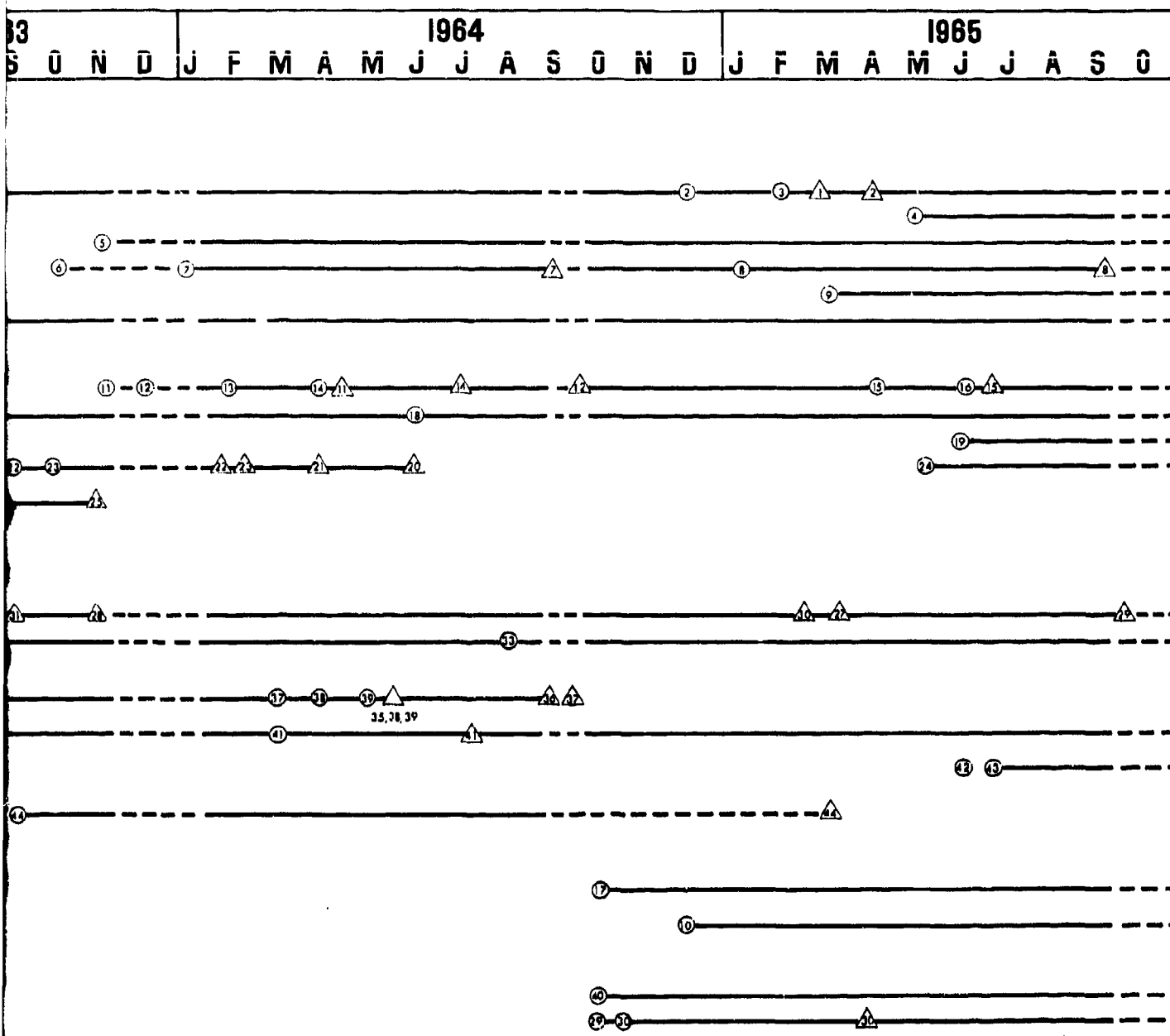


Figure 2-2. Schedule, Research



Legend for Figure 2-2

Identification of Research and Development Efforts

1. FITC Staining Methods
2. Methods Utilizing Labelled Polymer
3. Spinning Disc Research
4. Radioantibodies
5. Hypersensitivity
6. Virus Detection Utilizing Synthetic Particles
7. Virus Purification and Detection (UCLA)
8. Rapid Detection of Group A Arboviruses (U. of Utah)
9. Agglutination
10. Bacteriophage
11. L-Glutamate Dehydrogenase
12. Fermentation Matrix
13. Alkaline Phosphatase
14. C¹⁴-Glucose Fermentation
15. Cytochrome c Reductase
16. Transaminase
17. Chemiluminescence
18. Hemin Staining Methods
19. Nucleic Acid Reactions
20. NH₃ Detection by Electron Capture
21. Quenching of Luminescence
22. Quenching of Fluorescence
23. Phosphorescence of Proteins
24. Sialoresponsin Formation
25. Bioelectrochemical Methods
26. EPR
27. Electromagnetophoresis
28. Zone Electrophoresis
29. Thin-Film Electrophoresis
30. Magnetically Stabilized Electrophoresis
31. pH - Density Gradient Electrophoresis
32. Porous Electrode Electrostatic Precipitators
33. Electrostatic Spray Collectors
34. Electric Field Gradient Separation
35. Double Plate Impeller Separator
36. Density Gradient Centrifugation
37. Open Cell Foam Classifier
38. May Pre-Impinger
39. Cyclone Classifier
40. Liquid Partition
41. Concentration in Foams
42. Sonicating Washer-Concentrator
43. Vertical Filter-Concentrator
44. Atmospheric Background Properties

Section 3

SUMMARY

The technical accomplishments of the Research Program on BW Detection, Task II, are summarized in this section. For convenience, the sections in which the expanded discussion is located have been indicated in parentheses after the title of each task.

Following the task summaries, the dispositions of the various tasks are compiled in Table 3-1. This table identifies tasks as either originally proposed or added during the course of the program. It also gives briefly the most recent assessment of their possible usefulness, and indicates the termination of a number of the least promising.

The successful conclusion of this effort is the result of a well-organized program conducted by a highly qualified team under the direction of Space-General with active participation by top management for all team members. The major accomplishment of this program, in a general sense, was a demonstration that a broadly multidisciplinary group could be focussed on the problems of BW detection and could effectively prosecute a program which extended from basic research on one extreme to the production of practical operating devices on the other.

Summary details of these accomplishments follow.

FLUORESCCEIN ISOTHIOCYANATE STAINING (4.1.1.1)

A quantitative photographic method has been developed for measuring the emission of fluorescent antibody-stained organisms. The minimum exposure time necessary to give barely detectable images correlated well with visual estimates of intensity. Staining time and titer were related quantitatively at 25°C for Hyland fluorescent antiserum and B. globigii spores. Minimum exposure times of one second (4 + reaction) were recorded for spores stained for six seconds with undiluted antiserum. At an antiserum dilution of 1/200, 4 + reactions were observed for spores stained for two minutes.

Excitation with near UV instead of blue-violet illumination reduced background emission so that stained preparations evidenced a signal-to-noise ratio of 100/1 instead of 5/1. The intensities of the stained organisms were essentially identical in both cases.

During research on fluorescent antiserum production, kinetic studies confirmed that lyophilized conjugates were more stable than material stored at refrigerator temperatures, and that gamma globulin conjugation rates were independent of FITC purity. Rates with globulins of different animal species may differ significantly. Conjugation sites of two different affinities for FITC appeared to be involved in reactions of the fluor with globulins. Hyland laboratories produced reactive fluorescent antisera for BG in bulk quantities. Fluorescent antisera for B. globigii and S. marcescens yielded certain cross reactions which were eliminated by dilution and by pretreatment with normal rabbit serum.

IMMUNOFLUORESCENT METHODS UTILIZING LABELLED POLYMERS (4.1.1.2)

Polylysine (MW 63,000) was reacted with fluorescein isothiocyanate (FITC) to give a product which was used to stain B. globigii (vegetative) cells non-specifically. The molar fluor/polylysine ratio was between 10 and 16 to 1. Vegetative cells were more intensely stained than spores, and live vegetative cells appear to have greater affinity for the tagged polymer than dead cells. However, unless higher fluor/protein ratios are achievable, this method appears of limited utility.

SPINNING DISC DETECTOR (4.1.1.3)

The basis for a spinning disc detector has been demonstrated, utilizing polystyrene discs to which rabbit Anti-B. globigii globulin had been heat fixed at 51°C. The rotating discs picked up from suspensions an average of 22 BG spores per 100 μ^2 field, whereas only 0.07 atmospheric background particles per 100 μ^2 field were retained. In addition, no significant adherence of E. coli was detectable under the same circumstances. A systems analysis utilizing a fluorescent readout signal suggests that 1100 liters of air will be required to detect one organism per liter.

A prototype spinning disc collector with a 3.2-cm disc diameter was constructed for initial experimentation. Three alternative detector designs have been examined as possible components of an eventual detection system.

HYPERSENSITIVITY (4.1.2)

Monocytes sensitized to a number of different antigens were employed to test a detection scheme based on hypersensitivity. Increased uptake of trypan blue dye by the sensitized monocytes was shown in the presence of specific antigen. Monocytes from tuberculin positive human donors, a human subject sensitized to rabies virus contained in duck embryo, and animals sensitized with Bacillus globigii, Newcastle Disease virus, and Brucella suis were tested. Results indicated that 1/5 organism per liter of air could be detected.

Methods for large scale production and storage of sensitized monocytes were examined. Thus far, the method of choice for preservation appears to be gradual freezing, followed by cold storage. In a study of adaptation of the method to fluorimetric instrumentation, four fluorescent dyes were found more or less satisfactory, with acridine yellow the best. Monocytes treated with acridine yellow and deposited on microporous tape gave strong signals in an automated fluorescent scanning device.

BACTERIOPHAGE (4.1.3)

A bacterial detection method based on P^{32} -labelled bacteriophage attachment was evaluated. The literature indicated that specific phage were available or easily attainable for almost all potential BW agents. High titered preparations of P^{32} -labelled coliphage containing 10^{-8} to 10^{-9} uc per phage have been repeatedly prepared.

Detection sensitivity was shown to depend on reaction rates between phage and bacteria and on the efficiency of separating excess unattached phage from phage-bacteria complexes. Reaction kinetics showed that labelled phage at a concentration of approximately 10^8 /ml would attach to 10^4 bacteria

within 5 minutes, giving a readily measurable signal. Filtration was emphasized as a method for removing excess phage after reaction. The major problem was non-specific adsorption (NSA) of phage to the Millipore filter membrane, which was influenced by filtration media and procedures, state of the phage preparation, and filter characteristics. Under optimal conditions NSA was reduced to 0.004%, permitting easy detection of 10^4 bacteria/ml. Zonal centrifugation and liquid partition methods also appeared promising for separation.

Studies were initiated on detection of bacterial pathogens with labelled phage. Instrumentation of this method demonstrated continuous detection of 10^5 to 10^6 E. coli/ml, with detection of as few as 10^4 bacteria/ml appearing obtainable.

CHEMILUMINESCENT DETECTION SYSTEM (CDS) (4.1.4.1)

The detection sensitivity of the continuous chemiluminescent detection system is of the order of 10^3 to 10^4 B. globigii (vegetative) per minute at a concentration level of 10^4 /ml (Petroff-Hausser count). Further improvement in sensitivity appears possible through use of disrupted cells and a reactor cell design which insures more rapid and efficient mixing. A preliminary evaluation of the effect of background indicated that the luminescence of the blank is increased by less than 20 percent on going from filtered to unfiltered air. Nebulized B. globigii can be detected at a level of 10^4 /ml or 10^4 to 10^5 per minute.

Without modification, the detector can also be used for detection of virus carrier with a demonstrated sensitivity of 10^{-9} gm egg solids/ml (corresponding to 3×10^3 , 1μ or $30,5 \mu$ egg particles). HeLa cells (3×10^4) were also detectable by luminol chemiluminescence at an S/N luminescence ratio of 2/1 (in a static system).

ALKALINE PHOSPHATASE (4.1.4.2)

The present level of detection of a continuous fluorimetric assay system based on Na β -naphthyl acid phosphate as substrate is 10^4 to 10^5 B. globigii (veg)/ml (10^2 to 10^3 bacteria in a 4μ l. viewing cell) within a 3-minute

reaction time. Other organisms which were found to exhibit detectable phosphatase activity at a level of 10^4 to 10^6 bacteria per ml in a 5-minute reaction time include E. coli, S. marcescens, Leucothrix sp., S. cerevisiae, and P. fluorescens. Spores (10^5 to 10^6 /ml) of B. globigii and B. cereus also exhibit phosphatase activity without cell disruption.

L-GLUTAMATE DEHYDROGENASE (4.1.4.3)

The minimum number of bacteria detectable by the L-glutamate dehydrogenase reaction is about 10^6 BG in a 2-ml reaction volume. Increase of sensitivity by use of microvolumes is precluded by the low intensity of the DPNH fluorescence produced, and further work does not appear warranted.

CYTOCHROME C REDUCTASE (4.1.4.4)

The existence of the DPNH-cytochrome c reductase enzyme in B. globigii (vegetative) was established by a spectrophotometric technique, and this system was then investigated for its ability to serve as a free radical initiator of luminol chemiluminescence. Although 4×10^{-8} g of this enzyme could be detected by the luminol reaction, the reductase is at least two orders of magnitude less effective (on a weight basis) than catalase as an initiator of chemiluminescence.

TRANSAMINASES (4.1.4.5)

A fluorimetric assay technique for glutamate-oxalacetate transaminase activity has been found to be sufficiently sensitive to detect 2×10^4 /ml B. globigii or 80 bacteria in a 4 μ l volume. Further development appears justified.

FERMENTATION MATRIX (4.1.4.6)

A fermentation matrix shows the capabilities of 24 pathogenic and non-pathogenic bacteria for fermenting 23 substrates. Its purpose is to distinguish pathogens via their characteristic reaction patterns. A mathematical analysis of the concept showed that it might distinguish single organisms readily, but is distinctly less attractive for distinguishing one class or mixed

group from another (e.g., pathogens in general from non-pathogens). Hence, it is not now recommended for further development.

Concurrent experimental work demonstrated that fermentation is measurable with adequate sensitivity through changes in pH. In the model reaction of E. coli with glucose, bacterial concentrations of 3×10^4 to 10^6 per ml appeared detectable in test volumes of 200 μ l. The threshold of detectability lies at responses of a few-thousandths units of pH per minute. Further improvements in sensitivity might be realized.

C^{14} -LABELLED GLUCOSE (4.1.4.7)

A detection scheme based on the metabolic production by bacteria of $C^{14}O_2$ from C^{14} -labelled glucose has been used to detect as few as 5×10^4 B. globigii in a 10-minute reaction time. However, indications of appreciable activation lags with some bacteria led to termination of work on this principle.

NH_3 DETECTION BY ELECTRON CAPTURE (4.1.4.8)

Although possessing a high sensitivity (10^2 bacteria), the use of electron capture for detecting trace amounts of NH_3 by complexing it with an acid suffers from lack of reproducibility owing to the ease with which the latter is hydrolyzed or absorbed on various surfaces.

CHEMILUMINESCENT DETECTION SYSTEM BASED ON HEMIN TAGGING (4.1.4.9)

The sensitivity of chemiluminescent detection utilizing a hemin-tagged antibody conjugate was found to be approximately 10^5 bacteria (BG spores). Direct staining of agent with hemin chloride, however, permitted detection of either 10^4 BG spores or 10^4 LD₅₀ viral (NDV) particles in static and flow systems. Hemin tagging of embryonated egg, B. globigii (vegetative) or S. marcescens increased the respective chemiluminescent signals from luminol- H_2O_2 by about two- or three-fold (in a static system).

SIALORESPONSIN RELEASE AS AN EARLY DETECTION SCHEME OF VIRUS INFECTION (4.1.4.10)

Approximately 4000 hemagglutinating units of Newcastle disease virus (corresponding to approximately 10^8 LD₅₀ infections NDV particles) was detectable within 15 minutes after infection. A colorimetric assay method for neuraminic acid was utilized. Higher sensitivities for this method are cited by another investigator (Bogoch) for influenza PR8. A C¹⁴ tracer assay method is discussed which is capable of detecting 0.01 to 0.1 HA unit.

QUENCHING OF LUMINESCENCE (4.1.4.11)

Sulfhydryl groups in bacteria can decrease chemiluminescence of a luminol-diphenyl picrylhydrazyl system. This principle was used to detect 10^4 bacteria. Objection has been raised that this system lacks specificity, insofar as components other than sulfhydryl groups may cause quenching of the DPPH-luminol chemiluminescence. These efforts were terminated.

FLUORESCENCE QUENCHING OF EOSIN Y BY PROTEIN (4.1.4.12)

A method which involves quenching of Eosin Y fluorescence by protein was used to detect 10^5 bacteria/ml. It was not studied further because of inherent disadvantages.

PHOSPHORESCENCE OF PROTEINS (4.1.4.13)

Phosphorescence of proteins excited by ultraviolet light at liquid nitrogen temperature was used for detection of 10^6 B. globigii (viable, vegetative). Further studies are needed to determine effects of impurities on background, sensitivity, and multi-agent capability of this principle.

RADIOTAGGED ANTIBODY METHODS (4.1.5)

Antibody preparations have been successfully labelled with S³⁵-sulfanilic acid. Initial experimental data have been obtained on some of the properties of these preparations, their reaction with specific antigen, and level of non-specific adsorption to a microporous Millipore filter tape. It was determined that ~180 antibody molecules attach per Bacillus Globigii spore

per minute and that there were at least an average of 41,000 antigenic sites per organism. Preliminary experiments showed as little as 0.014 percent non-specific adsorption of antibodies to a microporous filter tape. It was estimated that at least 10^4 B. globigii could be detected with S^{35} -labelled antibody within a 5-minute period at a 2 to 1 signal-to-noise ratio.

NUCLEIC ACID METHODS (4.1.6)

It has been shown that both RNA and DNA can be efficiently concentrated from dilute solutions of 0.1M salt by single passage of the solutions through 0.1 ml beds of either strong or weak base anion exchangers at flow rates of 15 ml/minute. The beds can be washed with 1M NaCl without removing significant quantities of adsorbed nucleic acid, but treatment with dilute base readily elutes the nucleic acid from the polyamine exchangers, Amberlite IR-45 and CG-4B. Some preliminary evidence of uptake of Ca^{45} , dependent on amount of nucleic acid adsorbed to Amberlite CG-4B, has been obtained. The estimated detection sensitivity of the technique described is at least one bacterium per liter of air in 5 minutes or less. A modification which could increase the sensitivity, possibly to the point of detection of single virus particles, is discussed as well.

VIRUS DETECTION UTILIZING SYNTHETIC PARTICLES (4.1.7)

Several methods employing sensitized polystyrene latex particles were evaluated for detecting viruses. In the competitive inhibition principle, the sensitized beads were reacted either concurrently or sequentially with untagged virus (to be measured) and isotope-labelled virus. From 10^6 to 10^7 virus particles/ml could be detected by measurement of retained radioactivity.

Initial attempts to detect virus concentrated on beads with FITC-labelled antibody were unsuccessful, mainly because of the limited number of virus particles attainable per individual bead (1 to 2). A procedure utilizing radioisotope-labelled antibody could integrate counts from many particles, and give stronger signals.

AGGLUTINATION (4.1.8)

A method based on agglutination of antibody-coated particles in the presence of specific antigen has been evaluated. A number of methods for measuring agglutination have been considered, but experimental efforts have emphasized the use of a modified Coulter device for this purpose. The ability of this device to measure the concentration of particles of different sizes is based on changes in electrical conductivity caused by passage of the particles through a small pore through which an electrical current is generated.

It was estimated that as few as 10^4 virus particles could be detected by the use of the Coulter device and as little as 0.01 μg of egg protein was observed to cause a visible agglutination of sensitized latex particles within 5 minutes. Experimental evidence also indicates that background interference resulting from atmospheric sources can be held to a minimum. These factors, along with advantages in specificity, multi-agent capability, logistic aspects, and virus detection would appear to rank the method as one of the more promising for BW detection.

ELECTRON PARAMAGNETIC RESONANCE (4.1.9)

One possibility considered for detecting bacterial enzymes was the measurement of free-radical intermediates from enzyme-substrate reactions with electron paramagnetic resonance. Trials in the Varian laboratories failed to detect such intermediates by the EPR technique, and this approach was abandoned.

BIOELECTROCHEMICAL METHODS (4.1.9.2)

An amperometric detection method was developed by Magna, Incorporated. With improved techniques, it gave a characteristic response to B. globigii extracts of 10^{-16} amp/min per cell/ml. At the lowest measurable electrical signal, this corresponds to detection of 5×10^4 bacteria in 5 minutes. Intact cells gave even stronger responses than extracts. Neither general background particulates nor B. globigii spores masked the signals from bacterial extracts. This effort was discontinued to concentrate on other enzymatic methods which appeared easier to develop.

RAPID DETECTION OF GROUP A ARBOVIRUSES (4.1.10)

Basic studies were undertaken at the University of Utah on the processing and properties of Group A arboviruses.

In basic studies conditions were established for optimal propagation of WEE, EEE, VEE, Sindbis and Semliki Forest viruses. Suspensions of viruses were obtained in most instances which titered greater than 1×10^9 PFU/ml. In replication in chick embryo cells, all viruses reached maximum titers in 10 to 12 hours.

The amount and type of protein added to the growth media affected both the growth rate and stability of these viruses; 5 percent calf serum appeared to increase titers and decrease inactivation.

Liquid partition in a two-phase system, density gradient centrifugation, column chromatography, and adsorption to aluminum phosphate were evaluated for concentration and purification. In liquid partition, bottom phase suspensions in some instances contained infectivity titers greater than 1×10^{11} PFU/ml. All of the viruses exhibited apparent densities of 1.195 in the density gradient.

Antisera were prepared against the purified agents, examined by hemagglutination inhibition techniques, and almost all were highly reactive with a high degree of specificity. The most cross-reactive was the Sindbis virus.

STUDIES ON IMMUNOLOGICAL DETECTION OF VIRUS AND VIRUS CARRIER MEDIUM (4.1.11)

A study conducted at UCLA sought to develop methods for obtaining purified antigen and antibody preparations. These could increase specificity and sensitivity in the immunological detection of virus and virus carrier medium.

Fowlpox virus purified by absorption to and elution from cation and ion exchange resins was relatively free of contaminating materials and was both serologically and biologically active. Methods utilizing DEAE-cellulose resulted in the purest virus preparations. The preparative methods preceding column chromatography of the crude virus had little effect on recovery following chromatography. The passive hemagglutination test was of little or no value in the detection and characterization of virus antigen fractions. The complement

fixation, latex agglutination, and latex agglutination-fluorescence tests were very useful and specifically reactive.

Similar procedures were used to fractionate egg antigens. The various fractions were characterized and compared by two-dimensional double diffusion in gel. Antigens common to all of the above-mentioned materials were found prior to discontinuation of this effort.

MAGNETICALLY STABILIZED ELECTROPHORESIS (4.2.1.1)

The technique of magnetically stabilized electrophoresis has been developed to the point where rapid and selective separations of organisms from background and background simulants have been made. This development has been possible because of significant improvements in technique and instrument development. Some of the improvements include an added hydrodynamic flow to decrease the residence time and allow collection control, the use of ion exchange membranes to seal the electrophoretic zone from the electrode pockets where electrolysis products are formed, and a closed system in which sample injection and collection are coupled. In a run with B. globigii cells and Arizona road dust, 95 percent of the organisms were retained in the correct collection tube, while 98 percent of the background was found in the reject collector stream. A similar enrichment at flow rates as high as 1.2 ml/min was shown for BG with actual atmospheric background particulates.

THIN-FILM MICROELECTROPHORESIS (4.2.1.2)

Studies of continuous electrophoretic separation concluded with the recommendation for further exploratory development of the thin-film electrophoresis device studied in the Beckman laboratories.

A small vertical thin-film apparatus with transverse electric field was shown to perform accurately and reproducibility in measuring mobilities of reference particles, including bacteria. (Each type of particle zoned in a characteristic fine line.) As indicated by measured deflections, mobility was invariant with times of operation longer than one-half hour, with field gradient, and with time (or distance) of travel. This behavior appears to confirm the elimination of effects interfering with electrophoresis.

A scaled-up version cleanly fractionated 3- and 4-component mixtures of bacteria and inorganic particulates, injected at 0.25 ml/min. The patterns observed, with additional results on individual inorganics, suggest some hope for a general separation of bacteria from background. Separated bands were photographed. Improved methods of control were operated, including completely adjustable positioning for sample injection, positive elimination of contaminants from electrodes, a reliable system for lateral biasing of flow, and many other features.

ZONE ELECTROPHORESIS (4.2.1.3)

The Beckman laboratories devoted a limited effort to performing zone electrophoresis of bacteria through gels. An apparatus was constructed to operate on a microscope stage under closely controlled conditions, but zoning could not be achieved. Efforts were transferred to continuous thin-film electrophoresis.

ELECTROMAGNETOPHORESIS (4.2.1.4)

The electromagnetophoretic migration of various small particles was briefly examined. Movement occurs through an aqueous medium placed in an electromagnetic field, at velocities depending upon particle conductivity. Velocities of different particles several microns in diameter, e.g., pollens and iron powder, indicated conductivity differences. However, separations according to conductivity alone tend to be masked by the influence of particle size.

pH-DENSITY GRADIENT ELECTROPHORESIS (4.2.1.5)

The combined effects of pH and conductivity (density) gradients have been briefly examined for the selective separation of microorganisms from atmosphere debris. A "focusing" of the organisms into a thin line as predicted by Kolin was observed, but the concept was not examined other than in an initial screening since it was considered, following the screening, not to be sufficiently sensitive, selective, or rapid.

ELECTRIC FIELD GRADIENTS (4.2.1.6)

In the electric field gradient technique, particles containing a permanent or induced dipole will migrate in strongly non-homogeneous fields. A velocity of migration dependent upon the dielectric properties, particle size, etc., suggests a means for separating atmospheric debris from microorganisms. A number of particles simulating atmospheric background, and the bacterial simulants E. coli and B. globigii, were studied briefly and all migrated rapidly. However, further work was not carried out because other methods were considered more selective and offered a better chance of providing a practical separation device.

POROUS ELECTRODE ELECTROSTATIC PRECIPITATOR (4.2.1.7)

The Metronics porous electrode electrostatic precipitator was developed to provide a universal aerosol collector for BW detection systems. The unit depends on the principle, previously discovered by this group, that operation is most effective when precipitation is accomplished with an electric field parallel to the air flow, rather than with the conventional field normal to air flow. Collection of solids is achieved, without moving mechanical parts, in a dilute aqueous collecting fluid flowing through and over a horizontal porous glass disc anode. Potential gradients of 1 to 2 kv/cm are employed. Two units have been evolved on the present program through several model changes. The first has a capacity of about 1000 l/min, and collects the particulates into 5 ml/min of water. The second operates over the range 22 to 60 l/min air flow and 0.1 to 0.33 ml/min water flow. Both have collected 1.3 μ polystyrene spheres into water at efficiencies exceeding 90 percent, under optimal operating conditions. With microorganisms, collection efficiencies have been lower, and material balances on intake air versus exhaust air and collection water indicate some retention within the collector. In a recent test series, the 1000 l/min collector gave an average balance of 85 percent for 8 runs with FITC-stained BG spores. Means of further improvement are under study.

ELECTROSTATIC SPRAY COLLECTOR (4.2.1.8)

Promising results were shown in preliminary tests of a new electrostatic spray collector. This device uses for collection a fine spray produced by running the collecting water through a hollow electrode connected to a high voltage source. In the initial test, a collection efficiency of 39 percent was obtained for 3 μ latex with 45 l/min air flow and 0.5 ml/min water flow. The device is of interest because the particles (e.g. fragile vegetative cells) might be collected in the charged water droplets, without charging the bacteria directly in the high voltage field.

DENSITY GRADIENT CENTRIFUGATION (4.2.2.1)

The separation of bacterial fractions from atmospheric debris by the technique of density gradient centrifugation was briefly examined in the Beckman laboratories. Successful separations of bacteria from airborne background particulates were made, but the separations were not deemed sufficiently rapid for practical use. The research was therefore discontinued in favor of other methods considered more practical, rapid, and selective.

DOUBLE PLATE IMPELLER CENTRIFUGAL SEPARATOR (4.2.2.2)

A centrifugal separator based on a double-plate impeller, which was thought to provide efficient separation of large (> 5 to 8 μ) particles from bacterial aerosols has been described. A device of this type was constructed and evaluated, but was found unsuitable for use in a practical collection subsystem. The sharpness of separation is no better than in the simpler open-cell foam filter; operation of the device is very complex because of the interdependence of several variables; and the holdup appears significant for small quantities of particulates. Further work on this technique was stopped in order that more promising techniques might be studied.

INVESTIGATION OF PRIMARY SEPARATORS (4.2.2.3)

Four types of devices have been shown effective for use as a primary separator in a collection subsystem to remove efficiently the approximately 50 percent by mass of atmospheric background larger than 5 μ . Of the principles tested, impingement on the fibers of open cell polyurethane foam (commercial Scott Foam) appears most practical. Separation in a 60-liter/min air stream can be achieved with a pad 1.5 inches in diameter and 0.5 inch thick of the 60 pore/inch foam. The filter will retain about 90 percent of the 5.6 μ material of density 1.5, while holding only 10 percent of the 2.6 μ material. The pressure drop across such a filter is only 0.07 inch of water, and the capacity appears sufficient for several days of continuous operation. The cut size can be varied over reasonably wide ranges by changing the pore size and face velocity.

The May pre-impinger also appeared satisfactory for a small primary separator. Both the May pre-impinger and open-cell foam appear superior to a standard cyclone or a tortuous path of tubing.

COMBINED FORCE EFFECTS (4.2.3.1)

A continuous liquid partition separator was developed and operated successfully. The operating principle is the opposing distribution of bacteria and background particles between immiscible phases of certain aqueous polymer solutions. This behavior was shown to be general for a large number of species of bacteria. A continuous centrifuge was adapted for rapid disengagement of liquid phases, which pass out as two separate effluents. Total feed rates (sample + polymeric medium) of about 10 to 20 ml/min are attainable. The separating medium was a 2-phase solution of dextran and polyvinyl alcohol. With bacteria alone, more than 90 percent were retained in the dextran-rich effluent under continuous, steady-state conditions. For a binary mixture of bacteria and dust, favorable operating conditions gave a retention of > 90 percent bacteria and removal of >90 percent dust. Additional runs with P^{32} -tagged bacteriophage showed the technique to be adaptable to virus separation. The separator was also coupled with a porous electrode electrostatic precipitator to demonstrate continuous collection and separation of aerosolized BG spores.

An improved centrifugal separator was constructed, and will be operated on a successor program. It is designed to be a more efficient developmental tool than the original separator, having advantages of simplicity, compactness, minimal maintenance, short dwell time, and ease of change of operating variables.

CONCENTRATION OF PARTICLES IN FOAM (4.2.3.2)

A limited feasibility study indicates promise for a method of concentration/separation involving the collection of particulates in an aqueous foam. In tests with 1.3 μ polystyrene latex, 90 percent of the particles were found in the 25 percent of the liquid which was foamed. However, with BG spores, the ratio of concentration in foam to concentration in residual liquid was 1.7 to 3.2 in the initial experiment. A new method of producing foam electrolytically was presented. This may lead to a compact, easily controllable foam separator.

SONICATING CONCENTRATOR-WASHER (4.2.4.1)

A new method for concentrating or washing suspended particulates, which depends on simultaneous filtration and sonication of the suspension, has been demonstrated. Tests showed a quantitative recovery of BG spores; 41 counts per field were recovered out of 42 possible, after washing with 3125 volumes of liquid. Other tests showed, qualitatively, a retention of complexes of BG spores in a liquid concentrate, while unreacted antibodies were either filtered out or held on the filter. In any case, an easy scheme of separation is provided.

VERTICAL FILTER-CONCENTRATOR (4.2.4.2)

Metronics Associates demonstrated a concentrating device which removed water by continuous filtration along a vertical membrane strip. The recovery of suspended particles and liquid concentration factors varied widely with operating conditions. This approach to the problem of concentration was discontinued in favor of other approaches.

CHARACTERIZATION OF ATMOSPHERIC BACKGROUND (4.3)

The particles present in a normal atmosphere should not interfere with the detection of biological pathogens. A limited study of the characteristics of atmospheric particulates has contributed to an understanding of this need, particularly as applied to detection techniques such as the fluorescent antibody sensing technique (FAST).

Over half of the particulates by weight are larger than 5μ , the usual upper limit of size for oral infection, thus providing a basis for useful size separation of aerosol particulates entering a detector. Further, over 50 percent of the particles were shown to be water soluble on 4-second contact. Since Space-General collection schemes have been based on liquid collection-concentration-separation, it is clear that a large number of particles will be removed by solution on collection. A study of fluorescing properties revealed that, while about 30 particles/l of air will glow, the majority are very dim. The colors are predominately blue, with lesser quantities of orange, red, green, and yellow particles, and many are quite large. The particles which are of a color, brightness, and size potentially to interfere with the FAST technique appear to number substantially less than 1 per liter. The number of viable organisms sampled per liter of air ranged from 0.01 to 0.25 and were mainly spore-forming, gram-positive rods.

Table 3-1

DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
I. DETECTION			
A. Immunofluorescence			
1. FITC Staining	Provides methods for detecting all pathogens		
2. Labelled Polymer Methods		Promising, but initial trial showed little advantage	
3. Spinning Disc Techniques			Promising, selective collection method
B. Hypersensitivity	Sensitive, specific; readily capable of instrumentation		
C. Bacteriophage	Good basis for breadboard, for bacterial agents		
D. Chemiluminescence			Iron Porphyrins occur widely; detection principle is simple, sensitive; breadboarded
E. Enzymatic Methods			
1. L-glutamate dehydrogenase reaction		Fluorescence intensity too low	
2. Alkaline Phosphatase Hydrolysis	Enzyme is widely distributed; potentially sensitive method		

Table 3-1 (Continued)
DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
3. C ¹⁴ -Labeled Glucose Fermentation		Time lag in response of resting cells	Widely distributed in microorganisms; involves metabolic processes
4. Transaminases			
5. pH Fermentation Matrix		May detect bacteria via fermentative capabilities; complex method	
6. Cytochrome-c Reductase		Widespread enzyme; questionable sensitivity	
F. Other Biochemical Methods			
1. NH ₃ Detection by Electron Capture		Poisoning of detector prevents long-term operation	
2. Quenching of Luminescence by S-H groups		Other components of aerosols may give false positives	
3. Quenching of Eosin-Y Fluorescence by Protein		Operational time too long	
4. Phosphorescence of Proteins		Desired sensitivity not attainable	Selective staining; increases ability of chemiluminescence to detect viral agents; sensitive response
5. Hemin Staining			
6. Sialoresponsin Formation			Unique host-parasite response

Table 3-1 (Continued)
DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
G. Radioantibody Methods			Sensitive, selective with multiagent capability; low background interferences
H. Nucleic Acid Methods			Involves ultimate agent composition; simple radio tracer methods available
I. Virus Detection Utilizing Synthetic Particles			Flexible methods allowing multiagent detection
J. Agglutination			Rapid, simple technique with easy readout
K. Physical			
1. Bioelectrochemical detection		Comparatively unpromising	
2. EPR		Too insensitive to detect free-radical intermediates of bacterial enzymes	
3. Spectrophotometry, Fluorimetry			Provides indispensable comparisons of bacteria with interfering background
L. Rapid Detection of Group A Arboviruses (Utah)			General properties of Group A arboviruses indicated unique problems

Table 3-1 (Continued)
DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

Research Concept	Originally Proposed	Terminated	Added
M. Virus Purification (UCLA)		Objectives achieved	
II. COLLECTION, SEPARATION & CONCENTRATION			
A. Electrophoresis			
1. Magnetically Sta- bilized Electro- phoresis	Provides highly sensitive separations; selected for breadboarding		Too sensitive method for field utilization
2. Thin-Film Electro- phoresis			
3. Zone Electrophoresis			Not demonstrated
4. Electromagnetophoresis	Separations based on parti- cle conductivities, may be quite selective		
5. pH-Density Gradient Electrophoresis			Not sufficiently senti- tive, selective or rapid
B. Electric Field Gradient	Offers several separation principles based on con- ductivity and polarity; continuous mechanization possible		
C. Porous Electrode Electro- static Precipitators			Recovers micron-sized particles efficiently; qualified by operating experience

Table 3-1 (Continued)

DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
D. Electrostatic Spray Collectors			Promising method which avoids water distribu- tion aspects of impinge- ment on washed electrodes
E. Inertial Methods			
1. Double-Plate Impeller		Simpler devices give equally sharp separations, less holdup	
2. Density-Gradient Centrifugation		Continuous operations pose engineering problems	
F. Primary Separators			
1. Cyclone Classifiers		Useful, but not as simple as foam	
2. Open Cell Foam Classifier			Proven usefulness; simple, sharp separations; inexpensive
3. May Pre-Impinger		Has possibilities but not as simple as foam	
G. Combined Force Effects			
1. Liquid Partition			Highly selective in separating bacteria and viruses from background; rapid, reliable method
2. Concentration in Foams		Simple, novel method appeared selective in first trials, but with engineering problems	

Table 3-1 (Continued)
DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
H. Concentration by Filtration			
1. Sonicating Washer-Concentrator			Promising, reliable, useful in washing
2. Vertical Filter-Concentrator			Probably effective, little data
III. BACKGROUND CHARACTERIZATION		Supplied useful data; further investigations associated with individual detectors	
IV. BREADBOARD EVALUATION			
1. Chemiluminescence Detector			Based on porphyrin-luminol reaction, rapid, sensitive, non-selective, simple; rugged instrumentation
2. Bacteriophage Detector			Based on bacteriophage research; prototype utilization of tracer techniques, selective
3. Collection-separation Subsystems			
a. Porous Electrode Electrostatic Precipitator (PEEP) Liquid Partition			Based on two former research techniques; promises small, reliable methods for collection and separation of background from biological materials; no sampling rate limitations

Table 3-1 (Continued)

DISPOSITION OF TASKS ON EW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
b. PEEP-Thin Film Electrophoresis			Most promising combination for utilization of electro- phoresis in practical de- vices; for low sampling rate detectors

Section 4

TECHNICAL DISCUSSION

This section presents a summary of the technical accomplishments of the Research Program on BW Detection at Space-General Corporation during the period May 1963 through October 1965. The discussion has been subdivided in terms of its natural components:

- a. Detection Research and Development
- b. Research and Development Related to Collection, Separation, and Concentration Devices
- c. Properties of the Atmospheric Background

The objective of Detection Research and Development projects (Section 4.1) was to examine each technical approach for its feasibility in terms of the ultimate sensitivity, selectivity, multi-agent capability, and ease of instrumentation which might result. The discussion in this area reviews the progress of twenty-six technical tasks which were examined as potential bases for automated detectors. Included in this section are examinations of the breadboard operations which were carried out to study the feasibility factors associated with the Chemiluminescence (Section 4.1.4.1) and Bacteriophage (Section 4.1.3) systems. The methodology applied in this research falls into three main categories: (1) biological phenomena associated with host-parasite responses (Sections 4.1.1 to 4.1.3, 4.1.5, 4.1.7, 4.1.8, 4.1.10 and 4.1.11), (2) biochemical reactions (Sections 4.1.4 and 4.1.6), and (3) physical detection principles (Section 4.1.9).

Research and Development Related to Collection, Separation, and Concentration Devices (Section 4.2) had, as its primary concern, studies of the feasibility of auxiliary devices which would be required for effective operation of various detectors. In general terms, the major function of these devices, whether collectors, separators, or concentrators, is to prepare the aerosol

sample for monitoring by the readout principle. Seventeen techniques have been studied, four as breadboards. One collector, the Porous Electrode Electrostatic Precipitator (Section 4.2.1.7), and three separation principles, Liquid Partition (Section 4.2.3.1), Magnetically Stabilized Electrophoresis (Section 4.2.1.1), and Thin Film Electrophoresis (Section 4.2.1.2), were selected for breadboarding.

The objective of the study of Atmospheric Background Properties (Section 4.3) was to define the atmospheric particulate levels and properties which were of importance as the background against which a BW aerosol must be detectable. This effort was carried out chiefly during the first eighteen months of the program. Data collected indicated that the background problem is partly unique for each individual readout principle which is utilized and requires examination for each system developed.

For the convenience of the reader, all references cited in this section are placed at the end of the technical task discussion in which they are cited. References are indicated in text by superscript numbers in parentheses.

4.1 DETECTION RESEARCH AND DEVELOPMENT

4.1.1 IMMUNOFLUORESCENCE

4.1.1.1 FLUORESCCEIN ISOTHIOCYANATE STAINING

4.1.1.1.1 SUMMARY

A quantitative photographic method has been developed for measuring the emission of fluorescent antibody-stained organisms. The minimum exposure time necessary to give barely detectable images correlated well with visual estimates of intensity. Staining time and titer were related quantitatively at 25°C for Hyland fluorescent antiserum and B. globigii spores. Minimum exposure times of one second (4+ reaction) were recorded for spores stained for six seconds with undiluted antiserum. At an antiserum dilution of 1/200, 4+ reactions were observed for spores stained for two minutes.

Excitation with near UV instead of blue-violet illumination reduced background emission so that stained preparations evidenced a signal-to-noise ratio of 100/1 instead of 5/1. The intensities of the stained organisms were essentially identical in both cases.

During research on fluorescent antiserum production, kinetic studies confirmed that lyophilized conjugates were more stable than material stored at refrigerator temperatures, and that gamma globulin conjugation rates were independent of FITC purity. Rates with globulins of different animal species may differ significantly. Conjugation sites of two different affinities for FITC appeared to be involved in reactions of the fluor with globulins. Hyland laboratories produced reactive fluorescent antisera for BG in bulk quantities. Fluorescent antisera for B. globigii and S. marcescens yielded certain cross reactions which were eliminated by dilution and by pretreatment with normal rabbit serum.

4.1.1.1.2 INTRODUCTION

The objectives of the research on fluorescein isothiocyanate (FITC) staining were twofold: (1) derivation of optimal conditions for the immunological

reaction and its observation, and (2) preparation of conjugated antisera of maximal brightness and titer.

Work toward these objectives has contributed to and benefited from the originally closely related FAST Breadboard task. A requirement for a method for testing the scanning subsystem led to advances in preparation and observation of brightly stained spores on tape. The logistics of FAST detection lent emphasis to the importance of titer. A method for preventing non-specific adsorption of fluorescent antisera by tapes, which was developed under laboratory conditions, contributed significantly to effective operation of the breadboard. The need for quality reagents in quantity lots stimulated research on methods for their production. These are but a few examples of the mutually catalytic effects of the two tasks.

In general, work on improvement of reaction conditions has been carried out at SGC, while aspects of fluorescent antibody production have been studied by Hyland Laboratories. The methods and advice of Dr. Pital of the USABL have contributed very significantly to this research, and are gratefully acknowledged.

4.1.1.1.3 STATUS

4.1.1.1.3.1 OPTIMIZATION OF CONDITIONS OF STAINING AND OBSERVATION

The literature contains surprisingly little information on observation of stained organisms by vertical illumination and even fewer references on the use of porous tape in fluorescent antibody technology. Consequently, it has been necessary to develop optimum conditions for staining suspensions of BG spores, and E. coli and S. marcescens vegetative cells, and for depositing the stained cells and separating fluorescent reagent, without non-specific adsorption to the Millipore tapes. Equipment needed for microscopic observation also required development. On the other hand, procedures for staining organisms fixed to glass slides are well known. Thus, although tape methods have been developed to a very satisfactory state at present, the glass slide procedure was necessarily the method of choice during the greater part of this work. Appendices A.1 and A.2 contain the details of both procedures.

Modification of fluorescence microscopy techniques for observation of slide preparations led to pronounced improvements in the intensity of stained B. globigii spores. These improvements may be attributed to: (1) observation immediately after preparation and use of freshly harvested spores, (2) use of a 100X oil immersion objective with a numerical aperture of 1.10, a 15X ocular for visual observation (total magnification with 1.2X tube factor, 1800X), and a 10X ocular for photography, and (3) employment of UG1 and BG38 excitation and Leitz UV-absorbing emission filters in place of the Corning 5543 and Wratten 16 or BG12-Leitz blue-absorbing filter combinations. The filter substitution brings about excitation in the near UV region instead of in the blue-violet.

The differences between the BG-Leitz blue-absorbing and UG1-BG38-Leitz UV-absorbing filter combinations were reconciled quantitatively. The intensities of stained BG spores were essentially identical in both cases. However, the signal-to-background (noise) ratio with the BG12-Leitz blue-absorbing filters was only 5/1; with the UG1-BG38 and Leitz UV-absorbing filters this ratio was 100/1 as measured by minimum exposure times.

The minimum exposure time method has been a most useful procedure for quantitating fluorescent staining intensity, and thus is an important contribution to immunofluorescence technology. Generally, the photographs were taken to establish the exposure time necessary just to detect the stained organisms; this datum has been termed the "minimum exposure time." Quantitative results obtained by this procedure correlated well with visual estimates, as can be seen in Table 4-1. The visual estimates were made before pictures were taken.

The contribution of autofluorescence or reflectance was evaluated by determination of minimum exposure times (using 3000 speed film) for unstained spores. When the BG12 and Leitz blue-absorbing filters were used, spores were visible on films exposed for 75 seconds. Ninety seconds were necessary when the UG1-BG38-Leitz UV-absorbing combination was employed. In contrast to the latter observation, BG spores stained with fluorescein-tagged antiserum were detected in 0.25 to 0.5 second with the same optics.

Table 4-1

RELATIONSHIP OF INTENSITY, STAINING TIME, AND TITER
Staining Time, Minutes

Conjugate Dilution	0.1		0.25		0.5		1.0		2.0		10.0	
	A*	B**	A	B	A	B	A	B	A	B	A	B
0	4+	1	4+	1	4+	0.5	4+	0.5	4+	0.5	4+	0.5
50	3+	4	3+	2	4+	1	4+	1	4+	0.5	4+	0.5
100	2+	12	2+	8	2+	8	3+	4	3+	2	4+	1
200	1+	26	1+	20	2+	14	2+	6	4+	2	4+	1

* A: Intensity of stained microorganism by visual evaluation

** B: Minimum exposure times in seconds, 3000 speed film

Antigen: B. globigii spores heat-fixed on slides

Conjugate: Hyland F15 L5, FITC

Microscopy: 1800 X visual, 1200 X photographic, UGI-BG38-Leitz
UV-absorbing filters

Minimum staining times are mandatory for detection devices based on immunofluorescence. Data in Table 4-1 on the relationship of staining time (on glass slides) and titer at 25°C not only showed that brief staining intervals of the order of 2 minutes or less could be used, but also indicated a relationship between detection time and logistics. The visual data were reinforced by determination of minimum exposure times.

Studies on the time and temperature relationships of fluorescent staining were performed by the "wet" method, i.e., by reacting suspended bacteria with diluted antiserum. They indicated that, for practical purposes, negligible gains in intensity were realized by reacting the same FITC fluorescent antibody at temperatures greater than 25°C and at a titer of 1:25. The results also suggested that reaction times of less than 6 seconds should be employed during further experimentation on the Q_{10}^* of staining. The data are presented in Table 4-2.

*The temperature coefficient Q_{10} , is the ratio of the reaction rate at $T + 10^\circ\text{C}$ and $T^\circ\text{C}$.

Table 4-2

RELATIONSHIP OF INTENSITY OF STAINED BG SPORES TO STAINING TIME
AND TEMPERATURE AT AN ANTISERUM TITER OF 1:25

Temp. °C	Staining Time, Minutes					
	0.1	0.25	0.5	1	2	10
15	1+	1+	2+	3+	3+	4+
25	2+	2+	3+	3+	3+	4+
35	2+	2+	3+	3+	3+	4+
45	2+	2+	2+	3+	4+	4+

Probably the most important single factor in successful combination of Millipore filtration with staining of bacteria in suspension has been the solution of the serious problem of non-specific adsorption of fluorescent antiserum to the tape. This solution consisted of pretreatment of the tape with whole rabbit serum, or with 0.1 percent gelatin in 0.1M, pH 9.6 bicarbonate buffer. The result of this procedure is a dramatic improvement in signal to noise. In fact, when low dilutions of antiserum were used without pretreatment, stained spores could not be seen against the brightly fluorescing background. A second improvement was the use of pH 9.6, 0.5M buffer for rinsing and an "aftertreatment" with pH 9.6 glycerol saline mounting solution. With these improvements, signals from spores stained with 1/200 dilutions of antiserum for 3 minutes at 25°C were strong enough to be detected by the FAST Breadboard scanning system. The spores were easily visible when examined at 500X magnification under a Leitz monocular microscope provided with an above-stage darkfield mirror condenser and UG1, BG38, and Leitz UV-absorbing filters.

4.1.1.1.3.2 RESEARCH ON ANTISERA

High-titer antisera are of great significance for logistics and for operational reasons, including minimization of reaction times, antiserum

blanks, background readings, and cross reactions. Hyland's recommended procedure for preparation of fluorescent antisera is contained in Appendix A.3.

Initially, the rooster was used for production of antisera for both BG and P. tularensis. Later, antisera to BG were produced in rabbits and a goat. The improvement in brightness of anti-BG conjugates resulting from the use of rabbits was noteworthy. Hyland's immunization schedule is indicated in Appendix A.4.

An understanding of conjugation and conjugate dissociation rates was essential to improvement of the quality of fluorescent antisera. Work on these problems was carried out at Hyland Laboratories. Their conclusions may be summarized as follows:

Optimum dye/protein ratios lie between 15 and 24 micrograms of fluor per milligram protein. Below this range poor conjugation occurred, as evidenced by poor staining of spores. Above this range intensity of staining decreased, presumably owing to masking of antibody reactive sites.

Fluorescein isothiocyanate (FITC) conjugated with serum proteins at first-order rates when the percentage of protein equalled 1.5 and the ratio of reactants was 8:1. A break in the curves occurred at 100-200 minutes. The presence of the isothiocyanate moiety at either the para or the meta positions of fluorescein had little effect on rates. Assuming covalent bonding, the apparent order of numbers of binding sites was: bovine albumin > goat globulin > bovine globulin > rabbit globulin > chicken globulin. Dissociation was small, little influenced by temperature, and decreased eightfold from pH 9.0 to pH 4.6.

The break in the first-order curves for conjugation is in agreement with the literature indication that sites of two different affinities are involved. Therefore, (a) k_1 may represent the reaction of FITC with one set of globulins and k_2 the reaction with another, or (b) k_1 may represent covalent bonding of fluor to protein (formation of a thiocarbamide bond between fluor isothiocyanate and protein amino moieties) and k_2 the adsorption of fluor by protein. The data are presented in Table 4-3.

Table 4-3

FIRST-ORDER RATES OF REACTION OF FITC WITH VARIOUS SERUM PROTEINS
AT 5°C pH 9.0, IN CARBONATE-BUFFERED SALINE

Protein	Protein Conc., %	F/P Ratio	First-Order Rate Constants of Association, moles FITC/mole protein/minute		Relative Number of Sites Binding Covalently
			$k_1 \cdot 10^{-2}$	$k_2 \cdot 10^{-4}$	
Goat globulin	1.5	8:1	7.8	7.8	6
Bovine II (globulin)	1.56	8:1	4.3	4.7	5
Rabbit globulin	1.76	16.45:1	11.5	3.34	4
Chicken globulin	1.4	8:1	--	3.66	2
Bovine V (albumin)	1.5	8:1	5.05	1.9	10
Human 7S globulin	1.5	8:1	--	43.	10

The rate of reaction of pure gamma globulin (human 7S) with FITC appeared independent of the use of chromatographically pure material or of a grade containing two isomers. Also, significant differences in reaction rates between globulins of different species were noted.

Table 4-4 illustrates the comparative stability of three aliquots from a rabbit globulin conjugate, where one aliquot was frozen and subsequently thawed, the second was frozen, frozen-dried, and reconstituted, and the third was maintained in the liquid state at 5°C. Clearly, frozen-dried material dissociated less than the frozen and subsequently thawed conjugate. Frozen and thawed preparations demonstrated a loss of approximately one-third of the originally conjugated FITC (fluorescein isothiocyanate) six days after thawing. Only approximately 10 percent of reconstituted lyophilized serum showed dissociation after six days.

4.1.1.1:3.3 CROSS REACTIONS AND POLYVALENCY

Cross reactions and polyvalency of antisera are important problems for any detection system based on immunofluorescence. Cross reactions are desirable if they contribute to detection of more than one pathogen. In fact, antisera to individual pathogens may be mixed deliberately to bring about just such a capability. In this case, the sera are termed "polyvalent". Obviously, cross reactions are undesirable if non-pathogenic species are stained as brightly as pathogens.

Fluorescent antisera (rabbit) for Serratia marcescens cross-reacted significantly with Staphylococcus aureus but not with representatives of a number of other bacteria families. Antisera prepared in rabbits for B. globigii yielded significant cross-reactions with both S. aureus and a gram positive spore-former from the El Monte atmosphere. The evidence from experiments performed on glass slides is presented in Table 4-5. Cross-reactions or non-specific staining were eliminated by dilution, by pretreatment with rabbit serum, or by both measures. Sera divalent for SM and BG were prepared so that the final dilution of each component would be 1/10 and 1/50. These sera were successfully used to detect both SM and BG on experimental tapes, glass slides, and the FAST Breadboard.

Table 4-4

DISSOCIATION OF FITC FROM METHANOL-FRACTIONATED RABBIT GLOBULIN
AT 5°C IN PHOSPHATE BUFFER

Buffer Change *	Frozen-Dried		Frozen (-25°C)		Liquid	
	D ⁴⁹⁰ **	mg FITC ***	D ⁴⁹⁰	mg FITC	D ⁴⁹⁰	mg FITC
1	0.43	0.00133	0.72	0.00233	0.45	0.00128
2	0.37	0.00108	0.37	0.00108	0.37	0.00108
3	0.36	0.00103	0.28	0.00085	0.37	0.00108
4	0.32	0.00090	0.37	0.00108	0.35	0.00100
5	0.36	0.00103	0.34	0.00098	0.37	0.00108
6	0.33	0.00095	0.36	0.00108	0.37	0.00108
7	0.37	0.00108	0.33	0.00095	0.34	0.00113
8	0.36	0.00103	0.36	0.00103	0.35	0.00100
9	0.35	<u>0.00100</u>	0.38	<u>0.00113</u>	0.32	<u>0.00090</u>
Total mg FITC		0.00943		0.01048		0.00963
FITC added as internal standard		0.00900		0.00900		0.00900
Percent available FITC dissociated ****		9.86		33.6		14.4

* Buffer changed and scanned (490 mμ) at irregular intervals over six day period.

** Optical density at 490 mμ.

*** mg in 50 ml buffer.

**** Total of 0.00441 mg FITC in 3.5 ml sample, conjugated to 49.8 mg protein for F/P ratio (molar) of 4:1.

Table 4-5

CROSS REACTIONS OF B. GLOBIGLI
AND S. MARCESCENS ANTISERA
PRODUCED IN RABBITS

Organism	SM Antiserum		BG Antiserum	
	Dilution	Reaction	Dilution	Reaction
<i>B. globigii</i>	0	0	0	4+
<i>S. marcescens</i>	0	4+	0	0
<i>Corynebacterium xerosis</i>	0	0	0	0
<i>Chromobacterium violaceum</i>	0	0	0	1+
<i>Pseudomonas aeruginosa</i>	0	0	0	0
<i>Sarcina lutea</i>	0	0	0	0
<i>Flavobacterium</i> spp.	0	0	0	0
<i>Alkaligenes faecalis</i>	0	0	0	1+
			1/10	0
<i>Streptococcus faecalis</i>	0	0	0	0
<i>Streptomyces griseus</i>	0	0	0	0
<i>Mycobacterium phlei</i>	0	1+	0	0
	1/5	0		
<i>Neisseria perflava</i>	0	1+	0	0
	1/5	0		
	0+ Rabbit serum			
<i>Lactobacillus</i> spp.	0	0	-	-
<i>E. coli</i>	0	0	0	2+
			1/10	0
<i>S. aureus</i>	0	2+	0, + Rabbit serum	0
	1/5	2+	0	3+
	1/10	2+	1/10	2+
	0, + Rabbit serum	0	1/50	1+
			0, + Rabbit serum	1+
<i>Cl. sporogenes</i>	-	-	0	0
Gram positive spore former from atmosphere	-	-	0	3+

4.1.1.1.4 CONCLUSIONS

The research on methods for improving the signal output from fluorescent antibody-stained organisms has established the feasibility of such methods for utilization in rapid detection devices. A quantitative photographic method of measurement of the signal from fluorescent-stained particulates has been used to establish an optimum range of operating conditions for the staining reaction. In addition, methods of producing high titer antisera in bulk have been developed.

On establishment of a separate program for advanced development of the instrumented FAST (Fluorescent Antibody Staining Technique), research on applicable FA staining methods was transferred to that effort.

4.1.1.2 IMMUNOFLOUORESCENT METHODS UTILIZING LABELLED POLYMERS

4.1.1.2.1 SUMMARY

Polylysine (MW 63,000) was reacted with fluorescein isothiocyanate (FITC) to give a product which was used to stain B. globigii (vegetative) cells non specifically. The molar fluor/polylysine ratio was between 10 and 16 to 1. Vegetative cells were more intensely stained than spores, and live vegetative cells appear to have greater affinity for the tagged polymer than dead cells. However, unless higher fluor/protein ratios are achievable, this method appears of limited utility.

4.1.1.2.2 DISCUSSION

A major problem in instrumentation of fluorescent antibody methods is signal brightness. The number of fluorescent molecules of a label such as fluorescein isothiocyanate (FITC) which may be attached to an antibody limits the obtainable signal. This limit could be exceeded if a moiety containing more than one fluorescent molecule could be synthesized to antibody protein.

Two approaches have been briefly examined. Conjugations of fluorescein isothiocyanate (FITC) with poly-L-lysine and polyvinyl alcohol were studied as methods of achieving high tag concentrations on polymers which might be attached to protein. In the latter case, attachment of the fluor to the polymer was not achieved.

The reaction between polylysine (MW 63,000) and FITC was carried out at 25°C for two hours followed by 16 hours at 4°C. Sufficient FITC in acetone was added to react with 1/3 of the amino groups present on 1.6×10^{-7} moles polylysine in 0.15 ml pH 9.0, 0.2M, bicarbonate buffer. The reacted polymer was freed of FITC by rinsing with acetone until this solvent remained clear, and taken up in 0.3 ml pH 4.0 buffer. Biuret determinations indicated that the polymer was recovered effectively by this procedure. Polylysine was added at a concentration of 10 mg per 0.3 ml (final), or 30 mg/ml. Analyses indicated 22.5 and 35.6 mg/ml. Molar fluor/polylysine ratios calculated from the equation

in Nairn⁽¹⁾ were 16 and 10 for the analyzed protein values, indicating that only 10 to 16 of the 240 amino groups per polylysine molecule were tagged.

Three other conjugates were prepared by similar techniques except that sufficient FITC was added to react with 1/2, 3/4, and all the amino acid groups present. The resulting tagged polymers were insoluble in water.

Figure 4-1 is a 15 second exposure of B. globigii vegetative cells stained for 3 minutes with the FITC tagged polylysine. Staining was carried out on glass slides at pH 4.0 followed by rinsing at pH 9.0. Vegetative cells were more intensely stained than spores, and live vegetative cells appeared to have considerably greater affinity for the tagged polymer than dead cells.

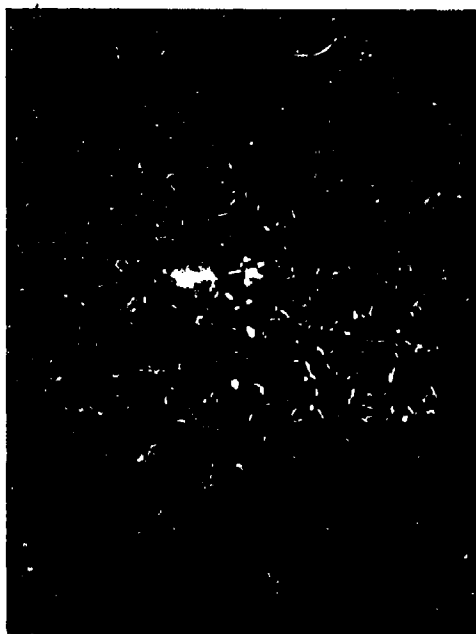
4.1.1.2.3 CONCLUSIONS

It is evident that stained cells are readily distinguishable. However, unless considerably higher fluor/protein ratios can be achieved, non-specific staining with tagged polymer does not seem a promising approach now that new methods for direct FITC staining are available. One possible improvement for this approach might be obtained by use of low molecular weight polylysine.

4.1.1.2.4 REFERENCE

- (1) Nairn, R. C., "Fluorescent Protein Tracing", E. S. Livingstone, Ltd, Edinburg, 1962.

Photography: 15 second exposure of 3000 speed film at 1200X minus demagnification factor of camera. Final magnification 610X. Illumination: Tungsten Darkfield



SGC/629

Figure 4-1. Bacillus globigii Vegetative Cells Stained with Fluorescein Conjugated Polylysine

4.1.1.3 SPINNING DISC DETECTOR

4.1.1.3.1 SUMMARY

The basis for a spinning disc detector has been demonstrated, utilizing polystyrene discs to which rabbit anti-B. globigii globulin had been heat fixed at 51°C. The rotating discs picked up from suspensions an average of 22 BG spores per 100 μ^2 field, whereas only 0.07 atmospheric background particles per 100 μ^2 field were retained. In addition, no significant adherence of E. coli was detectable under the same circumstances. A systems analysis utilizing a fluorescent readout signal suggests that 1100 liters of air will be required to detect one organism per liter.

Conjugation of BG antibodies to carboxymethyl (CMC) cellulose was demonstrated by examination of the adherence of BG spores to treated CMC strips. A prototype spinning disc collector with a 3.2-cm disc diameter was constructed for initial experimentation. Three alternative detector designs have been proposed as possible components of an eventual detection system.

4.1.1.3.2 INTRODUCTION

In concept, a spinning disk device provides a means of concentrating organisms from an aerosol suspension. It is so simple and effective that the problem of detection readout assumes secondary importance.

In use, a device is visualized consisting of a rotating disc to which antibody has been attached. The disc rotates at such speeds that agents which are added near the center in the fluid from a collector will specifically react with the antibody and be retained while other particulates pass over the sensitized surface and are removed at the edge with the liquid.

Modifications in the geometry of the conjugated surface may be made according to the degree of sensitivity desired. Antibody may be conjugated to bands of a moving microporous tape, to a thread, or to the points of a rotating single or multipointed configuration. Alternatively, antibody may be attached to solid particles, which may be used to concentrate viruses from a liquid phase, and enumerated by the appropriate instrumentation.

The device must also provide a means of pre-treating the sample stream to label the microorganism to be detected. Any number of signal-generating procedures may be applied, since specificity and sensitivity reside largely in the concentration step. For example, signal may be generated by addition of fluorescent or radioactive antibody, possibly purified by passage through columns conjugated to the specific antigen. The concentrated agents may be stained with dyes, such as acridine orange. Alternatively, they may be reacted with hematin to generate a luminescent signal (luminol), or with oxidase reagent (sulfanilic acid) for detection of rickettsia and bacteria in collector fluid to which β -naphthol and lysozyme have been added. Other similar approaches which generate a simply quantitated signal may be employed.

4.1.1.3.3 STATUS

The basic objective has been establishment of the feasibility of a BW agent detection system based on conjugation of specific antibody to circular bands on a rotating disc. This objective has been approached by (1) evaluating the principle by a brief systems analysis, then (2) testing the key assumptions in the systems analysis by laboratory experiments with model systems and prototype spinning disc device. The experiments were designed primarily to show sensitivity, though initial studies have been performed on multi-agent capability and background interference.

4.1.1.3.3.1 SYSTEMS ANALYSIS

The systems analysis indicated that the concept was feasible if a few assumptions were valid. This conclusion was developed as follows for a system with a simple line scan and fluorescent readout:

If S_1 is light from background plus organisms, and S_B is light from background alone,

$$S_B = Ab \quad (1)$$

where A is the area of the field and b = background brightness per unit area, and:

$$S_1 = n \sigma a + b (A - na) \quad (2)$$

where n is the number of organisms per field, a the area of one organism, and σ the organism brightness per unit area.

$$\text{Then, } \frac{S_1}{S_B} = \frac{n\sigma a + b(A-na)}{Ab} = n\left(\frac{\sigma}{b}\right)\frac{a}{A} + 1 - n\frac{a}{A} = 1 + \left(\frac{\sigma}{b} - 1\right)n\frac{a}{A} \quad (3)$$

Assuming a signal-to-noise ratio of 50, and a value for $S_1/S_B = 1.05$ (reasonable for a sector configuration having 100 sectors),

$$1.05 = n\left(\frac{49}{10}\right) + 1 \quad (4)$$

Thus, $n = 10$ organisms per 100 μ field.

At a 1-cm radius, the area of the band would be $3.14 \times 10^6 \mu^2$. The area of a 100 μ field is $0.78 \times 10^4 \mu$. Therefore, there would be 4.2×10^2 fields in the band area, and, with 10 organisms per field, 4.2×10^3 organisms would be required. Concentration of this many organisms would be cumulative over a period of 5 minutes. Thus a collection rate of 1.1×10^3 liters of air per minute (one organism per liter) would be necessary at a collection efficiency of 80 percent. Such rates and efficiencies are attained by the porous electrode electrostatic precipitator.

These results are highly encouraging in that they imply a small, inexpensive, sensitive, and selective detection device adaptable to the modular concept in automation. However, the two key assumptions have not yet been stated. These are (1) that antibody may be conjugated to the band in sufficiently high concentrations, and (2) that all or a high percentage of organisms collected will adhere to the band.

4.1.1.3.3.2 REACTION RATES

As the literature pointed out a number of times ^(1,2), no quantitative measurements on the rates of reaction between protein antigens and antibodies are available. This omission makes impossible any theoretical estimate of the appropriate spin velocities of the antibody-coated discs. The recently reported work by Froese and Schon ⁽¹⁾ with the hapten 4,5-dihydroxy-3 (p-nitrophenylazo)-2,7-naphthalene disulfonic acid (DHNS-NP) indicates that reaction rates for the combination of rabbit anti-p-nitrophenyl antibodies

with DHNDS-NP in solution are within 10 percent of the collision rate between the antibody and the dye. If this efficiency can be maintained for reaction between antigen and antibody on a surface, reaction times in the micro-millisecond range should be achievable. The actual time required for an antigen to be picked up by a surface-attached antibody will be a function of (1) antibody surface concentration, (2) liquid film thickness (and related flow properties of the surface layer), and (3) the reaction efficiency of collisions between antibodies and antigens.

It appears probable that the absolute rate of antigen attachment to the antibody surface will not be a major factor in development of the spinning disc method. However, the need for appropriate data to justify such a conclusion is obvious.

4.1.1.3.3.3 LABORATORY INVESTIGATIONS

One of the key assumptions of the systems analysis was that antibody may be conjugated to the band in sufficiently high concentrations. It follows that the materials of which discs are made will be an important factor in obtaining high percentages of adherence of agents, and, of course, will be an important factor in determining rotation rates and disc diameter. Laboratory experiments have been performed to establish the efficiency of antibody conjugation to surfaces and the operability of these surfaces in disc form in an experimental rotating disc device. Two approaches have been followed. The first involves conjugation of antibody to cellulose derivatives, which represent materials offering relatively high impedance to liquid flow. Polystyrene, a material offering low resistance to flow, was the material chosen for the second approach. Results are presented in the following sections.

Conjugation to Cellulose Derivatives - Conjugation of proteins to carboxymethyl cellulose (CMC) is well documented in the publications of Weliky, Weetall⁽³⁾, et al. Work at Space-General followed Weliky's precepts in principle, with experimental details modified according to verbal advice of Mr. H. Weetall. The protocols include (1) coupling of benzidine to strips of CMC paper (2.5 meq/cm^2) with N, N'-dicyclohexyl carbodiimide (DCC), (2) diazotization, and (3) coupling to antibody. Encouraging results were obtained when

the CMC-conjugated strips were dipped in a suspension of B. globigii spores (10^7 /ml), rinsed, and examined under vertical darkfield illumination (tungsten) at 500X. The spores were observed in areas measuring roughly 15 or 20 μ^2 , and occasionally were arranged in linear fashion. The globulin used for these experiments had a modified Pearlette precipitin titer of 1/40. In subsequent experiments, the unit processes were greatly improved and more effective globulin conjugation was demonstrated with fluorescent anti-globulin.

Spore Adherence to Antibody Conjugated Polystyrene Discs - The second approach has been to use a material offering low resistance to flow as the substratum for antibody attachment. Polystyrene seemed the logical choice in view of established methodology. The procedure for conjugation of antibody to polystyrene discs was identical to the heat fixing procedure used with polystyrene latex beads (Section 4.1.8).

Discs prepared in this fashion were tested for spore adherence by two methods: (1) by dipping in a spore suspension of known concentration for 10 minutes, followed by rinsing and staining with specific fluorescent antibody, and (2) by actual operation of an experimental spinning disc prototype device. This device is illustrated in Figure 4-2.

Immersion in Spore Suspensions - The results of experiments performed by the first method indicate that the minimum number of organisms per field needed for line scanning has been exceeded, although only 1.86 percent (or less) of the spores needed to cover completely a 1-cm² area were noted. No organisms were seen on any of the unconjugated controls.

The efficiency of spore adherence appears in a much more favorable light if calculated from consideration of the total antibody which may be attached. If antibody molecules are packed on end, 2.8×10^{17} molecules or 7.6×10^{-2} grams would be needed to cover a 1-cm² surface. Packed sidewise, 1.7×10^{16} molecules or 4.6×10^{-3} grams would be required. If the conditions of Test Number 1A (Table 4-6) pertain, the 0.3 ml globulin used amounted to 0.0024 g protein per cm², with both sides of a 2.55-cm diameter disc conjugated. This is 3 to 50 percent of the antibody needed to cover the surface, even if all of the protein had been globulin, had conjugated to the disc, and if none were

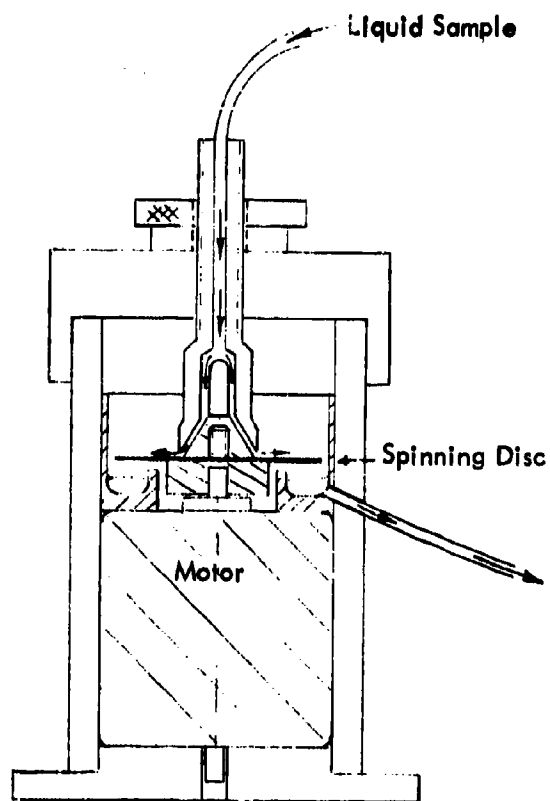


Figure 4-2. Prototype Spinning Disc Detector

Table 4-6

SPORE ADHERANCE TO POLYSTYRENE DISCS CONJUGATED
TO GOAT ANTI-B. GLOBIGII GLOBULIN

Test	Conjugation* (per disc)	Average per Field (255 μ)	Disc Diameter cm	Spores per cm ²	Theoretical Maximum per cm ²	Percent of Theoretical
I. A. Test	0.3 ml globulin*, 3 ml pH 8.2 glycine buffer, incubated at 51°C for 72 hours	120	2.55	6.1×10^5	10^8	0.6%
B. Control	Not Conjugated		2.55	0	10^8	
II. A. Test	As IA, incubated at 50°C for 49 hours	61	2.55	3.1×10^5	10^8	0.30%
B. Control	Not Conjugated		2.55	0	10^8	
III. A. Test	0.9 ml globulin, 2.4 ml buffer, incubated at 50°C for 20 hours	2.3	2.55	1.1×10^4	10^8	0.01%
B. Control	Not Conjugated		2.55	0	10^8	
IV. A. Test	As IA	376	1.5, covered with 1.1 cm disc	1.4×10^6	10^8	1.86%
B. Control	Not Conjugated		"	0	10^8	
V. A. Test	As IA	16	"	8.9×10^4	10^8	0.09%
B. Control	Not Conjugated		"	0	10^8	
VI. A. Test	0.5 ml globulin, 5 ml buffer, 72 hours at 51°C	53	2.55	2.7×10^5	10^8	0.27%
B. Control	Not Conjugated		2.55	0	10^8	

* Both sides of disc conjugated.

** Protein concentration, 1 through V, 68-80 mg/ml, not filtered; VI, 21 mg/ml, not filtered. Spore concentration in 5 ml suspension: 1.1 to 2.2×10^8 /ml.

denatured. Therefore, it is apparent that, on an antibody concentration basis, the observed percentage adherence of spores is rather encouraging.

The conclusions generated thus far are strong justification for improved antibody concentration methods. In this connection, the results in Table 4-6 indicate that the amount of globulin conjugated to the polystyrene was probably more a function of time interval of conjugation than of the range of concentration used.

The Spinning Disc Prototype - The results of the first experiments with the spinning disc prototype (Figure 4-2) exceeded expectations. The results (Table 4-7) indicated that an average of 22 BG spores adhered per $100 \mu^2$ area of a 3.2-cm diameter disc at feed rates of 10 ml per minute and a rotation rate of 700 rpm. No bacteria were retained by a non-conjugated disc. These discs were prepared by heat fixing rabbit anti-B. globigii to the surface at 51°C .

The adherence of background to discs was examined. A suspension of El Monte air background, collected at 1000 l/min into collection fluid flowing at 5 ml/min by a porous electrode electrostatic precipitator, was passed across similarly prepared discs operating under the same conditions. Examination of 44 fields showed that an average of 0.07 particles adhered per $100 \mu^2$ area.

Initial experiments with BG-sensitized discs over which E. coli suspensions were spun showed no significant adherence by the latter organism.

4.1.1.3.3.4 INSTRUMENTATION TECHNIQUES

The previous analysis suggests that 1.1×10^3 liters of air are needed for the detection of one organism/liter. This value has been utilized to establish tentatively the basis for a complete system. The following block diagram indicates schematically the interaction of the component parts:

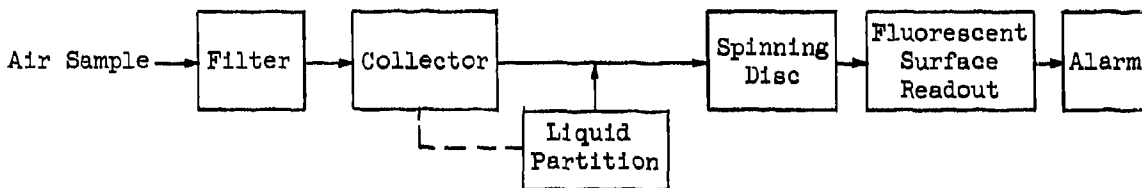


Table 4-7

B. GLOBIGII SPORE ADHERANCE TO A CONJUGATED POLYSTYRENE
DISC ROTATED AT 700 RPM

Disc Sensitized with Rabbit Anti-B.Globigii Globulin
Heat Fixed to Polystyrene

<u>Disc Number</u>	<u>Distance Between Counted Fields</u>	<u>Count</u>
I	1-2 mm	21, 14, 3, 118, 120, 52 0, 78, 15, 15, 24, 10, 42, 7
II	2-3 mm	27, 82, 190, 110, 117, 48 0, 45, 12, 25, 49, 60
III	2-3 mm	28, 33, 25, 159, 260, 122, 160 0, 69, 51, 124, 36, 16, 56, 52

Disc Diameter: 3.2 cm

Total fields observed: 43

Total spores present: 2454

Diameter of Fields: 256 μ

Average number of spores per field: 57

Average number of spores per 100 μ^2 area: 22

Concentration of spores in feed: 1.1×10^8 /ml

Feed rate: 10 ml for one minute

Rotation rate: 700 rpm

Conjugation: 0.5 ml globulin (21 mg/ml), 5 ml
buffer, pH 8.2, 72 hours at 51°C

Figure 4-3 shows three alternative designs for spinning disc collectors. It is estimated that total response time of an eventual system will fall well within a 5-minute period.

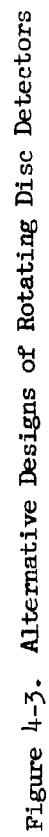
4.1.1.3.4 CONCLUSIONS

Initial experimentation has indicated that a sensitized-surface spinning disc is a highly promising principle on which to base a rapid detection device. Current estimates of sensitivity indicate that 1100 liters of air must be collected to detect one organism/liter. With collection devices currently available this requirement appears to pose no problem. The eventual practical success of this technique will probably be dependent on the surface concentration of active antibodies which can be achieved. A number of relatively simple readout methods may be utilized which involve fluorescence and radio-counting measurements. Logistics requirements appear small.

On the basis of analysis of available data, this detector principle should be developed further.

4.1.1.3.3 REFERENCES

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4.1.2 HYPERSENSITIVITY

4.1.2.1 SUMMARY

Monocytes sensitized to a number of different antigens were employed to test a detection scheme based on hypersensitivity. Increased uptake of trypan blue dye by the sensitized monocytes signaled in the presence of specific antigen. Monocytes from tuberculin positive human donors, a human subject sensitized to rabies virus contained in duck embryo, and animals sensitized with Bacillus globigii, Newcastle Disease virus, and Brucella suis were tested. Results indicated that 1/5 organism per liter of air could be detected

Methods for large scale production and storage of sensitized monocytes were examined. Thus far, the method of choice for preservation appears to be gradual freezing followed by cold storage. In a study of adapting the method to fluorimetric instrumentation, four fluorescent dyes were found more or less satisfactory, with acridine yellow the best. Monocytes treated with acridine yellow and deposited on microporous tape gave strong signals in an automated fluorescent scanning device.

4.1.2.2 INTRODUCTION

Hypersensitivity can be defined as an enhanced capacity to react to a subsequent exposure to a particular substance⁽¹⁾. When monocytes and other cells from a sensitized animal are reacted with a specific antigen, an increased permeability of the cell membrane results and absorption of dyes by the cell occurs readily. This phenomenon is the basis on which the principle of hypersensitivity can be used as a detection scheme.

This method has the advantages of the specificity of an antigen/antibody reaction, impressive sensitivity, rapidity, multiagent capability, and adaptability to automation.

Considerable experimental data have been obtained at SGC on each of the above-indicated properties of this detection principle. It appears that detection of less than one organism per liter of air is possible in an automated system. Detection of all types of BW agents including bacteria, viruses and

toxins appears possible. Detection of viruses and/or virus carrier appears especially promising in that concentration of the antigenic materials is not required as an intermediate step in detection.

A number of readout methods can be employed in instrumentation. For instance, a fluorescent dye can be used and fluorescence measured as in the present FAST system, or a radiotagged dye could be employed and increased radioactivity taken as a measure of reaction.

4.1.2.3 STATUS

4.1.2.3.1 SCOPE AND FEASIBILITY

Much information is available in the literature on the general phenomenon of hypersensitivity. It was indicated that this principle has considerable potential when used as a BW detection method, especially in terms of sensitivity of detection and multi-agent capability. As little as 10^{-15} grams of antigen can elicit a skin reaction, and infection hypersensitivity has been demonstrated in a number of diseases including brucellosis, typhoid, pneumococcal pneumonia, glanders, streptococcal infections, coccidiomycosis, histoplasmosis, mumps, and vaccinia. At times, infection hypersensitivity has been encountered in almost all bacterial and viral infections⁽¹⁾.

The literature is rather indefinite on the mechanism of hypersensitivity at the cellular level. Experimental work at SGC has thus been concerned with demonstrating that the delayed hypersensitivity phenomenon can take place at the cellular level and that monocytes can be employed for BW agent sensing.

In general, the feasibility of detection based on increased uptake of various dyes by sensitized monocytes was examined in terms of sensitivity, specificity, multi-agent capability, adaptability to automation, and various logistic aspects of reagent preparation and storage. The results of these experiments indicate that the use of sensitized monocytes in detection of various antigens is feasible.

Monocytes sensitized to a number of different antigens were employed in these studies. Thus, monocytes from tuberculin positive human donors, a human subject sensitized to rabies virus contained in duck embryo, and animals sensitized with B. globigii, Newcastle Disease virus, and Brucella suis were tested for dye uptake.

Most of the work thus far has been performed with monocytes from tuberculin positive human donors. In these studies, the monocytes were grown in cell culture by the "plasma clot method." Comparison of trypan blue uptake by monocytes from tuberculin positive and negative subjects indicated that significant differences did exist, as indicated by the statistician's t-test:

<u>"t" Value</u>		
PPD Positive with O.T.	3.47	Highly significant difference
PPD Positive, no O.T.		for 16 degrees of freedom
PPD Negative with O.T.	0.75	No significant difference
PPD Negative, no O.T.		

(PPD is purified protein derivative; O.T. is old tuberculin)

The results indicated the general feasibility of detection by this method and established the level of detection sensitivity which might be attained.

It has been calculated that 35 monocytes⁽²⁾ can react with 1 bacterium, and that 300 monocytes which accept stain require only 9 bacteria for reaction. On the basis of this, approximately 1/5 organism per liter of air could be detected when reasonable capabilities for a continuous reactor and collector are assumed.

The specificity of this reaction was tested by exposing tuberculin-sensitive monocytes to M. phlei and E. coli. Although dye uptake did occur with these organisms, the percent uptake of dye was less than with tuberculin. These results suggest that the reaction has specificity but further data are required to determine the degree of this specificity.

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It was demonstrated that monocytes from humans and animals sensitized to other types of antigens (B. globigii, Newcastle Disease Virus, rabies/ duck embryo mixture) had increased dye uptake in the presence of specific antigen. With B. globigii, the percentage reactivities were 55 percent with antigen and 1 percent without. These results need further confirmation but suggest that the method does have broad application to detection of multi-agents and that animals can be used for obtaining sensitized monocytes. Specificity was demonstrated also with monocytes sensitized to duck embryo extract. These monocytes did not react with chick embryo extract.

During these experiments, it became increasingly apparent that the major obstacles to adaption of this method for BW detection were concerned with logistics, i.e., production of large quantities of monocytes and conditions for suitable storage. Studies were therefore conducted to evaluate methods for isolating monocytes from animals, separating monocytes from blood constituents, growing monocytes in tissue culture, and for preservation of monocytes.

4.1.2.3.2 MONOCYTE PREPARATION

Most of the monocytes used in initial experiments were isolated from whole blood of sensitized humans or animals. In an attempt to obtain increased quantities of these cells along with perhaps less contamination by erythrocytes, procedures were investigated for isolating leucocytes by intraperitoneal washing. Initial results with those procedures were disappointing in that not as large quantities of leucocytes were obtained as had been anticipated.

Separation of erythrocytes from leucocytes was considered important since the presence of only a small number of red cells make it extremely difficult to obtain accurate counts of the monocytes. The albumin flotation method and methods employing phytohemagglutinin (PHA-M)⁽³⁻⁷⁾, Dextran^(5-8,9), polyvinyl pyrrolidone (PVP),⁽¹⁰⁾ and streptolysin O were evaluated for separating leucocytes from erythrocytes. PHA-M is a substance obtained from a species of bean that is used for agglutinating a large fraction of erythrocytes from a sample of blood containing an anticoagulant⁽³⁾. The other substances, Dextran and PVP, behave in a similar manner and also cause agglutination of the erythrocytes.

Except for using different amounts of reagent, all three substances are used in a similar manner. The reagent is added to the blood and after standing for a given time, either at room temperature or in an ice bath, the plasma is drawn off. Sometimes light centrifugation, 300 rpm/3 min, preceded the withdrawal of the plasma. In some instances Dextran was added to plasma rather than to blood⁽⁸⁾. As a result of these studies, it can be stated that there is not an appreciable difference between the agglutinating agents. The Dextran added to the plasma was better than the dextran added to the blood, which was slightly better than the PVP, and the latter was better than the PHA. The albumin flotation method was found to be not as efficient as the other methods for separating leucocytes from erythrocytes.

In addition to the above procedures, streptolysin O was also used in attempts to separate erythrocytes from leucocyte suspensions. It was hoped that streptolysin O could be added to a culture and selectively hemolyse the red blood cells and leave the leucocytes intact, but this did not prove to be true.

PHA-M, in addition to being an agglutinating agent for erythrocytes, also has been used in tissue culture studies to stimulate general cell growth as well as to encourage mitosis^(9,10,11). In subsequent studies on monocyte replication, PHA was found stimulatory to growth.

After removal of erythrocytes by the various methods, the plasma so obtained could be cultured directly by adding to tissue culture medium, but more often the plasma was centrifuged at 2000 rpm for 10 minutes to concentrate the leucocytes. With this procedure the concentrated leucocytes can be re-suspended with culture medium to a definite number of cells per given volume, usually 1 to 2×10^6 .

Experiments were conducted to determine optimal conditions for in vitro culture of monocytes. Various media were compared for growing monocytes. The various media used included Hanks, TC 199, Eagles media with glutamate, and NCTC109. Both fetal calf serum or plasma from which the monocytes were harvested (autologous plasma) were added to the various media.

Culture medium NCTC 109 is recognized to be superior to some of the more common media used in tissue culture work by virtue of its inclusion of several ingredients not present in the others. However, it is a more expensive mixture and, since large quantities of cells are to be grown, it was considered expedient to compare several media using cells prepared under identical conditions. TC199 media with fetal calf serum was included since it has been used successfully for growing lymphocytes⁽⁹⁾.

The results of these studies indicated that NCTC 109 medium was best for culturing monocytes, and Eagles, TC199, and Hanks media were inferior in the order given. It was also indicated that calf serum was superior to autologous plasma for supporting monocyte growth.

The control of pH changes during incubation is of the utmost importance. Since many tissue culture media contain sodium bicarbonate as one of their ingredients and, since during incubation some of the CO₂ escapes, it is well to have an incubator in which a constant stream of CO₂ is admitted into the incubator.

It was considered worthwhile to determine whether an air-tight culture flask might retard the escape of CO₂ and thus maintain the proper pH. To determine this, cells prepared by one of the above methods were introduced into Leighton screw cap test tubes, Earle plastic flasks with rubber stoppers, and rubber-sealed, aluminum-capped vaccine bottles, and incubated at 36°C. The use of vaccine bottles with sealed rubber and aluminum caps maintained a more uniform pH than did either of the other two culture medium flasks.

Methods for preserving monocytes have also been under investigation. Thus far, the method of choice appears to be slow freezing of the monocytes at a definite rate. A freezing method based on an apparatus suggested by Parker⁽¹²⁾ was used for a long term freezing experiment. In those methods the cells in 6 percent glycerol/Hank's solution contained in sealed ampules are immersed in alcohol which is contained in an aluminum vessel. The latter is immersed in turn in a 20 percent glycerol/water mixture which is contained in a mixture of CO₂ and alcohol. The temperature in the vessel containing the sample is reduced, at the rate of 1 degree per minute, until -15°C is reached,

after which the frozen ampules are stored in a CO₂ chest. Recent preliminary results indicate that monocytes can be stored in this manner for at least 16 days without significant loss in reactivity. In this time, the percent dye uptake (no antigen) increased from 0 to only 1.5 to 2 percent.

4.1.2.3.3 INSTRUMENTATION

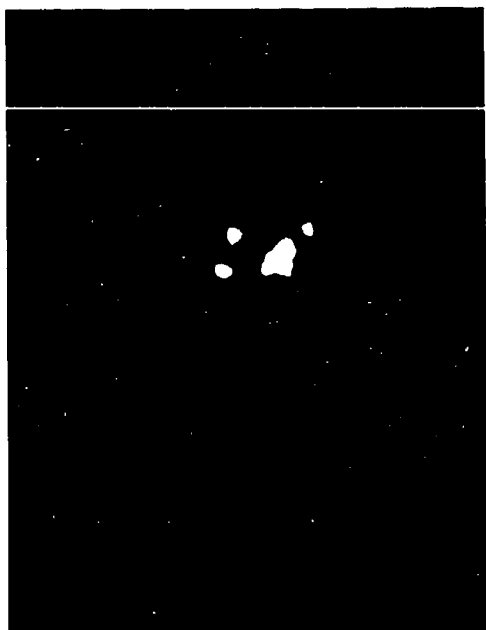
Studies were conducted to determine whether this method could be adapted to instrumentation such as the FAST Breadboard being developed under Contract DA 18-064-AMC-137(A). In these studies a number of fluorochromes were tested for uptake by monocytes. Four (auramine, eosin E, eosin B and acridine yellow) were found to be more or less satisfactory. Figure 4-4 illustrates monocytes stained with acridine yellow. Two points are noteworthy; the size and the intensity of the signal (1/5 second minimum exposure time) and the absence of background.

Monocytes were stained with 0.2 to 0.52 percent of the acridine yellow dye in the presence of antigen, filtered on a 0.8 μ Millipore tape, and examined under blue-violet excitation at 100X and 400X in the FAST breadboard scanner using a photomultiplier. The oscillograph tracings from this experiment are presented in Figures 4-5 and 4-6.

Visual observations confirmed that monocytes were being scanned. The preparation was remarkably free from other particulates visible at the 400X and 100X magnifications used. Also worthy of mention is the fact that Figures 4-5 and 4-6 were obtained using a 1 μ scan. The use of a 10 μ pore sized filter membrane was found to eliminate particulate debris and resulted in less background.

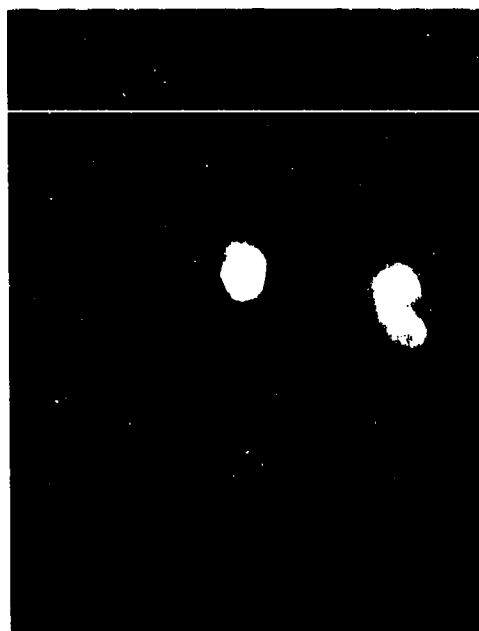
4.1.2.4 CONCLUSIONS

The results of these experiments indicate that the use of sensitized monocytes for the detection of BW agents is feasible. It was demonstrated that the method possesses sensitivity, specificity, multi-agent capability, and adaptability to automation. One of the most serious disadvantages of this method has been the logistic aspect, i.e., preparation of large



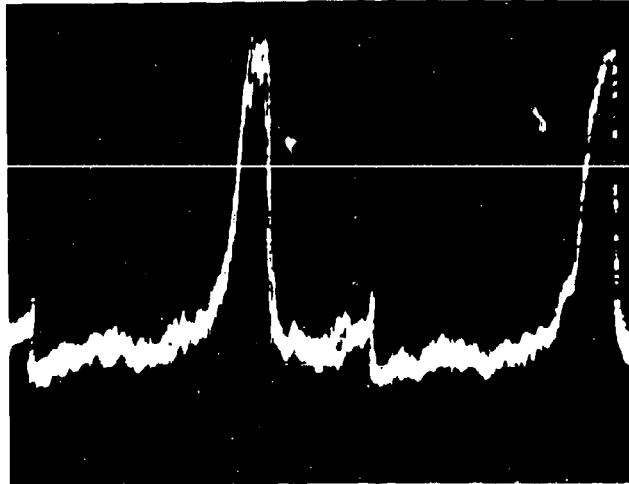
SGC/628

2 Second Exposure
3000 Speed Film, 125X



2 Second Exposure
3000 Speed Film, 610X

Figure 4-4. Monocytes Stained with Acridine Yellow



Signal

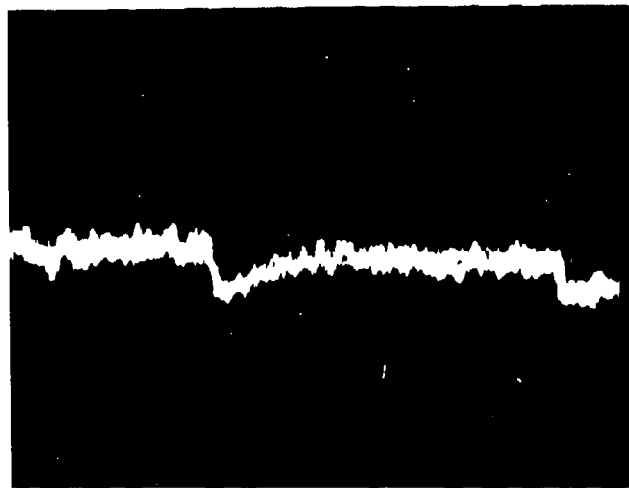
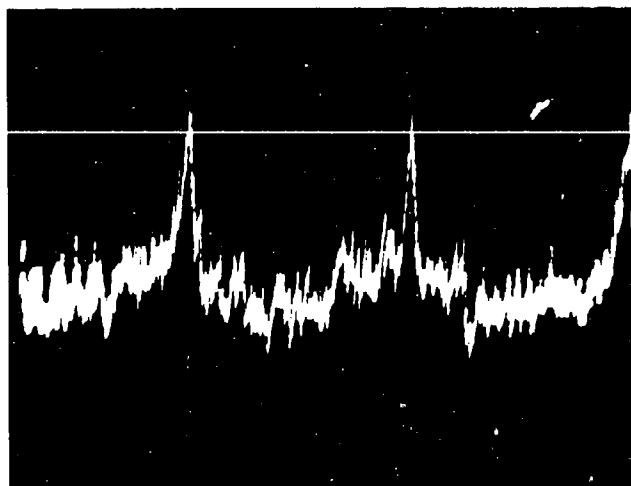


Figure 4-5. Fluorescent Signal from a Sensitized
Monocyte, 400X



Signal

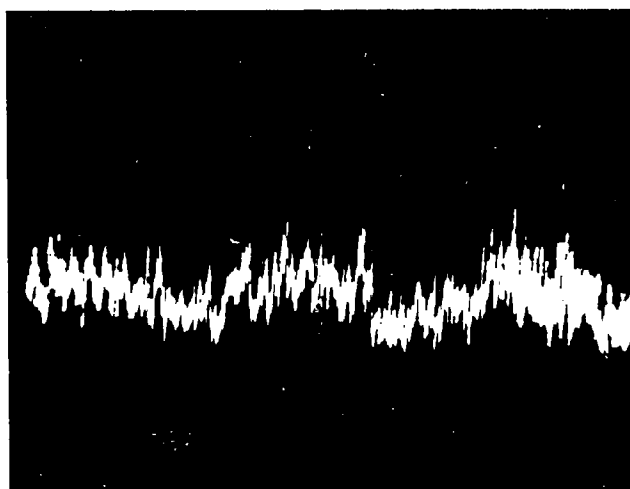


Figure 4-6. Fluorescent Signal from a Sensitized Monocyte, 100X

quantities of monocytes and use of suitable conditions for their storage. Even though data obtained thus far indicated that these problems may be solved, further experimentation is necessary before suitable methods can be developed for preparation and storage of monocytes.

4.1.2.5 REFERENCES

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4.1.3 BACTERIOPHAGE

4.1.3.1 SUMMARY

A bacterial detection method based on P^{32} -labelled bacteriophage attachment was evaluated. The literature indicated that specific phage were available or easily attainable for almost all potential BW agents. High titered preparations of P^{32} -labelled coliphage containing 10^{-8} to 10^{-9} μ c per phage have been repeatedly prepared.

Detection sensitivity was shown to depend on reaction rates between phage and bacteria and on the efficiency of separating excess unattached phage from phage-bacteria complexes. Reaction kinetics showed that labelled phage at a concentration of approximately 10^8 /ml would attach to 10^4 bacteria within 5 minutes, giving a readily measureable signal. Filtration was emphasized as a method for removing excess phage after reaction. The major problem was non-specific adsorption (NSA) of phage to the Millipore filter membrane, which was influenced by filtration media and procedures, state of the phage preparation, and filter characteristics. Under optimal conditions NSA was reduced to 0.004%, permitting easy detection of 10^4 bacteria/ml. Zonal centrifugation and liquid partition methods also appeared promising for separation.

Studies were initiated on detection of bacterial pathogens with labelled phage. Instrumentation of this method demonstrated continuous detection of 10^5 to 10^6 E. coli/ml, with detection of as few as 10^4 bacteria/ml appearing obtainable.

4.1.3.2 INTRODUCTION

The continuous detection method utilizing P^{32} -labelled bacteriophage is based on the ability of the latter to attach specifically to bacteria. This attachment is subsequently measured by determining the amount of radioactivity retained on a microporous tape following filtration.

The literature indicated that phages are available or easily attainable for all potential bacterial BW agents. In many instances phage systems were found already developed for distinguishing and identifying particular

groups of bacterial pathogens. It was therefore felt that phage of desired specificity levels could be obtained so that a phage system containing relatively few different types could be developed for detection of pathogenic bacteria as a group. This circumstance, coupled with the relatively low cost of production when compared to antibody production in animals, appeared to give this method a logistic advantage.

In evaluating this method, it was necessary to show that it had the potential of being sensitive, rapid, specific, adaptable to automation, and had the capability of readily detecting almost all important bacterial pathogens.

4.1.3.3 STATUS

4.1.3.3.1 SENSITIVITY AND INTERNAL BACKGROUND

4.1.3.3.1.1 PREPARATION, PURIFICATION, AND CHARACTERIZATION OF P^{32} -LABELLED PHAGE

T4D and T2L phage have been repeatedly labelled by growth in media containing their bacterial hosts (E. coli strains S/6 and B/5, respectively) and P^{32} as $H_3P^{32}O_4$. Final preparations have been usually obtained containing from 10^9 to 10^{10} phage/ml and 10^{-8} to 10^{-9} μ c per phage. It was determined that 80 to 90 percent of the radioactivity in these preparations resided in the phage.

Prior to preparing labelled phage, it was determined that the amount of P^{32} attainable per phage was theoretically limited to $\approx 10^{-8}$ μ c because of the inhibitory effect of the increasing concentrations of radioactivity on bacterial growth.

Although a comprehensive set of data was not obtained on the effect of age and storage conditions on the properties of labelled phage, the experimental evidence thus far indicates that storage at 5°C for 4 to 6 weeks in Tris buffer medium (pH 7.4) does not appear to decrease significantly their ability to attach to their host bacterium.

4.1.3.3.1.2 KINETICS

Prior to experimental work, the available data from the literature⁽¹⁻⁵⁾ were mathematically analyzed and it was determined to be theoretically possible to obtain significant adsorption to as few as 10^4 bacteria/ml within a short period of time.

Many experimental data were subsequently obtained on the kinetics of adsorption using phage T4D and T2L and their specific bacterial hosts (E. coli S/6 and B/5, respectively). The data have provided information on the concentration of phage and the time required for adequate detection of small numbers of both living and nonliving bacteria. The effect of temperature on the reaction rate was also investigated.

Two different methods were used to obtain data on phage adsorption. In one method adsorption was determined by measuring the P^{32} content of bacteria following reaction with P^{32} -labelled phage. In these experiments, appropriate concentrations of labelled phage and bacteria were reacted for varying periods of time and at different temperatures. The reactants were then filtered through a 0.45 μ Millipore filter membrane and radioactivity retained on the filter was measured by a gas-flow proportional counter. Control preparations containing labelled phage but no bacteria were treated in an identical manner. In the other method, adsorption was measured indirectly from the number of surviving bacteria following reaction with phage. In these experiments, varying concentrations of unlabelled phage were reacted with approximately 10^4 bacteria/ml at 30° and 40° C. After specified time intervals the reaction mixture was diluted in media containing antiphage serum and passed through pre-sterilized Millipore filter bacterial Field Monitors. The surviving bacteria were determined by direct count following incubation for 24 hours at 37° C. Unreacted bacterial and phage controls were treated in a similar manner.

Correlation of the Surviving Bacteria Method with the P^{32} Tracer Technique - It was shown that the data on phage adsorption by bacteria as determined by the P^{32} tracer technique and the surviving bacteria method are simply related if the assumption of a Poisson distribution remains valid over the range of experimental determinations.

The rate constant, K, may be expressed as

$$K = \frac{n}{Pt} \quad (1)$$

where n = the average number of phage per bacteria, P = phage concentration in numbers/ml and t = time in minutes. The quantity n can be measured either directly by radioactive methods or indirectly by the surviving bacteria methods. The equation

$$2.303 \log \frac{B_0}{B} = n \quad (2)$$

where B_0 and B equal the initial and final bacterial concentration, respectively, relates how n can be calculated from the surviving bacteria data.

Multiplicities and Rates of Adsorption to Live Bacteria - Figure 4-7 shows typical multiplicity data as a function of time which were obtained by both experimental methods at 40°C. The data indicated at phage concentrations of 4.0×10^8 and 4.0×10^7 phage/ml were obtained by surviving bacteria methods. The data at 1.0×10^8 phage/ml were obtained using the radiotracer technique. The linearity of these plots indicates that the adsorption reaction is pseudo first order in the presence of excess phage. By application of Equation (1) and using the slopes of the lines in Figure 4-7, second order rate constants have been calculated:

Rate Constants for Adsorption of Phage T4D by
E. coli (S/6) at 40°C.

Phage Concentration (no./ml)	Bacterial Concentration (no./ml)	Rate Constants, k , (ml/phage-min)	Method
4.0×10^8	1.7×10^4	2.3×10^{-9}	S.B.
1.0×10^8	1.0×10^5	2.8×10^{-9}	P ³²
4.0×10^7	1.6×10^4	2.8×10^{-9}	S.B.

The constancy of the value of the rate constant indicates that the reaction of phage T4D with E. coli (S/6) is a second order reaction, being a function of the concentrations of both phage and bacteria.

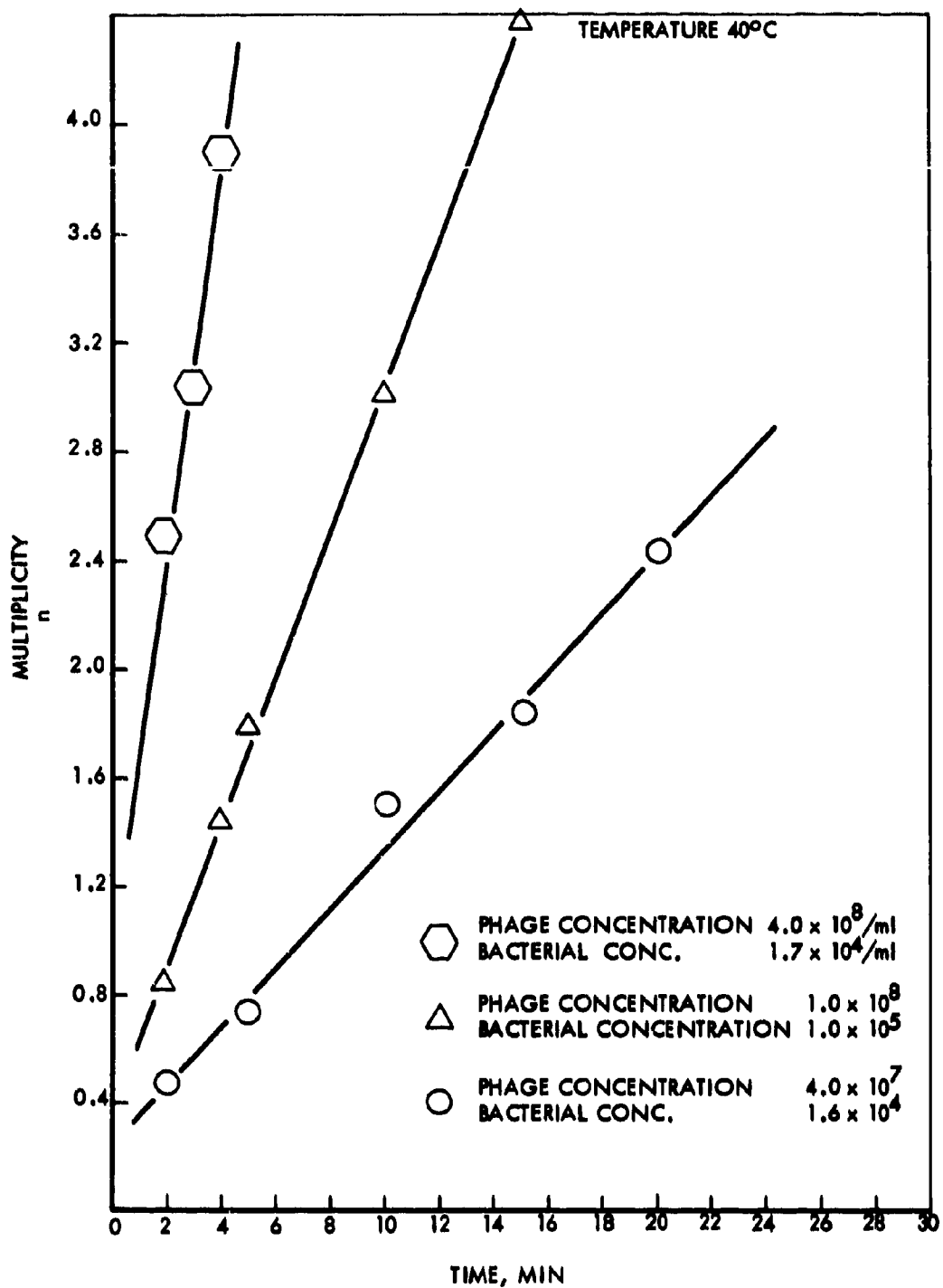


Figure 4-7. Adsorption of Coliphage T4D by E. coli (S/6), Multiplicity versus Time

The similarity of the K values obtained by two different methods furthermore would tend to increase the validity of the data and indicate the applicability of the P^{32} method for obtaining data on adsorption kinetics under conditions of excess phage.

Temperature - Kinetic data have been obtained at 25°, 30°, and 40°C. Figure 4-8 is a plot of the log of the specific reaction rates against the reciprocal of the absolute temperature. The linearity of the plot implies that only one reaction mechanism is operating in this temperature range. The activation energy obtained from the slope of the line is 19.4 kcal/mole, which is consistent with the values obtained during bond formation in other protein reactions. The value of the pre-exponential term is consistent for values usually obtained for reactions in solution.

Multiplicities and Rates of Adsorption to Ethylene Oxide-Killed Bacteria - The adsorptions of phage T2L on ethylene oxide-killed E. coli (B/5) and of phage T4D on ethylene oxide-killed E. coli (S/6) are shown in Figure 4-9, where multiplicity of attachment has been plotted as a function of time at 40°C. The rate constant for adsorption in the T2L-B5 system is almost ten times faster than that of the T4D-S/6 system. Saturation was achieved in the dead B/5 strain with 78 T2L phage. Saturation in the T4D-S/6 system required only about 9 phage.

Adsorption of Phage by Ethylene Oxide-Killed Bacteria

<u>Phage</u>	<u>Bacteria</u>	<u>Specific Rate of Adsorption (ml/phage-min)</u>	<u>Saturation (Phage/Bacterium)</u>
T2L	B/5	1.3×10^{-8}	78
T4D	S/6	1.9×10^{-9}	9.6

The data suggest that there are differences in adsorption between the different T phage systems and that the multiplicity of adsorption obtained with the ethylene oxide-killed E. coli is considerably less than that of the living organisms.

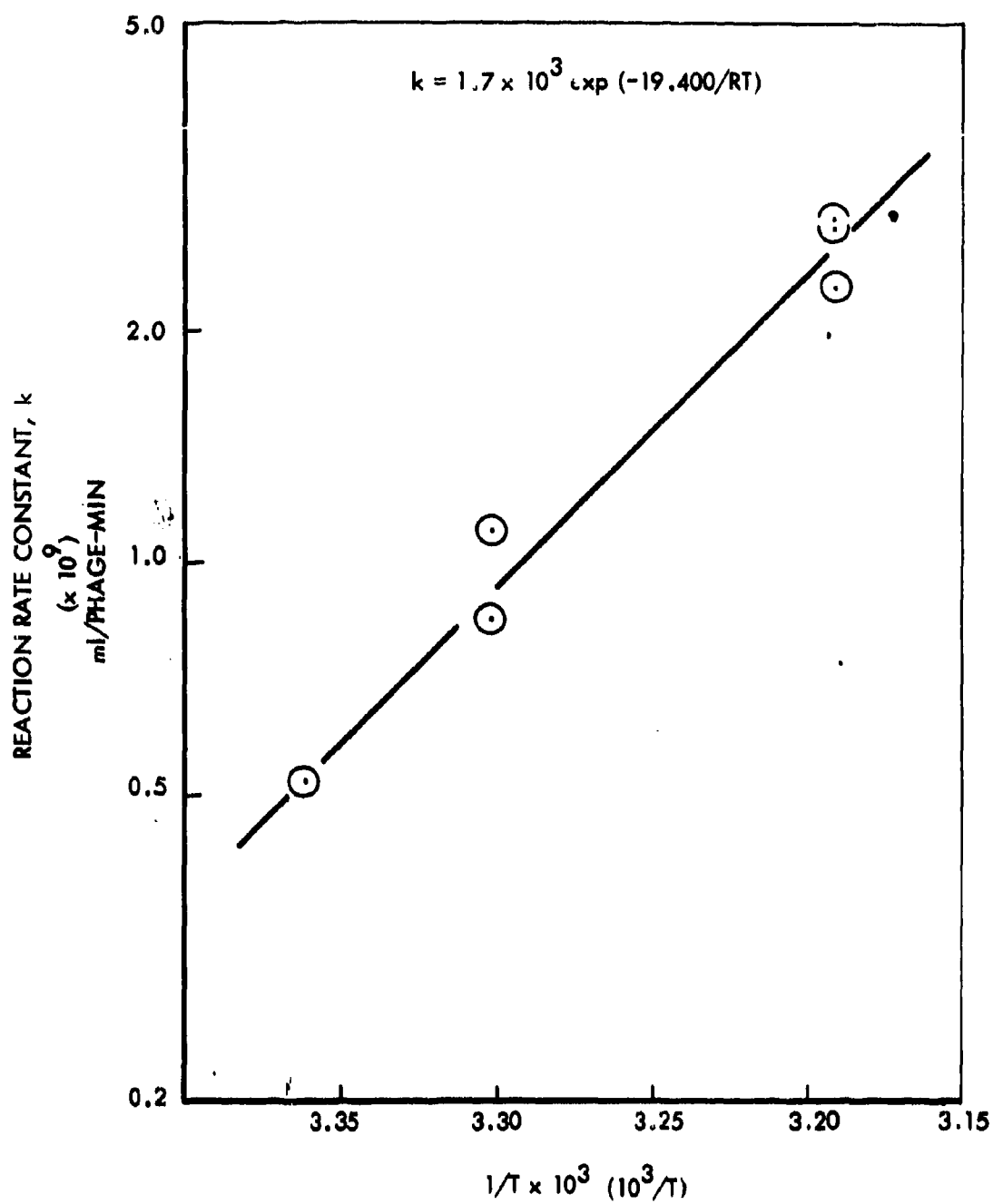


Figure 4-8. Adsorption of Coliphage T4D by E. coli
(S/6), Reaction Rates versus Reciprocal
of Temperature

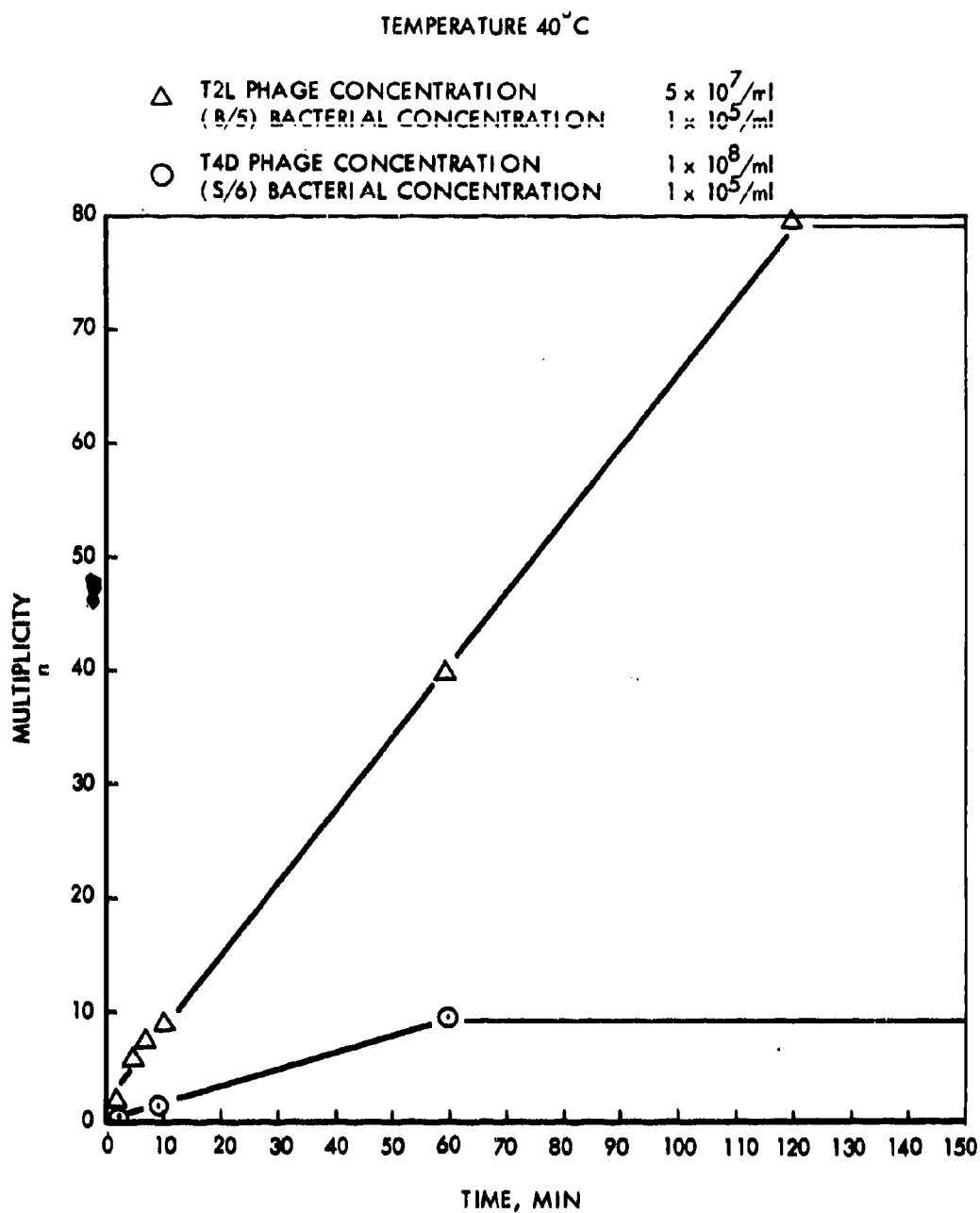


Figure 4-9. Reaction of Ethylene Oxide-Killed E. coli with Coliphages, Multiplicity versus Time

4.1.3.3.1.3 SEPARATION

4.1.3.3.1.3.1 FILTRATION

The various factors studied in terms of their effect on adsorption to the filter included alteration in filtration media and procedures, properties of the phage preparation, and characteristics of the filter. In general, the experimental procedures for determining adsorption consisted of first passing a buffer medium containing 0.1 to 0.5 percent Triton X 100 (synthetic non-ionic detergent, Rohm and Haas) through the filter followed by the labelled phage preparation (prefiltered) diluted 1/100 in the same medium. The filter was then washed with the same medium and the amount of radioactivity retained on it determined. The results from these experiments with the Millipore filter are summarized in a qualitative manner in Table 4-8.

From the data in Table 4-8 it was possible to select procedures by which the non-specific adsorption of phage to uncoated Millipore filter was reduced to between 0.05 and 0.1 percent. Specific data on amount of adsorption to fluorocarbon-coated filters are presented below.

Evaluation of the adsorption in the above experiments was based on a comparison of the individual results to a control. The percent adsorption for the latter varied between different experiments, presumably due to minor variations in the procedure and differences between the particular phage preparations used. It was therefore felt that the data would be best compared in a qualitative manner.

More quantitative data from recent experiments on the comparative adsorption properties of the Millipore and other types of filters are presented below:

<u>INFLUENCE OF FILTER CHARACTERISTICS ON NON-SPECIFIC ADSORPTION OF PHAGE</u>	
	<u>% Adsorption</u>
Millipore filter (0.45 μ)	0.08
Flotronics filter (stainless steel, 0.45 μ)	2.8
Gelman filter (0.45 μ)	0.3
Nuclepore filter (polycarbonate, 0.5 to 0.7 μ)	0.03

Table 4-8

FACTORS EFFECTING PHAGE ATTACHMENT TO THE MILLIPORE FILTER*

FACTORS TESTED	EFFECT ON ADSORPTION
I. <u>Alterations in Filtration Media and Procedures</u>	
pH	Increased at pH 6.0 and 9.0
Temperature	No effect
Non-ionic detergents	Decreased significantly, depending on type
Unlabelled phage	Increased or no effect, depending on amount added
Broth solutions	Variable
Urea	No effect
Salt concentration	Increased or no effect, depending on concentration
Mechanical backflush	Decreased
EDTA	Decreased
II. <u>Properties of Phage Preparation</u>	
Prefiltration	Decreased significantly
Age	Increased with age
DN-ASE treatment	No effect
Nos. of phage	Increased or no effect, depending on concentration
III. <u>Filter Characteristics</u>	
Ultra-thin (types Tw and Th)	Increased
Polysiloxan coated	Increased
Paraffin coated	Occlusion of filter
Fluorocarbon coated	Decreased
Increased pore size (0.8 μ)	No effect

* 0.45 μ Millipore filter (HA) used in all cases except where otherwise indicated

It would appear from the above data that the Nuclepore filter had the least amount of adsorption.

Recent data indicate that Millipore filters coated with fluorocarbon compounds (Teflon-like) may have decreased adsorption of phage to their surfaces. In these experiments, the type and amount of fluorocarbon used for coating the filters were varied.

Millipore filters (0.8 μ) were coated by placing them in a solution of a particular fluorocarbon compound dissolved in a liquid fluorocarbon or aqueous solvent. While still in solution, the filters were placed under vacuum to assure even distribution of the fluorocarbon, removed from the solution, and air dried. Adsorption to the filter was determined by the usual procedures. The results are presented below:

<u>Fluorocarbon Compound (3M)</u>	<u>Solvent (3M)</u>	<u>Concentration of Fluorocarbon Compound(α)</u>	<u>% Nonspecific Adsorption</u>
FC144	FC75	0.01	0.066
FC144	FC75	0.12	0.125
FC176	FC75	0.03	0.004
FC176	H2O	0.01	0.052
-	-	-	0.120

The results indicate that fluorocarbon compounds may have considerable effect on reducing phage adsorption to the Millipore filter, depending upon the type and concentration of compound used. Further experiments were conducted in which varying concentrations of different fluorocarbon compounds were used to coat the filters. In these experiments an additional solvent (such as CCl_4) was employed to dissolve the various fluorocarbon compounds. These experiments indicated that relatively minor alterations in the procedure used for coating the filters can cause differences in their adsorptive capacity, perhaps due to the amount of compound adsorbed to the filter membrane. It was furthermore interesting to note that CCl_4 treatment decreased the NSA about 3 fold. This would imply that there were CCl_4 -soluble substances on the untreated filter which increased adsorption.

4.1.3.3.1.3.2 OTHER SEPARATION METHODS

Zonal centrifugation, liquid partition, and electrophoresis procedures have all been considered as possible separation methods. A certain amount of experimental data has been obtained in the first two areas. The continuous liquid partition system, described elsewhere (Section 4.2.3.1), has not been used specifically as yet to separate phage from bacteria, but phage have been concentrated in one phase within a 5-minute period. It would appear that, with the use of a proper polymer phase system, liquid partition would have application in the separation of bacteria and phage. A modification of the hollow rotor zonal centrifuge as developed by N. G. Anderson (see Section 4.2.2.1) was considered as a method for utilizing the zonal centrifugation principle in a continuous way.

Initial experiments were conducted to determine the feasibility of separating phage from bacteria based on difference in densities or sedimentation rates of the particles. Separations were found to be much more successful when based on density differences than when based on differences in sedimentation rates through low-density media. Sucrose and a colloidal silica sol (Ludox AM, DuPont) were employed, for the most part, as density-gradient media. Two general approaches for separation were studied. One involved the use of a density-gradient solution and the other a "density shelf" procedure. In the latter instance, the material to which the samples were added was of the same density throughout instead of having a continuous linear gradient. This principle was based on the idea that if the shelf were dense enough to support all bacteria above it, the phage would distribute beneath it, allowing for separation.

Ludox AM proved to be the most suitable substance for separating phage and bacteria. Sedimentation of the silica particles resulted in a density gradient during centrifugation with a solidified mass appearing at the bottom of the tube. Almost all of the dense phage particles sedimented at a faster rate and were imbedded in the silica pellet. At least 95 percent of the bacteria remained in the supernatant 5 to 6 cm above the pellet. The use of Ludox AM with the continuous liquid partition apparatus could possibly result in an extremely efficient method for separating phage and bacteria. The results with

sucrose gradients were inconclusive, but indicated that separation may also be possible with this method. The density shelf procedure proved ineffective for separation purposes.

A summary of the results is presented below:

<u>Procedure</u>	<u>Media</u>	<u>Density</u>	<u>Estimate of Separation Effectiveness</u>
Sedimentation	Sucrose	1 to 10% and 1 to 20%	Poor
Density Shelf	Sucrose	1.154	Poor
Density Gradient	Sucrose	1.154 to 1.251	Inconclusive
Density Gradient	Ludox AM	1.18	Good

4.1.3.3.1.4 SUMMARY OF SYSTEM SENSITIVITY

The following table shows typical signal-to-noise ratios which have been estimated from data obtained from kinetic studies and from measurements of non-specific adsorption to the filter.

<u>Phage Conc.</u> <u>(nos/ml)</u>	<u>E. coli Conc.</u> <u>(nos/ml)</u>	<u>Multiplicity</u>	<u>Signal-to-Noise Ratio at 0.05% Non-specific Adsorption of Phage</u>	<u>Signal-to-Noise Ratio at 0.004% Non-specific Adsorption of Phage</u>
1 x 10 ⁸	10 ⁶	1.1	22	275
	10 ⁵	1.1	2.2	27.5
	10 ⁴	1.1	< 1	2.75
4 x 10 ⁸	10 ⁶	3.0	15	187.5
	10 ⁵	3.0	1.5	18.75
	10 ⁴	3.0	< 1	1.8

Increased sensitivity may be achieved by obtaining higher multiplicities or by decreasing the non-specific adsorption.

4.1.3.3.2 MULTI-AGENT CAPABILITY

It was determined from the literature that phage for almost all bacterial pathogens are either available from individual investigators or the

American Type Culture Collection, or readily obtainable by standard laboratory procedures. The following table is a representative list of bacterial pathogens for which phage are available.

<u>Bacterial Host</u>	<u>Source</u>	<u>Reference No.</u>
<i>Pasteurella pestis</i>	Gunnison	(7)
<i>Shigella</i> species	ATCC	(6)
<i>Salmonella</i> species	ATCC	(6)
<i>Listeria monocytogenes</i>	Sword	(8)
<i>Malleomyces mallei</i>	Smith	(9)
<i>Malleomyces pseudomallei</i>	Smith	(9)
<i>Brucella</i> species	McDuff	(10)
	Lazuga	(11)
<i>Bacillus anthracis</i>	Buck	(12)
<i>Corynebacterium diphtheriae</i>	Groman	(13)
<i>Vibrio comma</i>	ATCC	(6)
<i>Streptococcus</i> species	ATCC	(6)
<i>Mycobacterium tuberculosis</i>	Groman	(14)

Initial experiments have been conducted with a *Pasteurella pestis* phage obtained from Dr. J. B. Gunnison⁽⁷⁾. It was adapted to grow in a strain of *Shigella flexneri* (Boyd 103) to determine more readily the applicability of methods developed for the *E. coli* - T phage systems. Preliminary experiments were initiated to determine the amount of P³² label obtainable per phage.

4.1.3.3 OPERATION OF DETECTOR

A first-generation breadboard was constructed for detection of antigens with radioactive reagents (either phage, antisera, or labelled "stains"). It was first operated in a series of continuous runs using P³²-tagged bacteriophage reagent. Comparisons with predictions from laboratory experiments are both informative and hopeful.

The detector provides continuous pumping of sample and reagent streams, followed by mixing and reaction in a coil at a constant temperature. The reacted solution is then diluted with a suitable solution (depending on

whether phage or antiserum is used) before it is deposited by filtration on the moving tape. The tape is pretreated before filtration and washed after filtration. Deposits are counted continuously from both the top and bottom surfaces of the tape, and continuously recorded, using a Nuclear Chicago Actigraph 4-pi radiochromatogram scanner assembly. This instrumentation is completely described in an earlier Comprehensive Report⁽¹⁵⁾.

Settings of the Actigraph, rate meter, and recorder were as follows: tape speed at 3 inches/min; recorder speed at 3 inches/min; sensitivity counts/min on 1K, 10K and 100K scales; high voltage at 1000 volts.

The object of the test series was to characterize the sensitivity of continuous detection, and to explore the factors which increase it. Sensitivity depends both on minimal background radiation from non-specific adsorption and on efficiency of reaction between phage and bacteria. To examine background deposition separately, as well as with reaction mixtures, tests were performed with phage suspensions alone.

4.1.3.3.1 ILLUSTRATIVE TESTS

Examples of runs showing background, non-specific adsorption of the phage, and reaction signals of phage + bacteria are given in Figure 4-10. In the run 0.45 μ black Gelman tape was used. The phage reagent (T₄D) contained 2 to 3 x 10⁸ phage/ml at 5 x 10⁻¹⁰ μ c/phage activity. It was pumped in at a rate of 1.5 ml/min. The bacterial reagent (E. coli S/6) at a concentration of 2 x 10⁶ cells/ml was also pumped in at 1.5 ml/min. The mixture was reacted for 2 minutes at 40°C. The diluent and wash liquids were both continuously pumped at 12.0 ml/min. The traces show signal-to-noise ratios ranging from 2:1 to 4:1. The transition from phage reagent background to reaction mixture is unmistakable and the cessation of hot phage reagent flow shows a rapid recovery to normal background.

4.1.3.3.2 PHAGE RETENTION ON TAPE

Results of all continuous tests on non-specific adsorption of phage on tape are compiled in Figure 4-11. The retention of phage per square micron, p (nominally, the area covered by one bacterium), is plotted as a function of number of phage per minute, ϕ , through the tape on log-log scales.

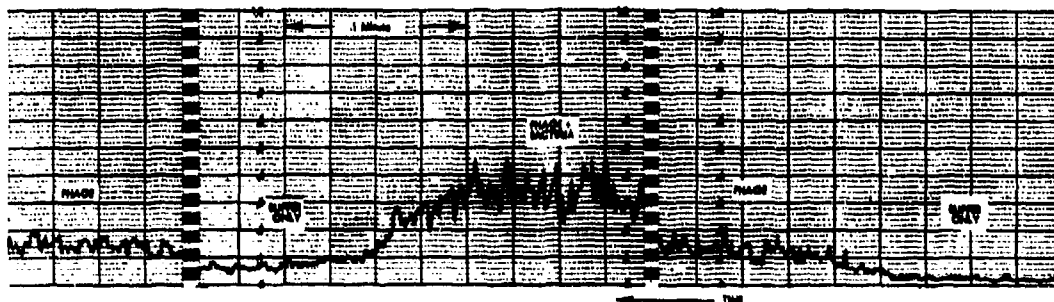
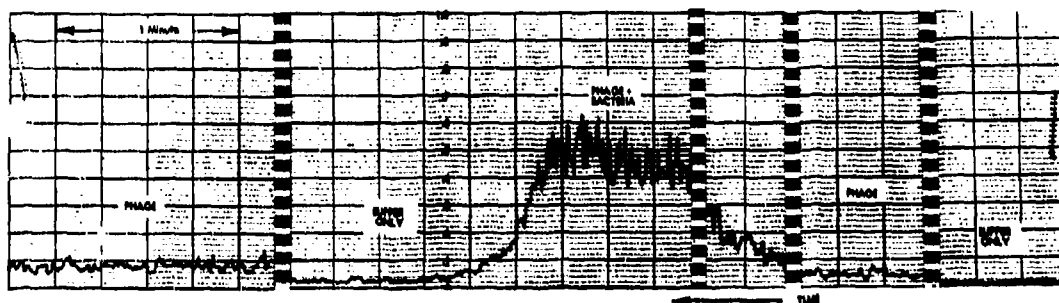
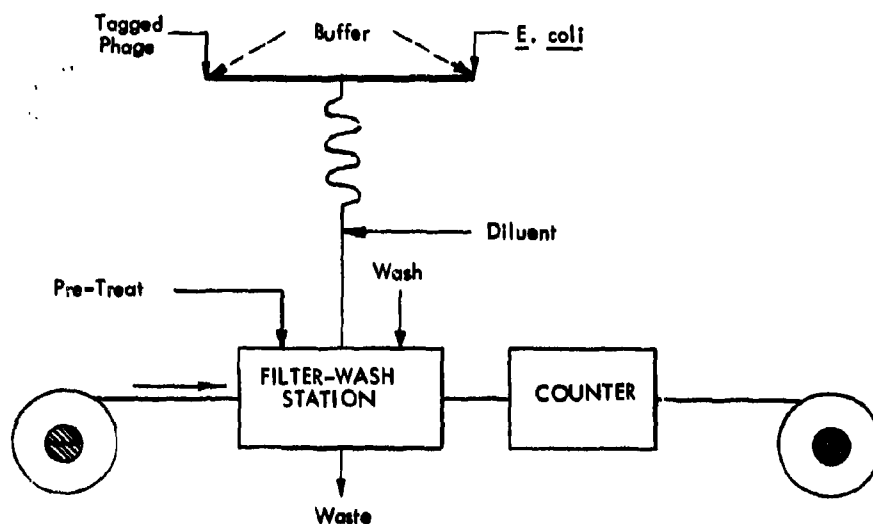


Figure 4-10. Continuous Detection with P^{32} -Tagged Bacteriophage

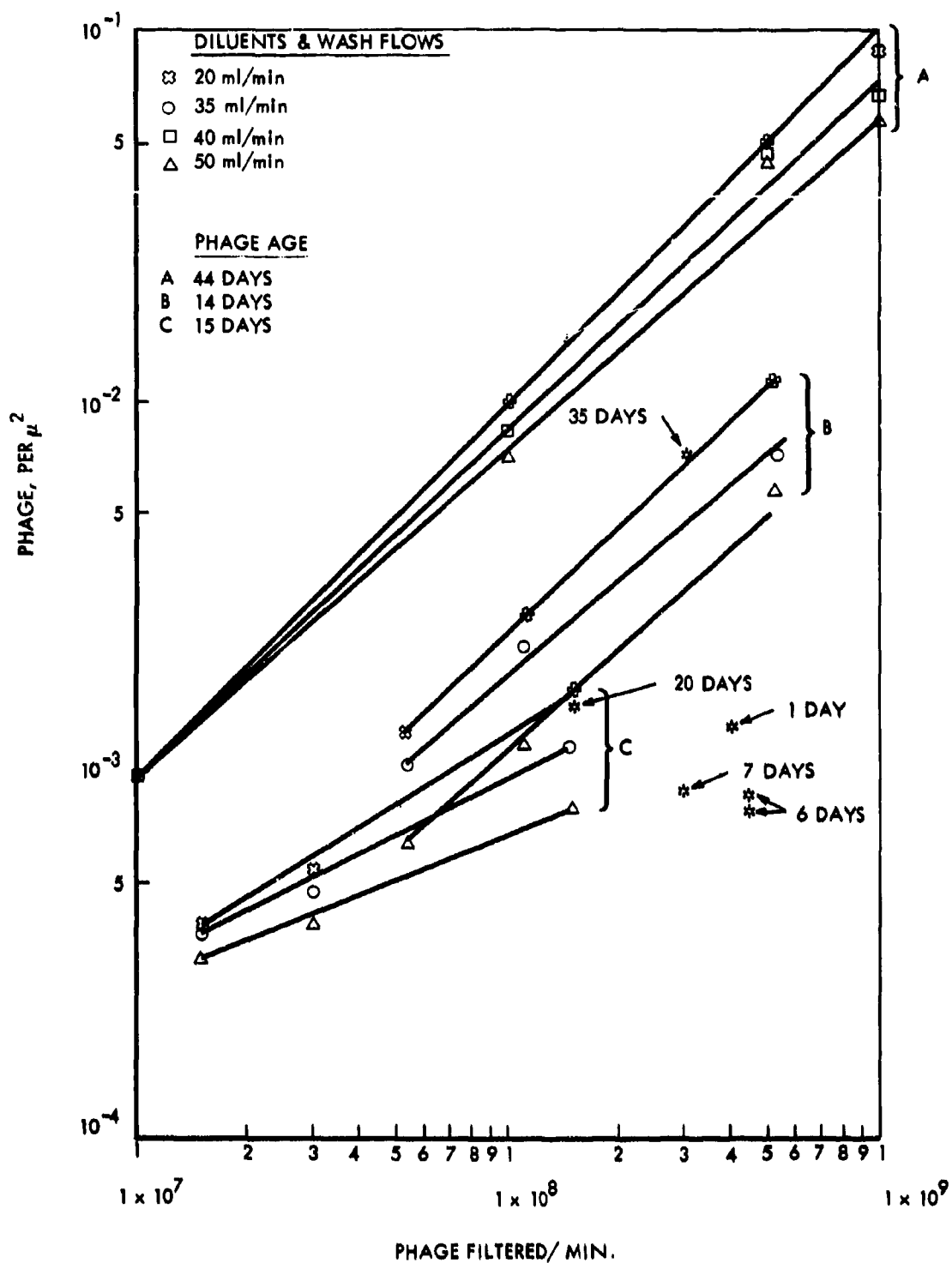


Figure 4-11. Adsorption of Phage to Tape in Continuous Runs

Among the many variables are phage preparation, tape composition, temperature, conditioning of reagent, etc. Results are shown for several test groups, each of which represents a certain kind of tape and a single preparation of phage. Within some groups, the effects of varying amounts of diluent liquid and wash liquid were explored, and are indicated here.

The following conclusions are drawn: (1) Major differences in phage retention as a function of phage throughput appear between groups. That is, they are attributable to changing types of tape or changing phage preparations and conditioning, and also to aging of phage. (2) Within one single group, there appears to be a large, though erratic, increase in comparative retention as age of the phage increases from 1 day to 35 days. Considering all points in general, those for old phage usually fall well above those for new phage. (3) As amounts of diluent and wash liquid are increased within each group, there are minor but consistent decreases of comparative retention. (4) The form of the variation of retention with phage throughput is of much importance. There is a general tendency for p to vary with ϕ roughly as a straight line with slope of unity, in series of tests with all conditions except ϕ held constant. This implies that phage retention is roughly proportional to phage throughput, as might be predicted. (5) However, the proportionality constant varies widely between groups, reflecting the wide spread of points discussed above. Thus, at a phage throughput of 10^8 per minute, retention on the tape varies approximately between 5×10^{-4} and 10^{-2} per μ^2 . If these values are multiplied by the area of deposition in one minute, $7.3 \times 10^8 \mu^2$, then the level of non-specific adsorption is 0.4 to 7 percent. This is much higher than the best values obtained in laboratory tests, and emphasizes the need for further development.

4.1.3.3.3 CONTINUOUS REACTIONS

E. coli were continuously reacted with tagged phage at different concentration levels, and the steady-state signals from the deposits were measured. Volumes of 0.5 ml each of suspensions of bacteria and phage were combined and reacted for 3 minutes at 40°C . Diluent was added to the reacted mixture at 40 ml/min; it was then filtered through CCl_4 -treated Millipore tape,

followed by a wash of 10 ml/min. The resulting signals (corrected to represent a 1-minute deposition area) for phage compositions of 5×10^7 to 5×10^8 per ml (20 to 21 day-old phage) and bacterial compositions of 5×10^5 and 2×10^6 per ml are shown below. Predicted values calculated from the known reaction kinetics (assuming multiplicities appropriate to large excesses of phage) are shown in parentheses.

COUNTS PER MINUTE ADJUSTED TO 1-MINUTE DEPOSITION AREA

Phage/ml (= Phage/min)	<u>Bacteria/ml = Bacterial/min</u>	
	<u>5×10^5</u>	<u>2×10^6</u>
5×10^7	2.6×10^3 (2.6×10^3)	3.4×10^3 (1.0×10^4)
	2.1×10^3 (2.1×10^3)	2.0×10^3 (8.2×10^3)
1×10^8	4.8×10^3 (4.0×10^3)	5.2×10^3 (1.6×10^4)
	3.2×10^3 (3.1×10^3)	3.7×10^3 (1.3×10^4)
5×10^8	1.3×10^4 (1.1×10^4)	1.5×10^4 (4.4×10^4)
	1.1×10^4 (1.1×10^4)	1.2×10^4 (3.5×10^4)

Measured signals agree consistently with predicted values at 5×10^5 bacteria/ml. However, they fall far short at 2×10^6 bacteria/ml. In contrast, a few other tests (not shown) gave actual counts far larger than predicted values, for bacterial concentrations greater than 10^6 per ml. Further experimental work is needed to resolve these disagreements*.

If the data for 5×10^5 bacteria/ml are considered reliable, then good sensitivity is attainable as soon as internal background (non-specific adsorption) can be reduced. Detection of 10^4 to 10^5 organisms per minute with these reagents appears to be a reasonable expectation, and this may be improvable with better reagents and reaction conditions.

* A reported agreement between actual and predicted values for the first experiments in Status Report No. 20 (15 August 1965) is in error.

4.1.3.4 CONCLUSIONS

The experimental data indicated that this method can be adapted to instrumentation and that such a system can meet the necessary qualifications for rapid detection of aerosolized BW agents. The inability of this method to detect viruses would seem to be balanced by the logistic advantages it offers for detecting bacteria. It further provides a principle of detection which is distinct and different from immunological and other approaches to detection.

It is felt that further studies on this method should be continued with the ultimate goal of using this principle in combination with a radiotracer method for virus detection.

4.1.3.5 REFERENCES

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The objective of the enzyme approach is to develop a detection scheme based on a constitutive enzyme common to most pathogens and which has a high sensitivity to readout. High concentration in the organisms and rapidity of reactions are also desirable features. Where required, the enzymatic activity should be detectable in spores or resting cells and should withstand freeze-drying, aerosolizing, and conventional techniques used for collection and concentration of bacteria. Low background interference for the system selected would be required to assure maximum sensitivity.

In assigning an appropriate physical method for detecting the products of an enzymatic or other biochemical reaction, emphasis was placed on utilizing those techniques with the ultimate in sensitivity, namely those involving chemiluminescence, fluorescence, phosphorescence, electron affinity, and radioactivity. The sensitivities of these methods are several orders of magnitude greater than those of conventional techniques involving colorimetric, spectrophotometric, or manometric measurements.

Sufficient work was done on each system to establish its sensitivity and to gain some familiarity with the problems that might be encountered in going from a static to an automated system. Based on this experimental survey, certain systems appeared to offer more promise than others and these were selected for more intensive study and automation.

The most attractive system at the present is that involving luminol chemiluminescence initiated by hematin iron originating in catalase or applied as a direct stain to the agent. By a combination of techniques, a single instrument can be used to detect bacteria (spores and vegetative), virus carrier, or virus itself. Aside from the demonstrated multi-agent capability and sensitivity (10^3 to 10^4 bacteria/min, 10^4 HeLa cells, 10^{-9} gm embryonated egg, 10^4 LD₅₀ NDV infectious particles), this method has the advantages of rapidity of response (reaction in less than 1 second), relative ease of automation, and minimum background interference. Fabrication and testing of a breadboard model based on this approach is described.

Two other methods which appear as promising candidates for breadboarding and involve formation of a fluorescent product are those based on monitoring of transaminase or alkaline phosphatase activity. Both of these enzymes are

ubiquitous in nature. Other desirable features of these two systems include the reported presence of transaminase activity in rickettsiae and the ability to detect alkaline phosphatase activity in bacterial spores without sonication. The present level of detection of B. globigii by monitoring of transaminase activity is 10^4 BG/ml (or $< 10^2$ (total) in a $4 \mu\text{l}$ viewing cell), of alkaline phosphatase activity, approximately 10^5 /ml (or 10^3 in a $4 \mu\text{l}$ cell).

An interesting method for detecting virus within 15 minutes after infection, based on sialoresponsin formation, was also briefly examined. This approach to early virus detection appears very promising provided sialoresponsin can be established as a general phenomenon of virus infection and if a sensitive assay method can be developed.

Other systems which have been examined but which appear less promising, for various reasons, include protein quenching of Eosin Y fluorescence, quenching of DPPH*- luminol chemiluminescence by sulfhydryl groups in bacteria, phosphorescence of proteins at low temperature, monitoring of C^{14}O_2 generated by metabolism of radioactive glucose by bacteria, detection of NH_3 in enzymatic reactions by electron capture, monitoring of fluorescent DPNH formed by L-glutamate dehydrogenase activity in bacteria.

Details of the work in each of these areas are presented below.

4.1.4.1 CHEMILUMINESCENT DETECTION SYSTEM (CDS)

4.1.4.1.1 SUMMARY

The detection sensitivity of the continuous chemiluminescent detection system is of the order of 10^3 to 10^4 B. globigii (vegetative) per minute at a concentration level of 10^4 /ml (Petroff-Hausser count). Further improvement in sensitivity appears possible through use of disrupted cells and a reactor cell design which insures more rapid and efficient mixing. A preliminary evaluation of

* DPPH is 2,2-diphenyl-1-picryl-hydrazyl

the effect of background indicated that the luminescence of the blank is increased by less than 20 percent on going from filtered to unfiltered air. Nebulized B. globigii can be detected at a level of 10^4 /ml or 10^4 to 10^5 per minute.

Without modification the detector can also be used for detection of virus carrier with a demonstrated sensitivity of 10^{-9} gm egg solids/ml (corresponding to 3×10^3 , 1μ or $30,5 \mu$ egg particles). HeLa cells (3×10^4) were also detectable by luminol chemiluminescence at an S/N luminescence ratio of 2/1 (in a static system).

4.1.4.1.2 INTRODUCTION

The chemiluminescent detector, built and operated under the current program, records continuously the luminescence produced by action of iron porphyrin (in bacterial catalase and egg carrier) on alkaline luminol and hydrogen peroxide. Aside from the simplicity in instrumentation required for continuous detection, the detector has a very rapid response time (seconds or less), high sensitivity, and multi-agent capability (bacteria and virus carrier). Preliminary experimental evidence presented below indicates that background interference is minimal.

Results obtained with both static and flow systems are described and a design for an improved version of the CDS which emphasizes compactness, low weight, and simplicity of design is presented.

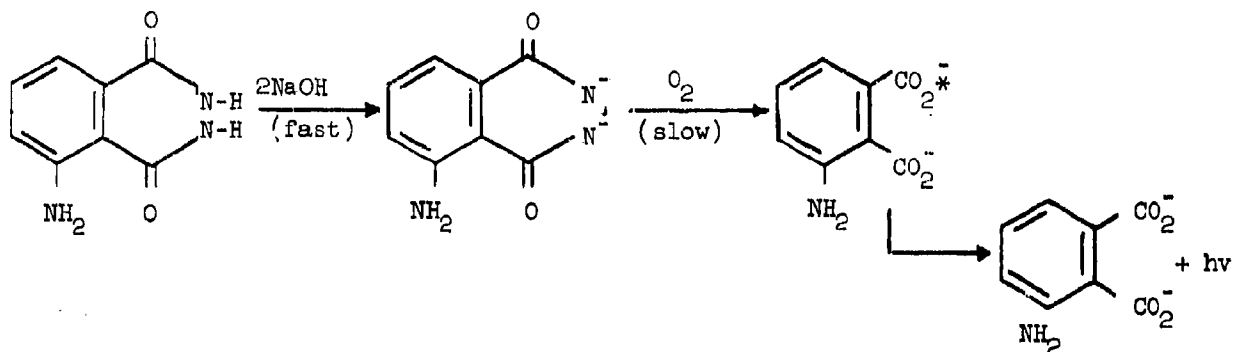
4.1.4.1.3 STATUS

A discussion of the theoretical and experimental evidence in each of these areas is presented below.

4.1.4.1.3.1 MECHANISM OF LUMINOL CHEMILUMINESCENCE

A clearer understanding of the mechanism of hematin iron catalysis of the chemiluminescence of luminol would considerably aid in the present efforts to automate the method and to increase its sensitivity for the rapid detection of bacteria. Several points concerning the interrelationships of the reacting species are of interest in this regard, namely, those determining the pH dependence of the chemiluminescent reaction itself and those affecting the decomposition of H_2O_2 .

A mechanism proposed by White^(1,2) for the chemiluminescence which accompanies the irreversible oxidation of luminol in alkaline solution is shown below:



The dinegative ion of luminol ($K_2 = 10^{-13}$) reacts with oxygen to form ultimately an excited singlet state of the amino-phthalate ion which emits a photon. Evidence that the aminophthalate ion is the light-emitting species is found in the good match between the emission spectra of luminol chemiluminescence and the fluorescence of sodium aminophthalate. On this basis, the efficiency of luminol oxidation should increase with increasing pH. However, it was shown by Seliger⁽³⁾ that the pH dependence of the chemiluminescence quantum yield closely parallels that of the fluorescence yield of the aminophthalate ion, with both reaching a maximum around pH 11 and decreasing rapidly in more alkaline solution. A comparison at Space-General of the light intensities produced at three different pH values (luminol in pH 10.4 (bicarbonate buffer), pH 11.3 (0.1N Na₂CO₃), pH 12.4 (0.1N NaOH) showed a maximum intensity at pH 12.4. (In the last case, addition of H₂O₂ to the luminol actually serves to lower the pH to a value of 11.2 for the final reaction mixture.) There may be some advantage in carrying out the luminol reaction at an even higher pH and including a second fluorescent species whose fluorescence yield is also high in this region, and thus produce better signals through sensitized fluorescence.

The data of Neufeld, et al⁽⁴⁾, established that hematin is an effective catalyst of luminol chemiluminescence and that the signal observed with bacteria is a function not only of their hematin content, but the accessibility of the hematin moiety for reaction with H_2O_2 , as well. The pH dependence of the hematin-catalysed chemiluminescence is parallel to that discussed above for the luminol chemiluminescence generally, and led Neufeld to suggest that enzymatic decomposition of H_2O_2 by catalase was not a factor in the chemiluminescent signal produced by this enzyme. Recent studies on rotary dispersion of catalase indicate that denaturation of the protein moiety occurs outside the pH range of 3.5 to 10.5, thus supporting Neufeld's contention that hematin iron and not catalase per se is the active agent in initiating chemiluminescence with bacteria.

Free radical catalysis has long been implicated in the chemiluminescence of luminol (e.g. White⁽⁵⁾) and many attempts have been made to demonstrate the direct participation of radicals in the luminol reaction. White, et al⁽²⁾, however, could not obtain EPR signals indicative of free radical formation during oxidation of luminol, even when their reactions were carried out at -60° . In the case of hemin-catalysed reactions, Jordan and Bednarski⁽⁶⁾ found that the oxidation and reduction of hemin in 0.1M KOH proceeds by a two-electron transfer. Both of the above observations argue against a free radical mechanism for the luminol reaction. It was observed at Space-General, however, that relatively high concentrations of catalase ($10^{-5}M$) in alkaline luminol produce a slight EPR signal which slowly decays on addition of H_2O_2 (see Section 4.1.9.1). This may possibly be explained on the basis of generation of radicals by catalase and subsequent quenching of these by H_2O_2 . In this case it is necessary to invoke formation of a non-radical, electronically excited state of the O_2 generated during H_2O_2 decomposition by the hematin iron to account for chemiluminescence. One possibility is that O_2^* resulting from reaction of either O_2 or H_2O_2 with free radicals or from H_2O_2 decomposition by hematin can form a triplet state adduct, such as that suggested by White and Bussey⁽²⁾, on collision with luminol. This adduct could then rearrange to an excited singlet and decay with emission of a photon, in accordance with reaction sequence given by (1) above.

4.1.4.1.3.2 STATIC SYSTEM

Sensitivity - In addition to the studies cited above, a static system was used to define some of the parameters involved in luminol chemiluminescence before proceeding to a flow system. The light measuring equipment included an RCA 1P21 photomultiplier tube, a Kepco high voltage supply (operated at 750V, 5 milliamps) and a Sanborn DC amplifier pen recorder (Model 320).

The initial studies cited below employed the following reaction mixture (referred to as reagent mixture A^{*}). It was selected on the basis of study of the effect of H₂O₂ and luminol concentration on the peak height ratio (S/N^{**}).

0.25 ml luminol solution (0.2 mg luminol/ml
in 0.1N NaOH, pH 12.4)

1 to 50 µl of a bacterial suspension

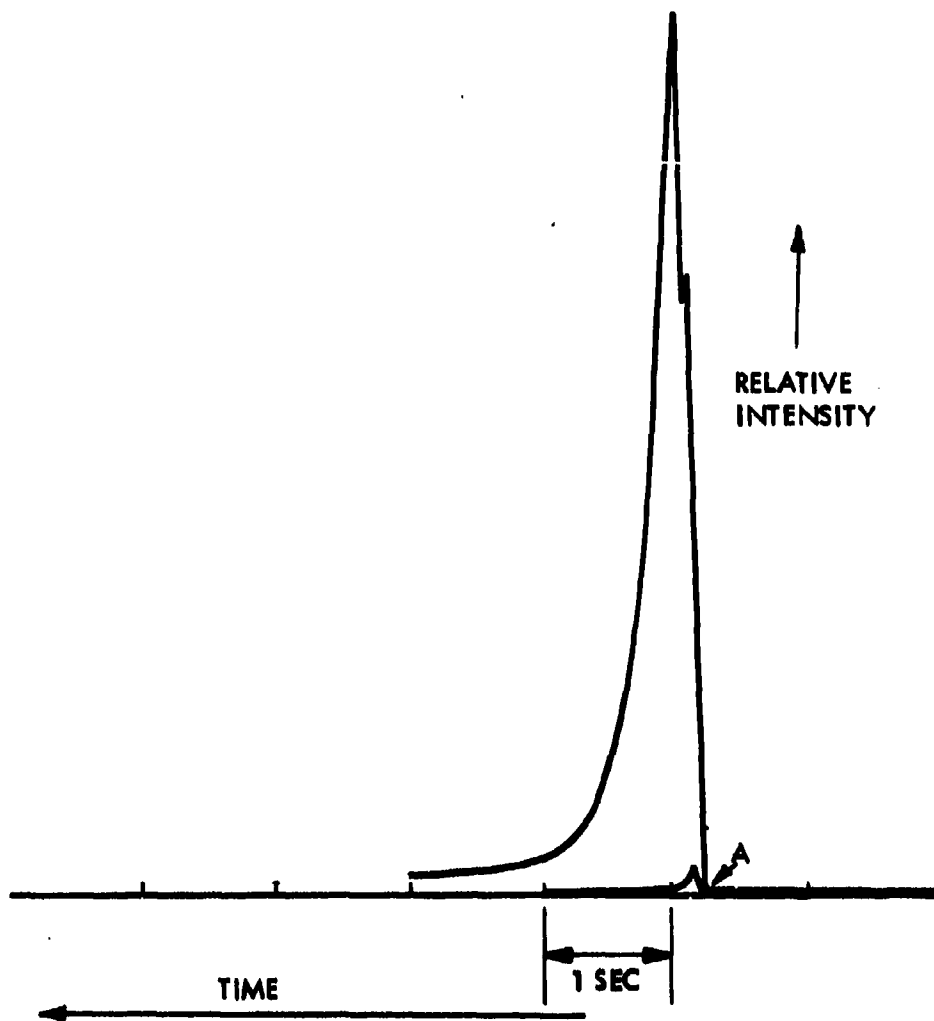
0.05 ml 3% H₂O₂

A more detailed study later indicated that lower blanks and higher S/N ratios could be obtained using a lower luminol concentration (0.0625 mg/ml) and by addition of EDTA (disodium ethylene diamine tetraacetate), 1 mg/ml, to the luminol and H₂O₂ solutions, respectively (referred to as reagent mixture B).

In a typical experiment, the solution containing catalase or bacteria is added to the reaction tube containing the alkaline luminol solution. The reaction tube (10 x 75 mm) is mounted in a light-free chamber. The H₂O₂ is then rapidly injected into the reaction mixture through a black cloth draped over the tube opening. No chemiluminescence is observed until the final addition of the H₂O₂. Rate of mixing is a very important variable. Maximum light intensities are achieved on rapid injection of the H₂O₂. A blank, run without bacteria being present, exhibits a comparatively low-intensity light emission. A typical trace obtained with and without bacteria is shown in Figure 4-12. Maximum light intensity is reached within 0.5 second after injection of the H₂O₂, followed by an exponential decay. Data obtained for several microorganisms using reagent mixture B are shown below.

* Reagents are prepared fresh daily.

** S/N = chemiluminescent intensity ratio of Sample (bacteria + luminol + H₂O₂)/
Blank (luminol + H₂O₂).



UPPER CURVE: 0.05 ml 3% H_2O_2 (+ EDTA) INJECTED AT POINT A
 INTO 0.25 ml LUMINOL (.0625 g/l IN 0.1 N NaOH + EDTA)
 CONTAINING 1×10^{-6} B. globigii

LOWER CURVE: BLANK (LUMINOL + H_2O_2)

Figure 4-12. Chemiluminescence as a Function of Time
 (Static Run)

<u>Microorganism</u>	<u>No. of Bacteria*</u>	<u>S/N</u>
<u>B. globigii</u>	4×10^4	2.3/1
<u>E. coli</u> 026B6	2×10^5	4.8/1
<u>E. coli</u> B/5	2×10^5	3.7/1
<u>S. marcescens</u>	8×10^4	3.3/1

No increase in sensitivity was observed either by changing the order of addition of ingredients or by use of certain salts such as KCN which are reported to enhance luminescence. The relation between maximum light intensity, plotted as (S-N), and bacterial concentration is shown in Table 4-9 and Figures 4-13 and 4-14 for B. globigii (veg) and S. marcescens. Proportionality of response to bacterial concentration is also reported by Neufeld for a number of microorganisms. No significant response was obtained with B. globigii spores (4×10^6).

Effect of Temperature - The effect of temperature on S-N and S/N is shown below. The results are only approximate since a precise temperature control was difficult to maintain at temperatures other than ambient.

<u>Temp. of Reactants</u> <u>°C, ($\pm 2^\circ\text{C}$)</u>	<u>Light Intensity</u> <u>(in Arbitrary Units)</u>			
	<u>H₂O₂</u> <u>+ Luminol (N)</u>	<u>H₂O₂ + Luminol</u> <u>+ Bacteria** (S)</u>	<u>(S-N)</u>	<u>S/N</u>
9	9	42	33	4.7/1
24	15	64	49	4.3/1
39	23	112	89	4.9/1

These results, consistent with those obtained using a flow system (described later), indicate that although the value of S-N increases with temperature, increase in S/N is not very significant above ambient. This would indicate there is

* Based on Petroff-Hausser count. Bacteria were all 16 hour cultures grown on TGY (tryptone, glucose, yeast) agar slants buffered to pH 7.4 with Tris buffer, centrifuged and washed twice with PBS with final suspension in PBS.

** 2.4×10^5 viable B. globigii (6.4×10^2 spores) were added to each (reagent mixture A).

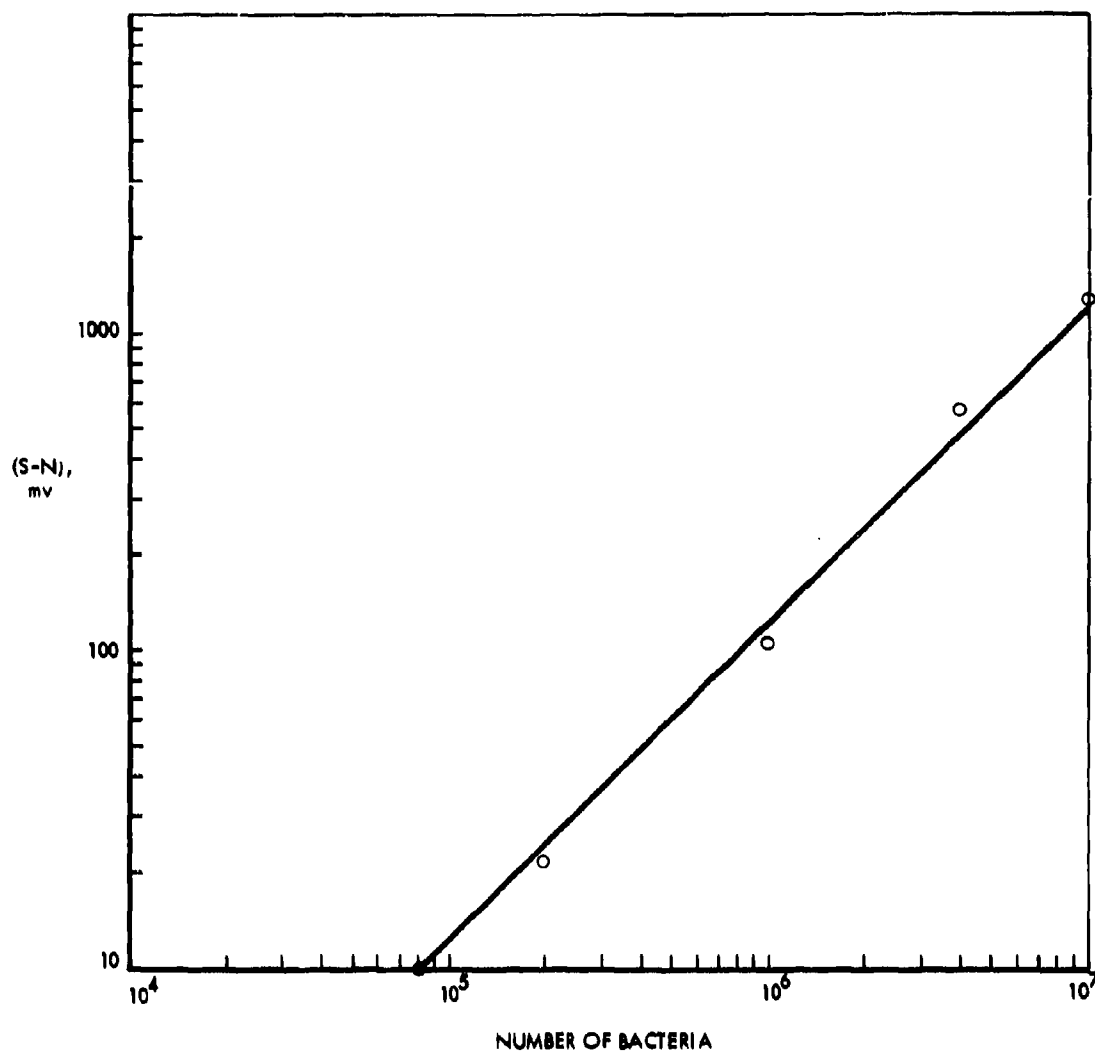


Figure 4-13. Chemiluminescence as a Function of B. globigii Count

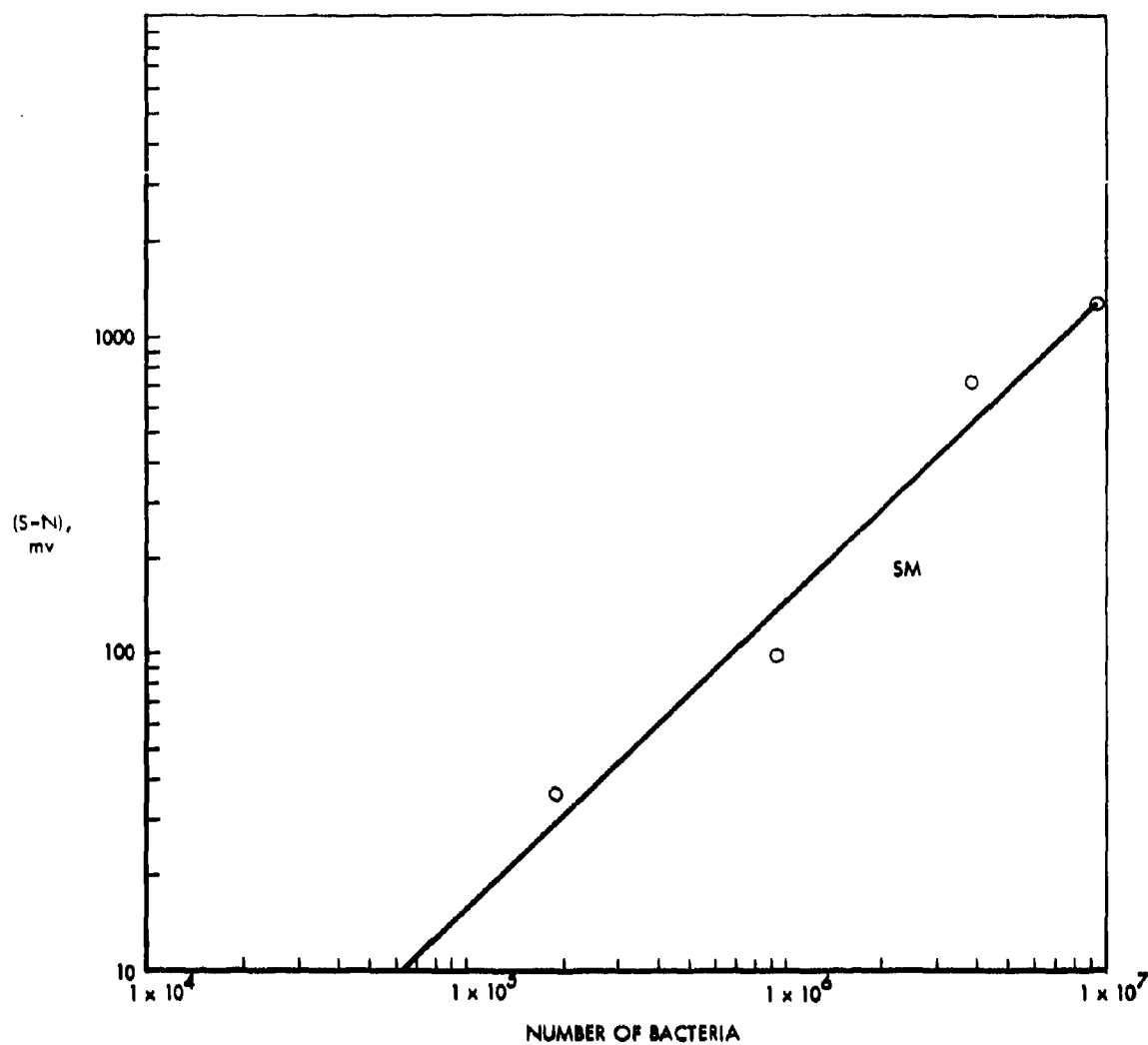


Figure 4-14. Chemiluminescence as a Function of S. marcescens Count

no particular advantage in operating above ambient temperature since S-N can be more conveniently increased by amplification. The data do indicate that a temperature control for the reaction cell is desirable or, alternatively, the use of dual streams (sample vs control) would be required to offset any temperature fluctuations.

Table 4-9

CHEMILUMINESCENCE AS A FUNCTION OF NUMBER OF BACTERIA*

<u>Microorganism</u>	<u>No. of Bacteria**</u>	<u>(S-N)</u>	<u>S/N</u>
<u>B. globigii</u>	4.0×10^4	5	2.3/1
	3.0×10^4	10	3.5/1
	2.0×10^5	22	6.5/1
	1.0×10^6	148	38/1
	4.0×10^6	596	150/1
	1.0×10^7	1296	325/1
<u>S. marcescens</u>	7.6×10^4	9	3.3/1
	1.9×10^5	36	4/1
	9.5×10^5	98	9.2/1
	3.8×10^6	723	61/1
	9.5×10^6	1264	106/1

Effect of Bacterial Viability on Luminosity - Studies were made to determine whether E. coli, killed by subjecting them to various conditions of heat and ultraviolet exposure, would retain their ability to initiate chemiluminescence. The activities were determined by the luminol reaction with the following results:

* Reagent mixture B, static run.

** Petroff-Hausser count.

	<u>Luminescence Ratio S/N*</u>
Untreated (live) <u>E. coli</u>	18/1
<u>E. coli</u> heated 15 min at 100°C**	10/1
<u>E. coli</u> irradiated 5 min with UV**	22/1

It is evident that even though the E. coli failed to reproduce themselves after being subjected to heat and UV radiation, they still retained the ability to initiate luminol chemiluminescence. These results reflect the innate stability of the iron porphyrin moiety, which is believed to be the actual catalyst for the luminescence.

Effect of Cell Disruption on Chemiluminescence - Although sonication of B. globigii failed to improve the chemiluminescence, disruption of the bacteria with the French cell led to about a threefold increase in signal. A bacterial suspension of B. globigii (vegetative, 1×10^9 /ml total (Petroff-Hausser), 8×10^8 /ml viable (4×10^6 /ml spores)) was passed through a French Press (previously cooled to 0°C) at 20,000 psi. The total viable number in the effluent, checked by plate count, was 5×10^5 /ml, indicating reasonably efficient disruption. Without attempting to separate the undisrupted fraction, or cell walls, an aliquot was selected for testing with luminol both with and without further addition of H_2O_2 . An undisrupted bacterial suspension of the same initial concentration was used for comparison. The results are tabulated below. The bacterial concentration indicated is based on the original Petroff-Hausser (total) count.

	<u>Sample/Blank Luminescence Ratio</u>	
	<u>Without H_2O_2</u>	<u>With H_2O_2</u>
5×10^7 (disrupted cells)	~ 1.5 mv/0.5 mv (3/1)	3000 mv/50 mv (60/1)
5×10^7 (whole cells)	~ 1.5 mv/0.5 mv (3/1)	1000 mv/50 mv (20/1)

* Average of several runs; same number of E. coli added to each (reagent mixture A used).

** E. coli failed to grow in a culture medium after heating for 15 minutes at 100°C or irradiating for 5 minutes with UV.

The data indicate that, in the absence of H_2O_2 , a slight signal is observed at this bacterial level on injecting the disrupted bacteria into luminol; however, the signal (as well as sample/blank ratio) is enormously higher when used in conjunction with H_2O_2 . The intensities of the blanks (blank consisting of PBS solution injected into luminol) for the runs without H_2O_2 are approximate since they are difficult to measure accurately with the present recorder due to the low light levels. Disrupted bacteria in the presence of H_2O_2 leads to about a threefold increase in signal. These results were confirmed with a continuous flow system (to be described later) when a 250 percent increase in signal was observed on using bacterial cells disrupted by a French Press.

Effect of Mixed Indicators on Luminescence - A recent report indicates that marked increases in chemiluminescence can be achieved by use of mixed indicators of fluorescein and luminol⁽⁸⁾. The luminol serves to catalyze the chemiluminescence of fluorescein by serving as an oxygen carrier to the substrate and thus facilitating the formation of fluorescein peroxide. An examination of a 1:1 molar mixture of luminol and fluorescein in 0.1N NaOH indicates no particular advantage over luminol itself for although there is at least a twofold increase in light intensity with mixed indicators compared to luminol, the background signal (without catalase but with H_2O_2) is proportionally higher. The mixed indicator system also appears to be less stable, resulting in a greater variability from day to day.

Effect of Reagent Composition on Chemiluminescence - One of the problems associated with automating the hematin-luminol reaction is the extreme rapidity of the reaction. The result is that rate of mixing becomes a critical factor affecting the reproducibility of the reaction. A means of increasing the time interval between mixing and light emission or, alternatively, of stabilizing the light emission against rapid decay would obviously be desirable. The effect on luminescence of substituting a luminol-perborate solution in place of the luminol- H_2O_2 currently being used was investigated. The former has been used in an analogous chemiluminescent reaction for detection of nerve gases, the nerve gas ostensibly serving the same function as catalase does in the present system⁽⁹⁾. The time required to reach peak intensities for the luminol-perborate-nerve gas system has been reported at about 5 to 15 seconds. An examination of several

variables indicated that, although the shape and peak height of an intensity versus time plot will vary depending on volume of reactants and order of addition, the time to reach maximum chemiluminescence was virtually instantaneous (less than one second) when either catalase or bacteria was used with a perborate-luminol reagent mixture*.

Effect of Other Agents on Luminol Chemiluminescence

a. Two components frequently used in the Litton collecting device were evaluated for their effect on chemiluminescence.

- (1) Wetting Agent: Addition of Brij 35 (0.1 percent) to a bacteria suspension in luminol (with EDTA) was found to decrease the S/N ratio (i.e., from S/N = 11/1 (without Brij 35) to S/N = 4/1 (with added Brij 35)).
- (2) Glycerol: It was found that the intensity of luminescence obtained with a glycerol-luminol system was only about one-third that of an analogous water-luminol system**. However, the ratio of sample-to-blank light intensities (S/N) was about the same for both liquids, at the same bacterial concentration. In addition, the luminescence for the glycerol system lasts for minutes instead of seconds.

b. Chelating Agents: Further increases in sensitivity of the luminol reaction were sought by reducing the blank reaction through use of more effective metal chelating agents than EDTA. Data shown below indicate the effect of using sodium salicylate (1 mg/ml) in place of EDTA as an additive in both the luminol and the H_2O_2 . Also, the effect of replacing the NaOH (known to contain trace amounts of metal impurities which would contribute to the blank) by aqueous NH_4OH (2N, pH 12) was evaluated both with and without bacteria. The results are summarized below.

* Other components included pyridine and trisodium phosphate.

** Luminol (0.25 g/liter) in 0.1N NaOH (aqueous) vs Luminol (0.25 g/liter) in 0.1N NaOH (1:1 glycerol: water).

	<u>Relative Blank Value</u>
Standard Luminol Solution (+EDTA)	1.0
Luminol Solution (without EDTA)	2.4
Luminol + Sodium Salicylate	1.6
Luminol + NH_4OH + EDTA (no NaOH)	0.5

However, with a sample containing bacteria, a comparison of the sample/blank (S/N) luminescence ratios of the luminol + NH_4OH vs luminol-NaOH (both contain EDTA and the same amount of bacteria) gave:

	<u>Luminescence Ratio (Static System)</u>
NH_4OH -Luminol Solution	30 mv/20 mv = 1.5/1
NaOH - Luminol Solution	225 mv/40 mv = 6/1

The data indicate that, although the standard NaOH-luminol solution gives a higher blank than NH_4OH -luminol, the luminescence ratio for the former is considerably greater.

Evaluation of Atmospheric Background - The level of detection of bacteria by the luminol- H_2O_2 -reaction is of the order of 10^5 bacteria using the static system. Before attempts were made to improve the sensitivity even further, it was of interest to determine the magnitude of the signal that would be produced by the bacteria or other agents normally present in the atmosphere. A Gelman sampler was used to collect the particulates from 10^5 liters of air onto a glass-fiber filter. The latter was then macerated for five minutes in a Waring blender (sterilized) with 100 cc of sterile water, filtered through a fritted glass disc (10 μ pore size) to remove inorganic debris, and the filtrate was centrifuged at 14,000 rpm to concentrate the bacteria. The supernatant liquid was poured off and the residue reconstituted to a 1 ml volume with sterile distilled water. A glass-fiber filter through which air had not been drawn was similarly processed, and served as a control. Aliquots were removed for testing of bacterial activity by the luminol reaction. Two separate runs were made and the aliquots, representing 1.7×10^5 and

2.5×10^5 liters of El Monte air*, each gave a signal which was no greater than that of a control (a chemiluminescence intensity of about 3 units was observed for both the sample and control. These results, consistent with results obtained later using an integrated collector-detector to sample the air, indicate that the background contribution to luminol chemiluminescence is not a major problem.

Detection of Virus Carrier by Luminol Chemiluminescence (Static System) - An indirect method investigated for detecting virus entailed monitoring the hematin iron activity in the carrier medium (very often chick embryo) by luminol chemiluminescence.

Initial tests were concerned with studying the effect of age of embryonated egg on hematin activity. For the test, fertile eggs (White Leghorn) were kept for prescribed periods in a humidified incubator at 37°C . As required, eggs were removed, candled, weighed, and added to a known amount of sterile distilled water in a sterile blender (whole egg contents rather than just the chick embryo were used in these preliminary tests). The contents were blended for five minutes and then brought to the proper dilution (with sterile distilled water) prior to test. Substitution of either Difco buffer (pH 7.2) or Tris buffer (0.1 M pH 10.4) for distilled water in these instances had a negligible effect on the hematin activity. The standard luminol- H_2O_2 system was used to check for hematin iron activity**. The data shown in Table 4-10 and Figure 4-15 indicate that detectable hematin iron activity occurs after the fourth day and increases progressively until a maximum is reached in about 11 to 15 days. The minimum quantity detectable at this point was about $6 \times 10^{-7}\text{g}$ whole egg or $2 \times 10^{-7}\text{g}$ egg solids (approximately 26 percent egg solids in whole egg). A further increase in sensitivity was observed on using luminol- H_2O_2 reagents containing added EDTA, thus:

* A total viable count made on the aqueous concentrate in one instance was 0.32 cells per liter of air or approximately 8×10^4 live organisms in 2.5×10^5 liters of air, the sample size used for testing catalase activity.

** Concurrently the changes in alkaline phosphatase activity (by Na β -naphthyl acid phosphate method) and ATP content (by luciferin-luciferase test) were examined up to 11 days. The absence in this time interval of both of these components was indicated using a $1 \times 10^{-4}\text{g}$ whole egg sample.

Table 4-10

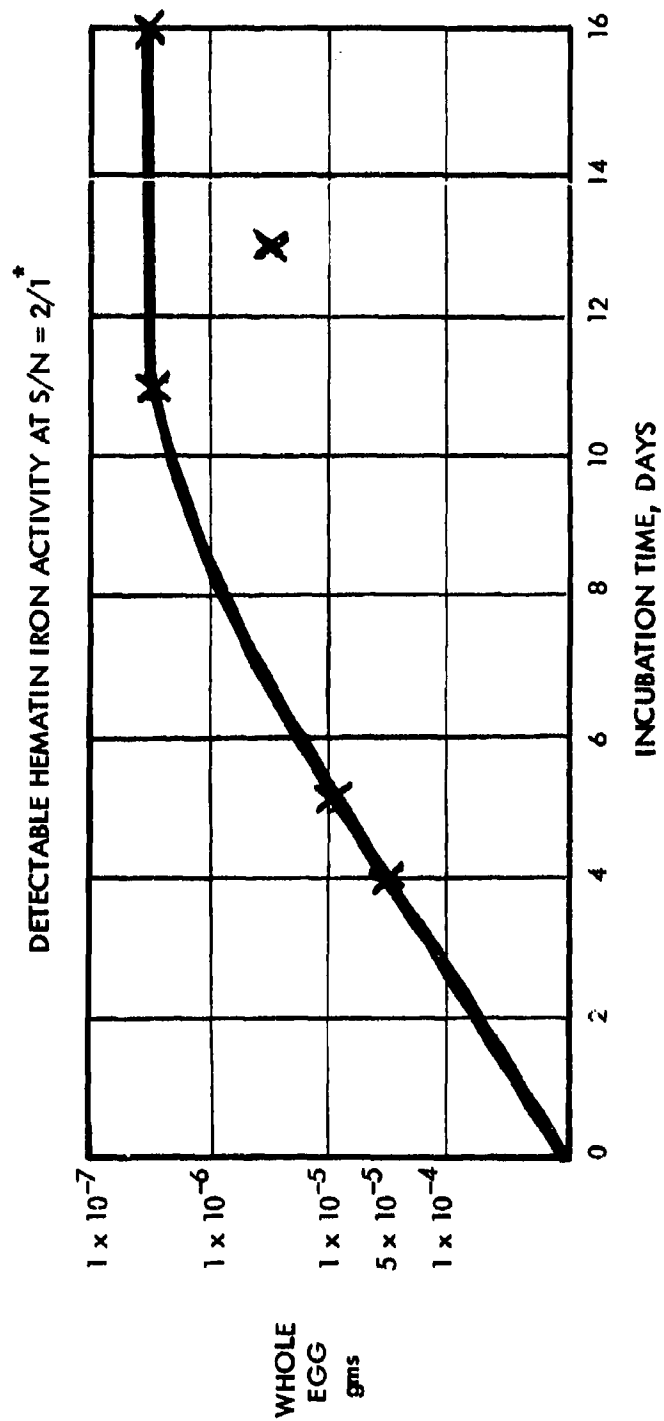
HEMATIN IRON ACTIVITY OF EMBRYONATED EGG

Incubation Time, Days at 37°C

0	1	4	5	8	11	14	15

(Sample/Blank
Luminescence)

Whole Egg Sample, g							
1×10^{-4}			5:1	-	124:1, 153:1	67:1	-
5×10^{-5}	1:1	1:1	2:1	-	47:1	-	-
1×10^{-5}	-	-	-	1.8:1	-	8:1 15:1	23:1
1×10^{-6}					1.7:1, -	1.4:1	2.5:1
6×10^{-7}					1.3:1 2.1:1	-	2.1:1
3×10^{-7}					- , 1.5:1	-	1.7:1



* By Luminol Reaction (In Absence of EDTA)

Figure 4-15. Hematin Iron Activity in Embryonated Egg

Chemiluminescence Ratio (S/N)

Sample weight	6×10^{-8}	1×10^{-7}	2×10^{-7}	4×10^{-7}	6×10^{-7}
(whole egg, g):	12/6	16/6	33/6	50/6	84/6

Within the limits of accuracy of the static method the results indicate rough linearity between concentration and luminescence. The results indicate that as little as 6×10^{-8} g whole egg or 2×10^{-8} g egg solids can be detected. The latter figure corresponds to about 3×10^4 1- μ egg particles or 3×10^2 5- μ egg particles.

This study was extended to other tissue cultures (Salk heart and HeLa cells) and it was found that although 14-day old Salk heart tissue cells (4×10^4 cells) did not show any appreciable signal over that of a control, 7-day-old HeLa cells (sonicated and unsonicated) produced the following results:

<u>Number of HeLa cells (7-day)*</u>	<u>S/N Luminescence Ratio</u>
3×10^4 (unsonicated)	2.0/1
3×10^4 (sonicated)	1.7/1

It is evident that sonication did not improve the response. The age of the HeLa cells is another important variable which should be explored.

Luminol Reaction on Tape - At the current state of the art, a bacterial concentration of approximately 10^4 /ml** is required for the luminol reaction. Since the PEEP collector produces 10^3 /ml from an air sampler containing 1 bacterium per liter of air, a concentration step would obviously be required between collector and detector. A concentration technique which was investigated entailed filtering the aqueous bacterial suspension on a Millipore tape and adding a reagent mixture of luminol + H_2O_2 directly to the tape and

* The trypsinized HeLa cells were centrifuged and washed twice with PBS and re-suspended in PBS. The last washing was used as blank for reaction with luminol. The latter procedure was found necessary since trypsin itself was found to produce luminol chemiluminescence to some extent.

** Based on sensitivity obtainable with the flow system.

observing the luminescence. In a specific application of this technique, 5 ml of an aqueous suspension of 5×10^4 E. globigii in PBS was filtered through a Millipore disc (1/2 inch diameter) and washed with 5 ml of sterile water. The Millipore disc was placed face down in a sterile, flat-bottom glass tube mounted over a photomultiplier in a light-free housing. A premixture* of luminol and H_2O_2 (0.05 ml) was then rapidly injected onto this disc and the light output recorded on a strip chart recorder. A Millipore disc similarly processed, but without bacteria in the PBS, served as a control. Instantaneous chemiluminescence occurred on contact of the disc by the luminol- H_2O_2 premix with the intensity of the sample disc being up to twice that of the control. Reproducibility was poor in these initial trials owing to the difference in area wetted by reagent from sample to sample. This variability could be minimized by confining the bacteria to a smaller area and the use of an appropriate wetting agent.

Brief Resume (Static System) - Sensitivity of detection of micro-organisms is of the order of 10^4 to 10^5 cells (vegetative). E. globigii spores do not initiate luminol chemiluminescence. Although no advantage is to be gained by operating at a higher reaction temperature, a threefold increase in sensitivity can be achieved by use of disrupted bacterial cells. Bacteria (E. coli) rendered non-viable by heat and UV treatment still were able to initiate luminol chemiluminescence. No significant increase in sensitivity was observed on use of mixed indicators (luminol + fluorescein), substitution of sodium salicylate for EDTA, or NH_4OH for NaOH. The addition of Brij 35 to collecting fluid was found to lower the S/N ratio, of glycerol to lower the intensity of the signal and blank proportionately. The background contribution to luminol chemiluminescence contained in 10^5 liters of air (Gelman sampler) was found to be insignificant.

It was shown that virus carrier (2×10^{-8} g egg solids and 10^4 HeLa cells) can be detected by luminol chemiluminescence.

The feasibility of conducting the luminol reaction on tape was also demonstrated.

* Luminol- H_2O_2 premix contained 0.1 gm luminol, 10 ml 10% NaOH, 25 ml 3% H_2O_2 , 100 ml distilled water, and 1 mg EDTA/ml of solution.

4.1.4.1.3.3 CONTINUOUS CHEMILUMINESCENT DETECTOR SYSTEM (CDS)

Description of Apparatus - Although the chemiluminescent detector used on the present program has undergone a certain amount of continuous development, essentially two distinct versions have been used. The basic difference is in the type of photomultiplier tube employed. The first used an RCA 1P21 tube which could be moved along the axes of the reaction cell to permit capturing the Maximum light output under any set of conditions; the latest version employs an EMI 9558B end-window tube (operated variably at 1000 or 1100 volts).

The principal advantages of the EMI over the RCA tube, apparent at this time, are (1) the higher sensitivity of the former permits operation at a lower gain setting resulting in less drift, and (2) the larger cathode sensing area permits capturing the maximum light output of the reaction without necessity of a positioning device. The greater sensitivity of the EMI will also be of advantage at such time as the background luminol- H_2O_2 reaction can be reduced to the level of the dark current of the photomultiplier tube.

The current model of the continuous CDS is shown illustrated in Figure 4-16.

The pumping unit, mounted in a single box, consists of three separately controlled, motor-driven syringes capable of delivering 0.01 to 25 ml/min of the respective reagents (hydrogen peroxide, bacteria in luminol, and luminol control). The continuous reactor, in its simplest form, is a narrow glass tube in which the reagents are combined, and is mounted directly in front of the EMI photomultiplier tube. The sensing circuit is shown in Figure 4-17. A light-free metal housing protects the reaction zone. A small lamp, which is operated well below its power limitations, is also contained in the housing and provides a known, independent energy source for calibrating the detector. An alarm circuit (Figure 4-18) which can be preset to operate over a range of 0 to 50 microamperes was also incorporated in the detector. The alarm contains a microammeter which electronically activates two relays, one which is activated when the current exceeds a preset, but variable value, and the other when the current drops below another preset but variable value. Figure 4-19 is a typical recorder trace obtained with the current unit.

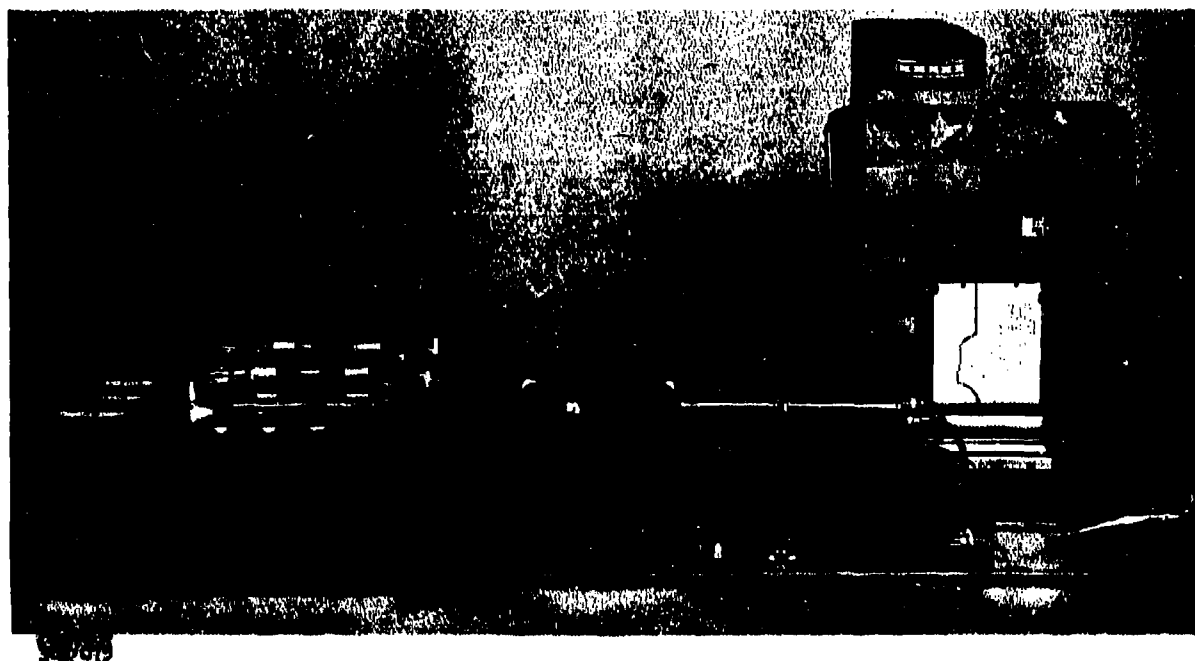
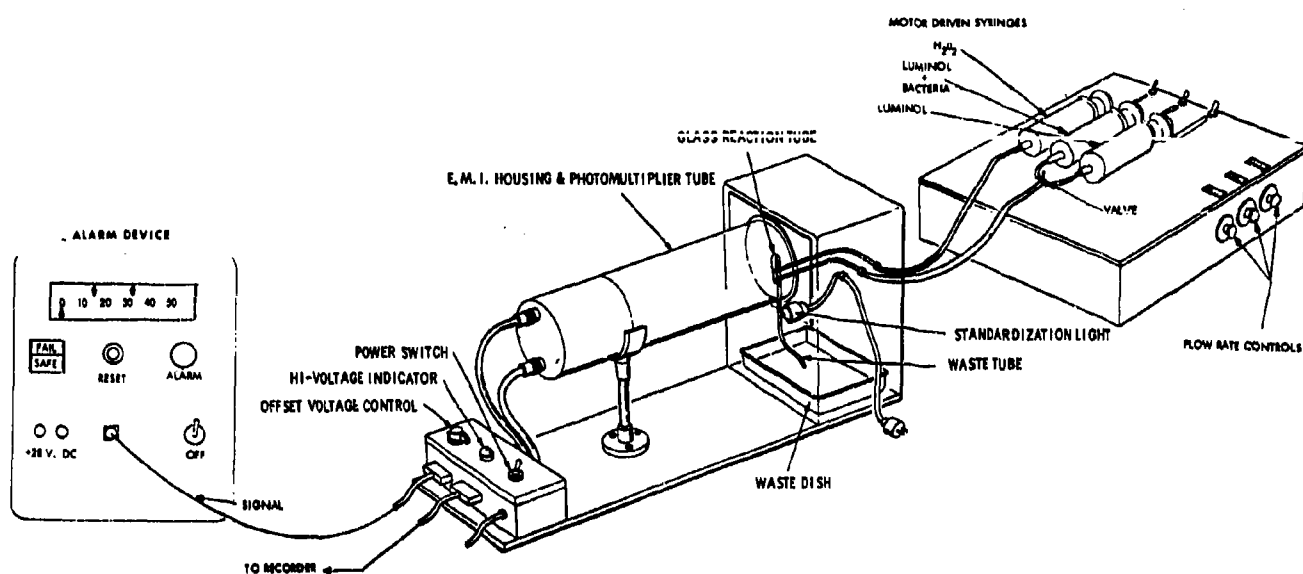


Figure 4-16. Chemiluminescence Detection System

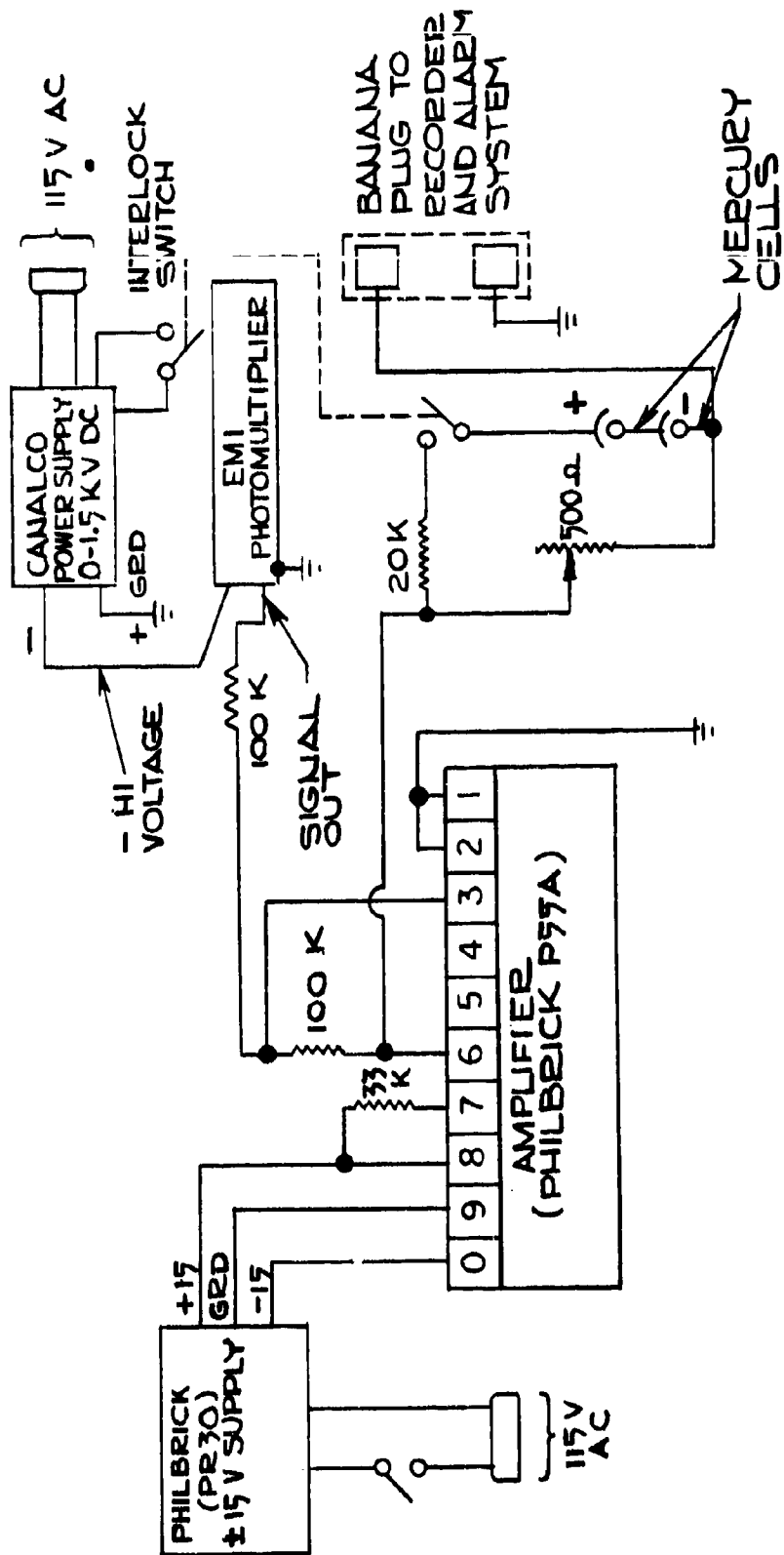


Figure 4-17. Sensing Circuit for EMI 9558B Tube

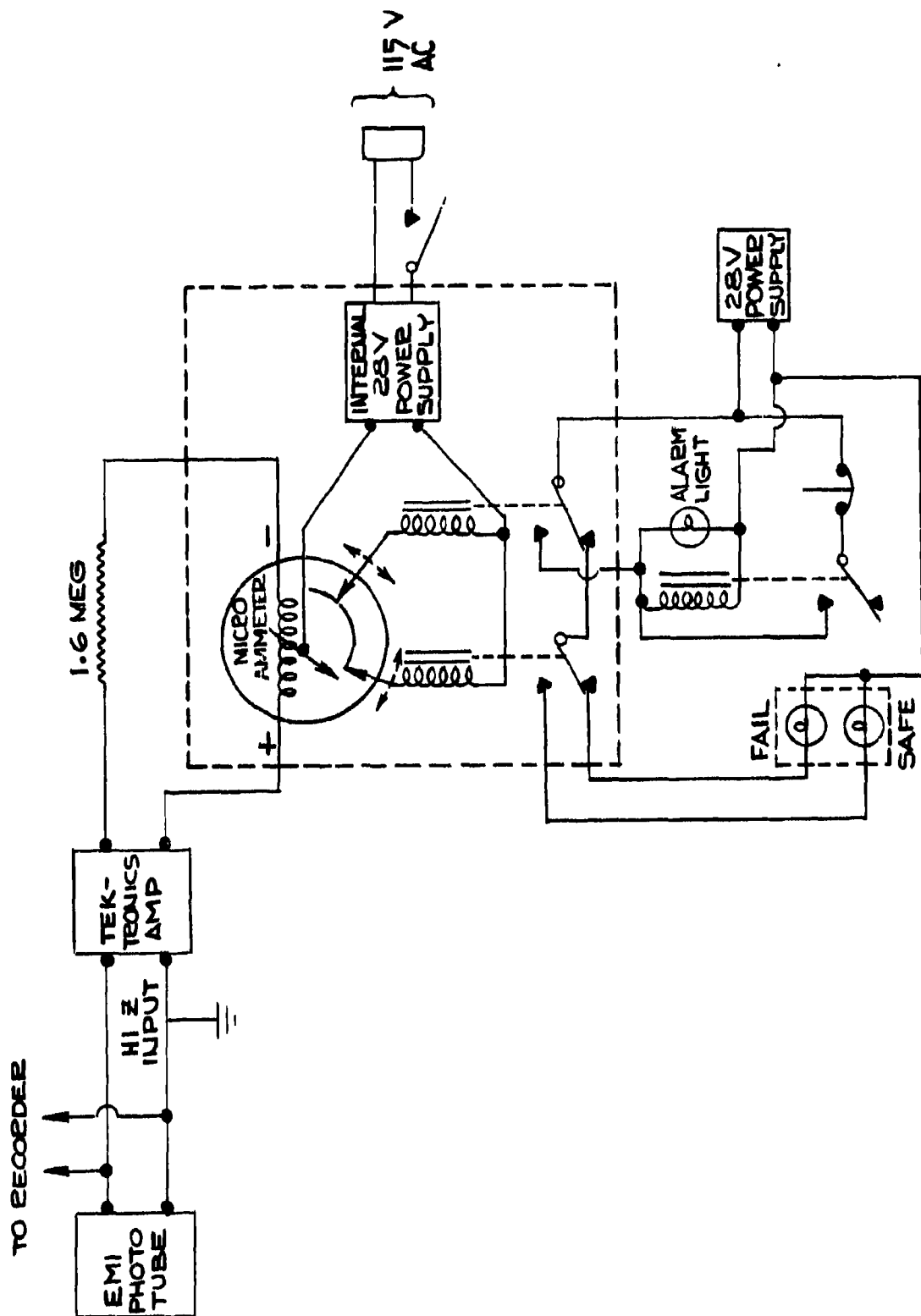


Figure 4-18. Alarm Circuit for Chemiluminescent Detection

Chart Speed: 1 mm/sec
Scale Sensitivity: 0.5 mv/mm
Reagents:
(1) 2×10^4 BG/ml in luminol solution, 0.33 ml/min
(2) 3% H_2O_2 , 0.066 ml/min
(3) Luminol solution, 0.33 ml/min
Type Reactor: U-Tube

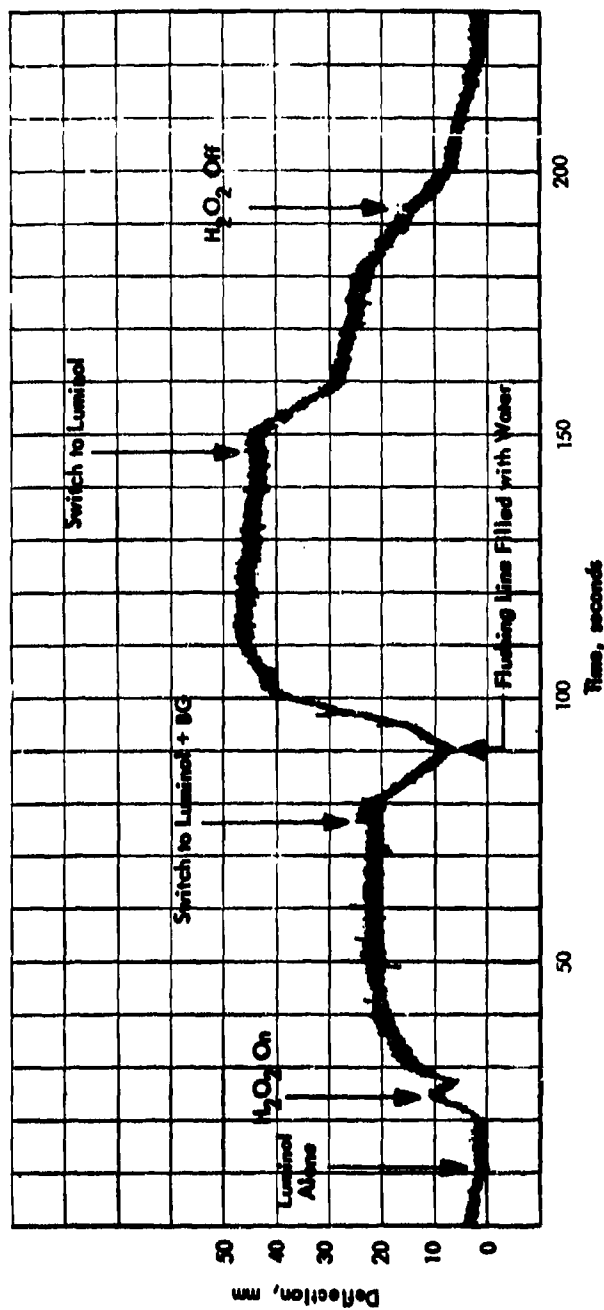


Figure 4-19. Example of Chemiluminescence Detector Recorder Trace

Sensitivity - The intensity of chemiluminescence observed under flow conditions is a function of several parameters, notably reaction cell geometry, flow rate, and concentration of reagents. Some idea of the sensitivity attainable with the present system under a given set of conditions* with two different photomultiplier tubes** is illustrated by some representative runs shown below:

Photomultiplier Tube	Total Conc. BG/ml***	Total BG/min	S mv	N mv	(S-N) mv	S/N
RCA 1P21	7×10^4	2×10^4	10.5	5	5.5	2.1/1
	3×10^4	1×10^4	9	5	4	1.8/1
	2×10^4	7×10^3	8	6	2	1.3/1
EMI	3×10^4	1×10^4	35	15	20	2.3/1
	1×10^4	4×10^3	25	15	10	1.7/1
	6×10^3	2×10^3	21	15	6	1.4/1

The sensitivities in the table above appear comparable with values of 10^3 to 10^4 B. globigii/min being attainable with both tubes. A plot of S-N against the bacterial concentration in Figure 4-20 indicates approximate linearity between these two parameters.

Process Variables Study - The results of a study of process variables indicate that further improvement in the present sensitivity of 10^3 to 10^4 BG vegetative cells per minute can be expected when all the favorable factors of reactor design, reagent concentrations, and use of disrupted cells are properly coordinated.

* U-type reactor. Flow rates: luminol (0.25 g/l); 0.36 ml/min; $3\% \text{ H}_2\text{O}_2$ 0.03 ml/min, (1 mg EDTA/ml) in each of the solutions.

** Each set run on a different day.

*** Based on Petroff-Hausser count.

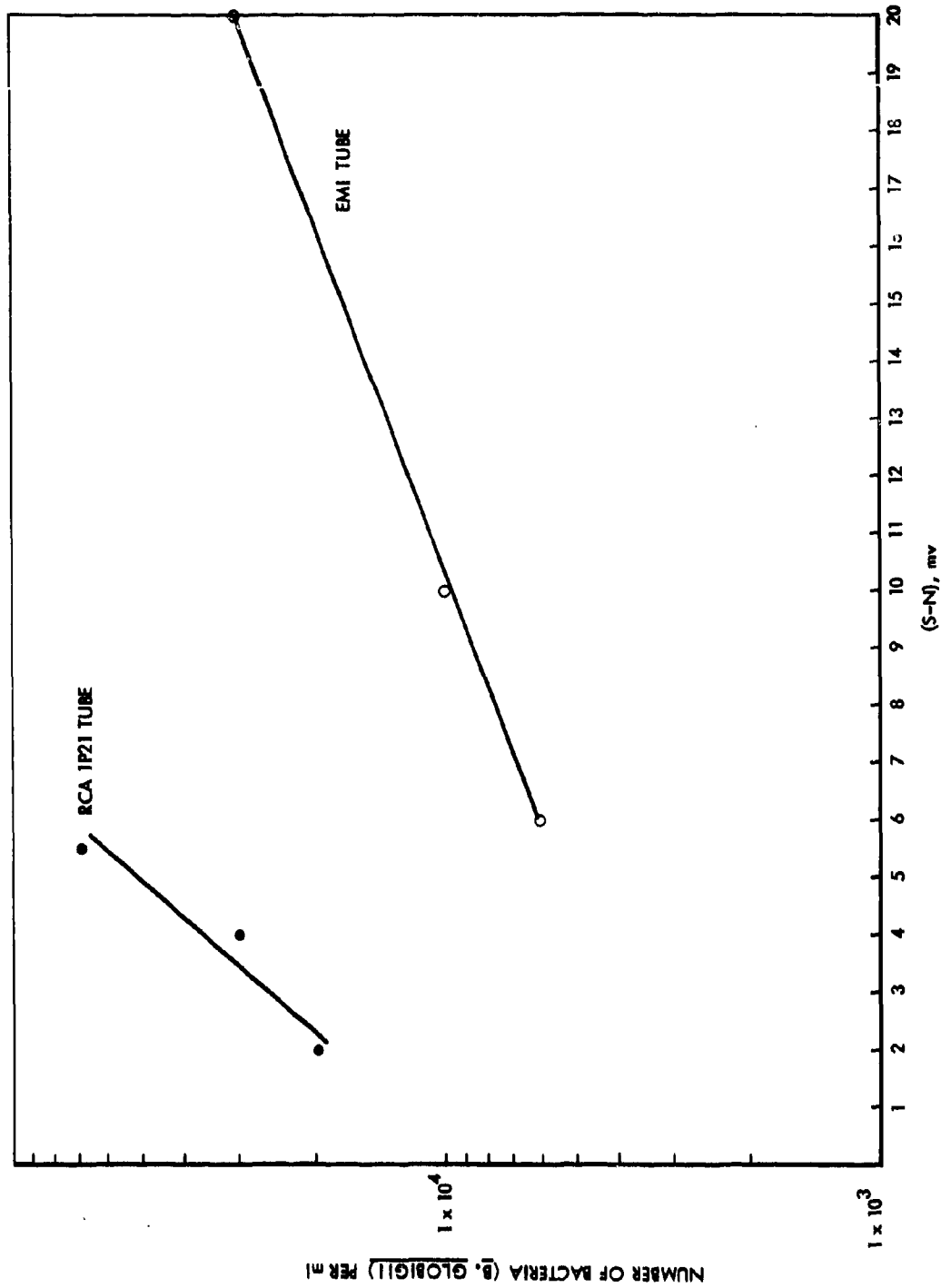


Figure 4-20. Chemiluminescence as a Function of Bacterial Concentration

A brief summary of the results obtained in a study of process variables is given below:*

Effect of Luminol from Different Vendors

Conditions: 1×10^5 BG (viable)/ml, 0.45 ml luminol (.25 g/l)/min,
0.026 ml 3% H_2O_2 /min, U-type reactor, RCA tube

<u>Vendor</u>	<u>S/N</u>
Eastman	4/1
Aldrich**	6/1
Variton**	2/1

In a comparison of luminol from several vendors, the Aldrich luminol generally seemed to give the highest S/N ratio. Whereas the Eastman and Variton luminol were bright yellow powders and produced yellow solutions in 0.1 N NaOH, the Aldrich product was an off-white and produced a comparatively colorless solution. Infrared traces of the Aldrich and Eastman powders (KBr pellet) (Figures 4-21 and 4-22) were quite similar.

In all subsequent runs, the Aldrich luminol was used.

Geometry of Reactor - The optimum reactor design is one which insures rapid mixing in a concentrated zone with a minimum holdup volume. Of several configurations (Figure 4-23) briefly examined, the two which appear to give the highest S/N ratios are the U-tube and the bulb-type reactor. The latter reactor was ultimately selected based on evidence for better mixing obtained from time-exposure photographs of the chemiluminescence reaction. Chemiluminescence in the U-tube (as well as in the straight tube) was found to extend beyond the photomultiplier window. Moreover, persistent streamlines appeared to be present, indicating poor mixing. In the bulb-type reactor the premixed streams of BG in luminol solution and hydrogen peroxide enter through

* Results were obtained under a variety of conditions (i.e., different photomultipliers, reagent and bacterial concentrations, and different reactors) and, while a comparison of results within a particular set is valid, cross comparisons may not be.

** Aldrich Chemical Company (Wisconsin); Variton Co. (Calif.).

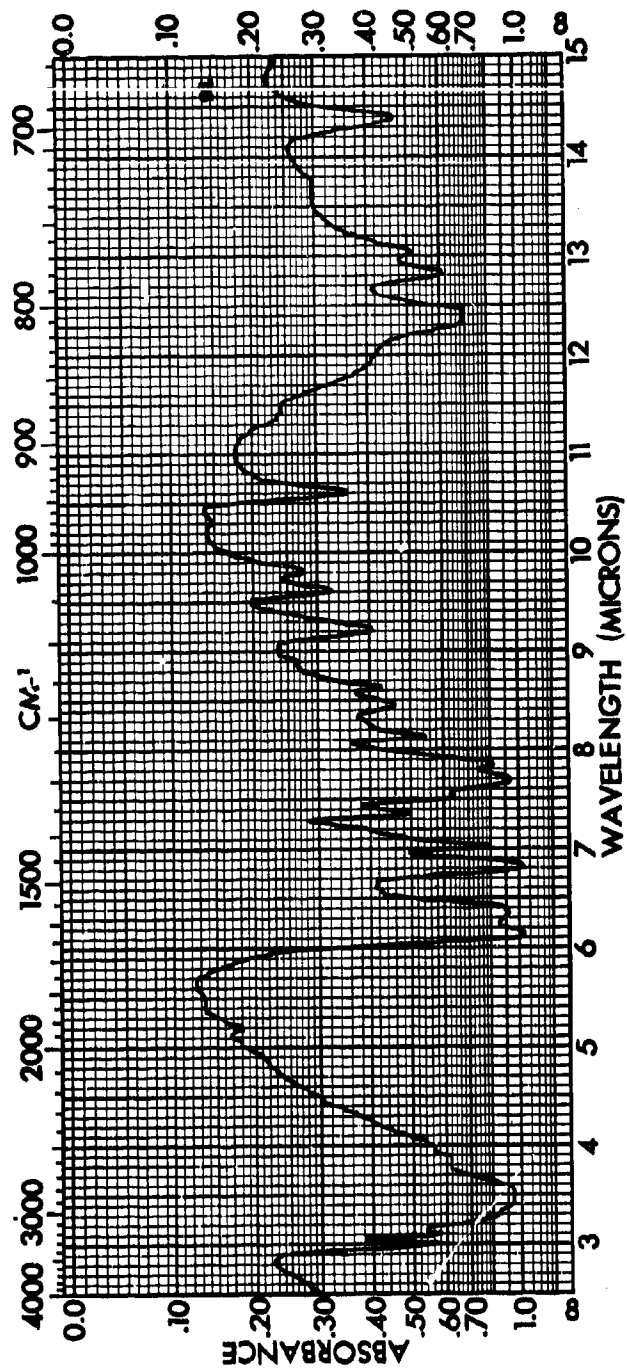


Figure 4-21. Infrared Spectrogram (KBr) of Eastman Luminol

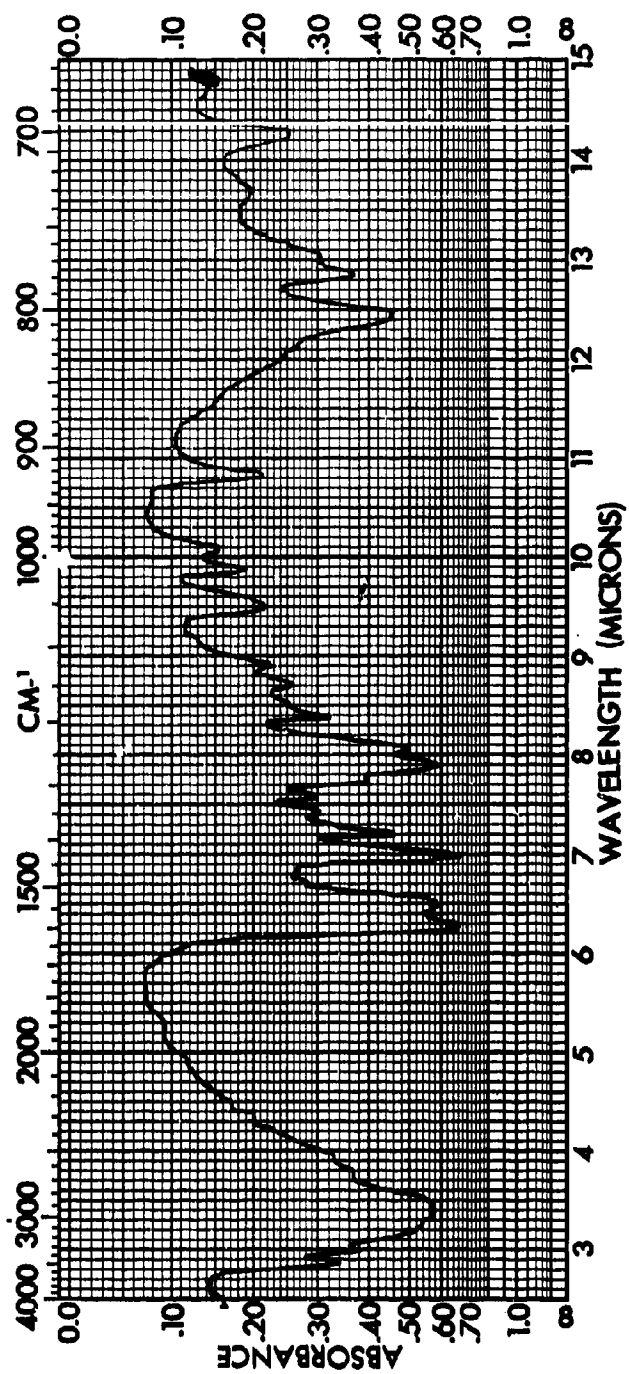


Figure 4-22. Infrared Spectrogram (KBr) of Aldrich Luminol

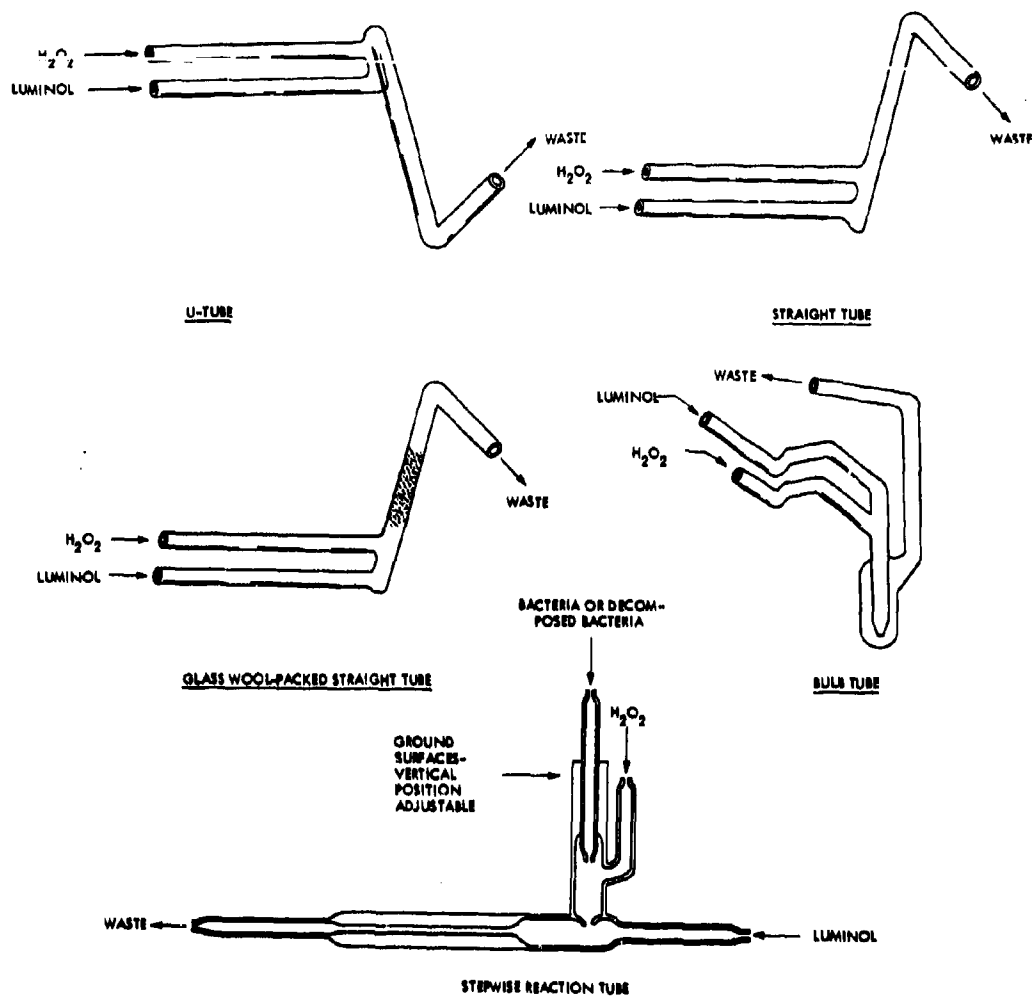


Figure 4-23. Configurations of Chemiluminescence Detector Reactor Tubes (All Fabricated from 4-mm OD Pyrex Tubing)

a downspout terminating in a 0.5-mm orifice, and are deflected off the bottom of the bulb (0.9 mm o.d. by 24 mm high), with high turbulence. The reacted mixture rises through the bulb, and then out to the waste receiver; the entire passage of the mixed sample through the downspout and bulb is viewed by the photomultiplier.

Time exposure photographs of the continuous reaction confirmed the superior mixing in the large bulb reactor. Luminescence was confined to a small region of turbulence in the lower part of the bulb.

The optimum luminol/ H_2O_2 ratio appears to be a function of cell geometry and the highest S/N ratio for the bulb reactor, at a total flow of 6 ml/min, is in the luminol concentration range of 0.0625 to 0.125 g/liter, a H_2O_2 concentration of 1.5 percent, and a luminol/ H_2O_2 flow rate ratio of 5:1 to 20:1. Further studies are required to more clearly establish the optimum conditions.

The relation between total flow rate and signal intensity is illustrated by the data below for the bulb type reactor.

Flow Rate (r) ml/min	S mv	N mv	S-N mv	(S-N)/r	S/N
0.4	134	69	65	162	1.9/1
2.0	360	57	303	152	6.3/1
4.0	530	94	436	109	5.6/1
6.0*	549	129	420	70	4.3/1

Standard Reaction Conditions: (1) $9-10 \times 10^4$ B G/ml in 0.125 g luminol/l in 0.1N NaOH with 1 g EDTA/l, and (2) 1.5 percent H_2O_2 containing 1 g EDTA/l. Volume ratio (1)/(2) = 10/1, EMI at 1100 volts.

It is evident that although S-N continues to increase with increasing flow rates, the maximum value for S/N as well as the maximum rate of increase of S-N is reached at 2 ml/min. The approximate constancy of S-N/r up to that point also indicates that the optimum flow rate for this particular

* Average of 3 runs.

reactor is 2 ml/min. An increase in S-N with increasing flow rate would be expected to occur so long as two conditions are fulfilled; namely (1) mixing is rapid and complete, and (2) the residence time of reactants is greater than the decay time for the luminescent reaction. Apparently both of these conditions are fulfilled up to a flow rate of 2 ml/min for this bulb. To operate a bulb of this design effectively at 6 ml/min (the output flow of the 1000 lpm PEEP collector), it would be necessary to lengthen the residence time within the reactor (i.e., by increasing the reactor volume). This is more clearly illustrated by a comparison of the following sets of data for two reactors of the same design but differing in volume.

Flow Rate ml/min	Small Bulb				Large Bulb			
	S	N	S-N	S/N	S	N	S-N	S/N
0.4	119	74	45	1.6/1	134	69	65	1.9/1
6.0	114	69	45	1.6/1	549	129	420	4.3/1

At the low flow rate of 0.4 ml/min, the luminescence efficiency S/N appears to be comparable; however, at 6 ml/min no further increase occurs for the small bulb, whereas the S/N does increase with the larger bulb. With the small bulb, although mixing is rapid and complete, at the higher flow rate, the decay time for the luminescence is greater than the residence time.

Effect of Reaction Temperature on Luminescence - The effect of reaction temperature on luminescence is summarized by three sets of data obtained under a variety of conditions; the results are, however, consistent.

t°C	Static Run*		t°C	U-Tube** 0.4 ml/min		t°C	Large Bulb Reactor** (5.4 ml/min)	
	S-N	S/N		S-N	S/N		S-N	S/N
9	33	4.7/1	0	25mv	5.2/1	0	125mv	3.8/1
24	49	4.3/1	22	39	6.6/1	22	450	6.6/1
39	89	4.9/1	40	44	6.5/1	40	725	6.8/1

* 2×10^5 viable BG

** 1×10^5 BG/ml (Petroff-Hausser)

In all three cases, although S-N increases with increasing temperature, S/N shows no appreciable increase above ambient.

Estimated activation energies for each set of data over this temperature range are as follows:

	<u>S-N</u>	<u>N</u>
Static	6 kcal/mole	9 kcal/mole
Bulb	8	7
U-Tube	2	1

The reaction in the U-tube is evidently diffusion-limited and undoubtedly incomplete within the viewing zone, as evidenced by the lower values. The values obtained for the static system and the bulb-type reactor are comparable and indicate that not only is mixing equally as effective in both, but all of the reaction is occurring within the viewing zone for both systems. The essentially similar values for the S-N and N plots suggest that the same reaction mechanism is operative for both. Additional tests under more controlled temperature conditions are required to verify this point.

Detection of Egg Carrier in a Flow System - Continuous detection of egg carrier was also demonstrated using the chemiluminescent breadboard.

<u>Run</u>	<u>Sample</u>	<u>Concentration</u>	<u>Luminol Flow Rate, ml/min</u>	<u>Luminescence in Millivolts</u>		
				<u>S</u>	<u>N</u>	<u>S/N</u>
7-12	14-day embryonated egg	2×10^{-8} g* whole egg/ml	3.6	135	40	3.4/1
		$= 5 \times 10^{-9}$ g egg solids/ml	0.36	58	30	1.9/1

* Sample aliquot added to luminol syringe in testing in the flow system; bulb type reactor used.

In obtaining this data, fourteen-day old embryonated chick egg was mixed in a sterile Waring blender with sterile water and an aliquot removed for testing with luminol- H_2O_2 in the conventional manner. The 5×10^{-9} g egg solids/ml (or 2×10^{-9} g/min) would correspond to approximately 3×10^3 μ particles or thirty 5μ particles of egg solids.

Background Evaluation - An evaluation of the effect of atmospheric background on the chemiluminescent reaction was initiated using the integrated system shown schematically in Figure 4-24. Bacteria, nebulized against either outside El Monte air or air drawn through an absolute filter, are collected downstream by the 1000 l/min PEEP. The PEEP fluid effluent is metered at 5 ml/min into a mixing chamber where the proper dilution occurs with an incoming stream of alkali luminol (0.36 ml/min)*. The solution is then fed into a bulb-type reactor where reaction with H_2O_2 (1 ml/min) occurs (total fl w 6.36 ml/min). Preliminary results obtained with two different PEEP fluids are summarized below. The bacterial concentration at detector (column 2 below) was estimated based on an experimentally determined average value** of 51 percent for the nebulization train efficiency (from point of nebulization to PEEP entry) and 55 percent collector efficiency (from entrance to PEEP to liquid effluent from PEEP) giving an overall efficiency of 28 percent. These efficiencies were determined using FITC-stained B. globigii spores, at a 1000 l/min air sampling rate. A method for obtaining bacterial counts directly during the course of an actual detector run would be more desirable. Results obtained are tabulated below.

* Alkali luminol solution contains 0.93 gram luminol and 15 grams EDTA in 1 liter of 1.5N NaOH (feed rate 0.36 ml/min) H_2O_2 is 1.5 percent and contains 1 gm EDTA/liter (flow is at 1 ml/min).

** Average of five separate determinations for nebulization train efficiency and average of eight separate determinations for collector efficiency.

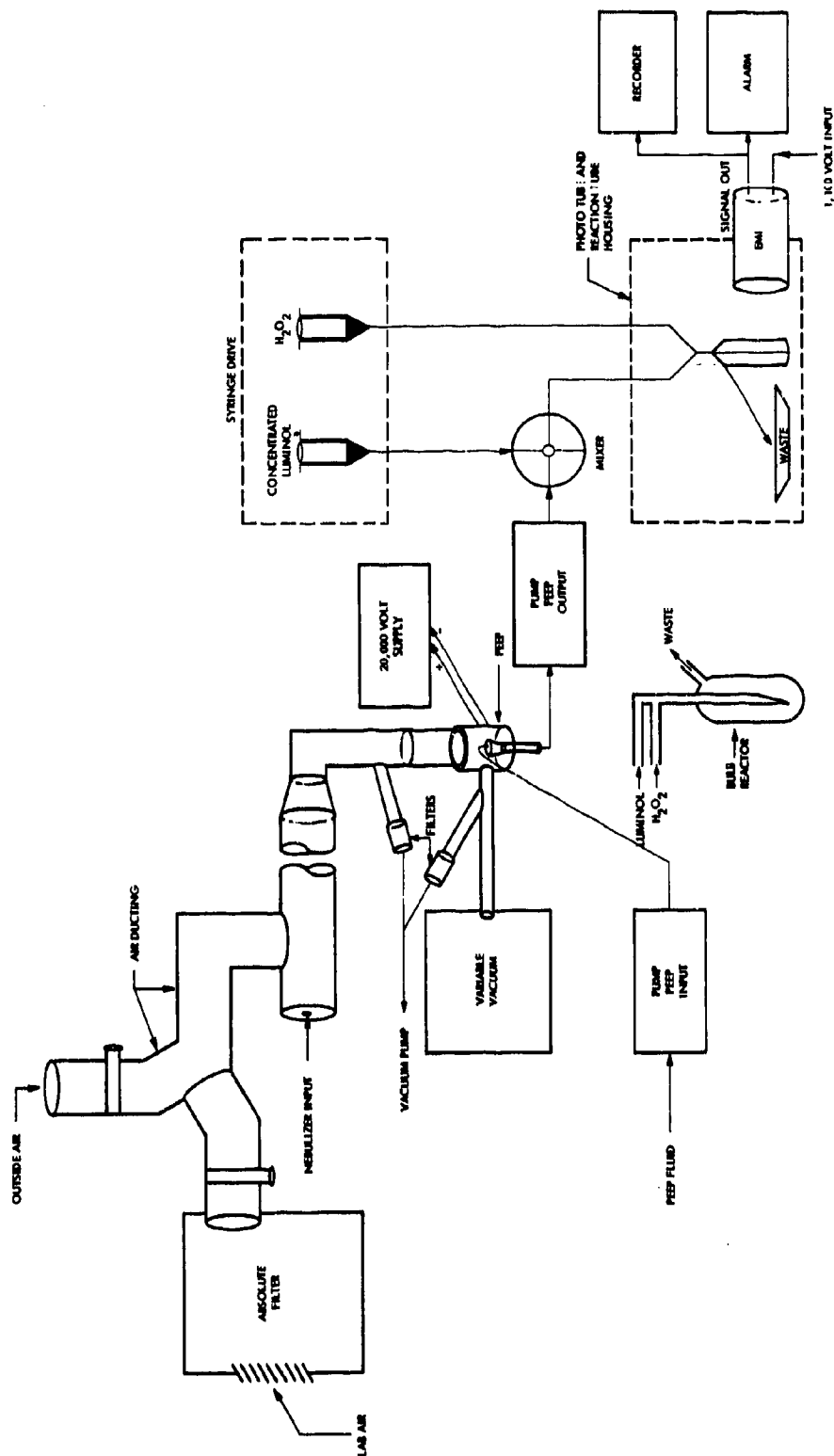


Figure 4-24. Chemiluminescent Detector Flow System

Run No.	Collecting Fluid	Bacterial Concentration at Detector	<u>Signal</u>		
			S	N	S-N
43	0.4% Saline & Photo-Flo(0.5 ml/1)	5×10^3 /ml 3×10^4 /min	230-200=30 mv (Filtered Air)		S/N = 1.1/1
44	0.4% Saline & Photo-Flo(0.5 ml/1)	5×10^4 /ml 3×10^5 /min	360-230=130 mv (Filtered Air)		S/N = 1.6/1
	0.4% Saline & Photo-Flo(0.5 ml/1)	5×10^4 /ml 3×10^5 /min	400-270=130 mv (Unfiltered Air)		S/N = 1.5/1
45	0.4% Saline & Photo-Flo(0.5 ml/1)	5×10^5 /ml 3×10^6 /min	630-230=400 mv (Filtered Air)		S/N = 2.8/1
46	Distilled Water & Photo-Flo(0.5 ml/1)	7×10^3 /ml 4×10^4 /min	690-490=200 (Unfiltered Air)		S/N = 1.4/1
49	Distilled Water & Photo-Flo(0.5 ml/1)	8×10^3 /ml 5×10^4 /min	740-420=340 (Unfiltered Air)		S/N = 1.8/1
47	Distilled Water & Photo-Flo(0.5 ml/1)	6×10^4 /ml 4×10^5 /min	1200-500=700 (Unfiltered Air)		S/N = 2.4/1

The data indicate that a bacterial concentration of approximately 10^4 to 10^5 /ml (or per minute) is required for a sizable signal. A plot of S-N vs bacterial concentration (nebulized against filtered air) shown in Figure 4-25 is seen to be approximately linear. The actual contribution of background to the signal is shown in Run No. 44. In this run, started on filtered air, a switch to unfiltered air was made half-way through the nebulization. The 40 mv increase (in both signal and blank) observed is the contribution of background to the luminescence and is seen to represent less than 20 percent of the blank signal (luminol + H_2O_2). The reason for the consistently higher blank (and sample) signals observed with distilled H_2O + Photo-Flo compared to saline + Photo-Flo is not known at the present time.

In a separate experiment in which a B. globigii suspension in PEEP fluid (0.4 saline + Photo-Flo) was metered to the detector under identical conditions except that it by-passed the PEEP, the sensitivity was shown to be higher by approximately one log. This would indicate that passage of the

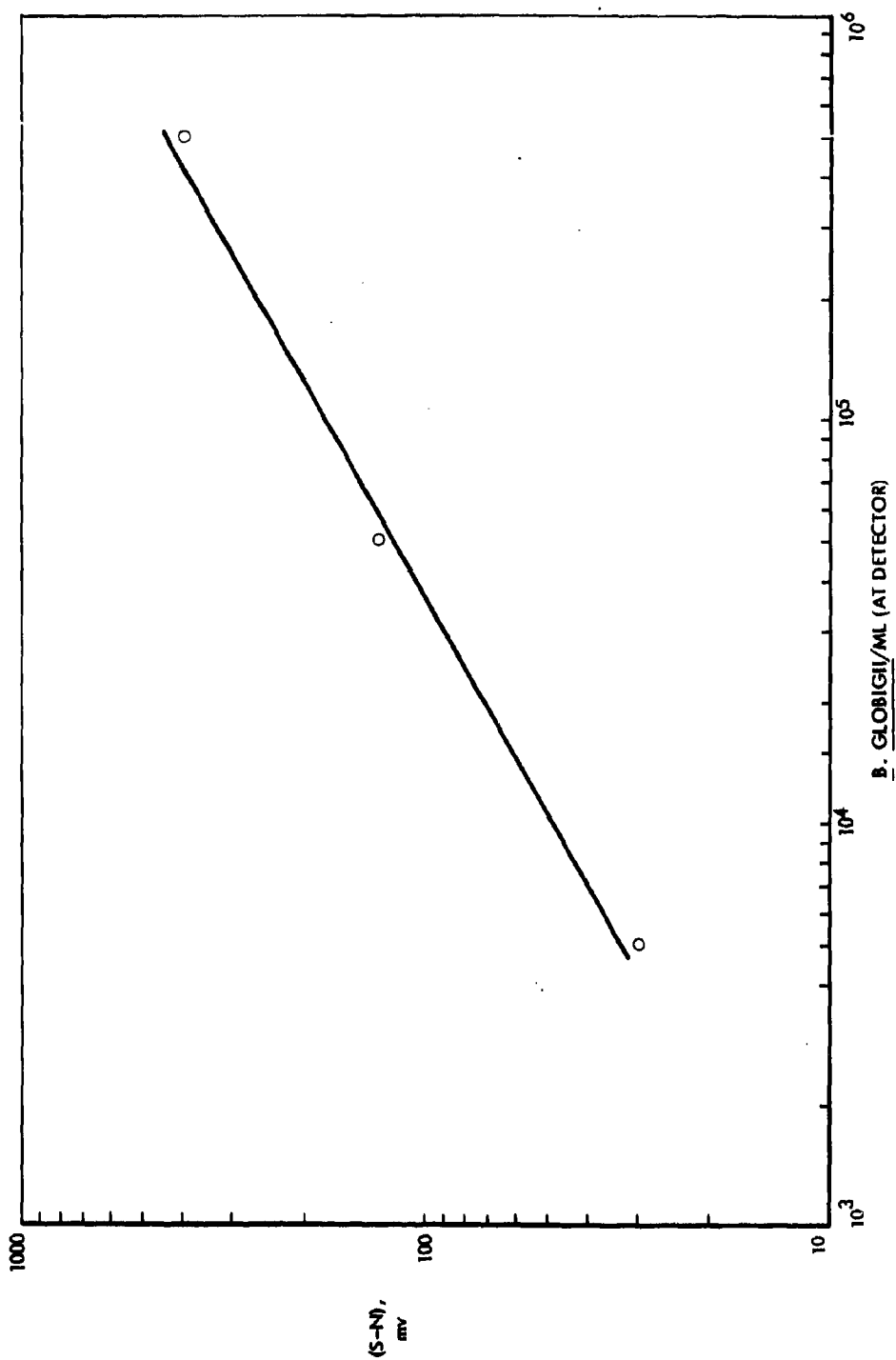


Figure 4-25. Chemiluminescence versus Bacterial Concentration (Against Filtered Air)

bacterial suspension through the PEEP tends to deactivate the bacteria to some extent. It is quite plausible that under the high voltage conditions in the PEEP, ozone, oxides of nitrogen, and even H_2O_2 are being formed. These strong oxidizing agents would be expected to have a deleterious effect on the bacteria. Inclusion of minute amounts of a reducing agent in the PEEP fluid may offset this effect and improve sensitivity. Additional tests are obviously needed to define the sensitivity of this system against a number of different agents.

Improved System Design - While the current device is a detector only, an improved breadboard was considered which will incorporate all elements of a complete system from air sampling, to disruption of bacteria, to readout in a continuous system. The improved chemiluminescence detection system is visualized as portable and will stress compactness, low weight, and simplicity of design. The portable feature will facilitate testing under a variety of field conditions and atmospheres.

The improved chemiluminescence detection system (Figure 4-26) would include the following elements:

- a. Foam Filter - The air samples will be drawn through a commercially available foam filter, sized to exclude particles above approximately 5μ size. This can be varied to large diameters if proven desirable.
- b. Collector - The current 1000 l/min PEEP will be used in conjunction with a dilute solution of an electrolyte and Photo-Flo surface active agent as the collecting medium. The latter has no adverse effect on detector sensitivity.
- c. Continuous Filter and Washer - This unit, described in another section of this report, removes interfering solutes by washing. It also serves to concentrate the sample.
- d. Crusher - The catalytic activity of bacteria is increased sizably by disrupting them prior to reaction. The method selected over several possible alternatives is continuous crushing in a mill by the oscillating motion of small quartz spheres having about a 50 micron diameter. This technique is recommended on the basis of research by a Space-General consultant⁽¹⁰⁾. The vibration amplitude is adjustable; a typical displacement value is estimated at 7 mm/stroke.

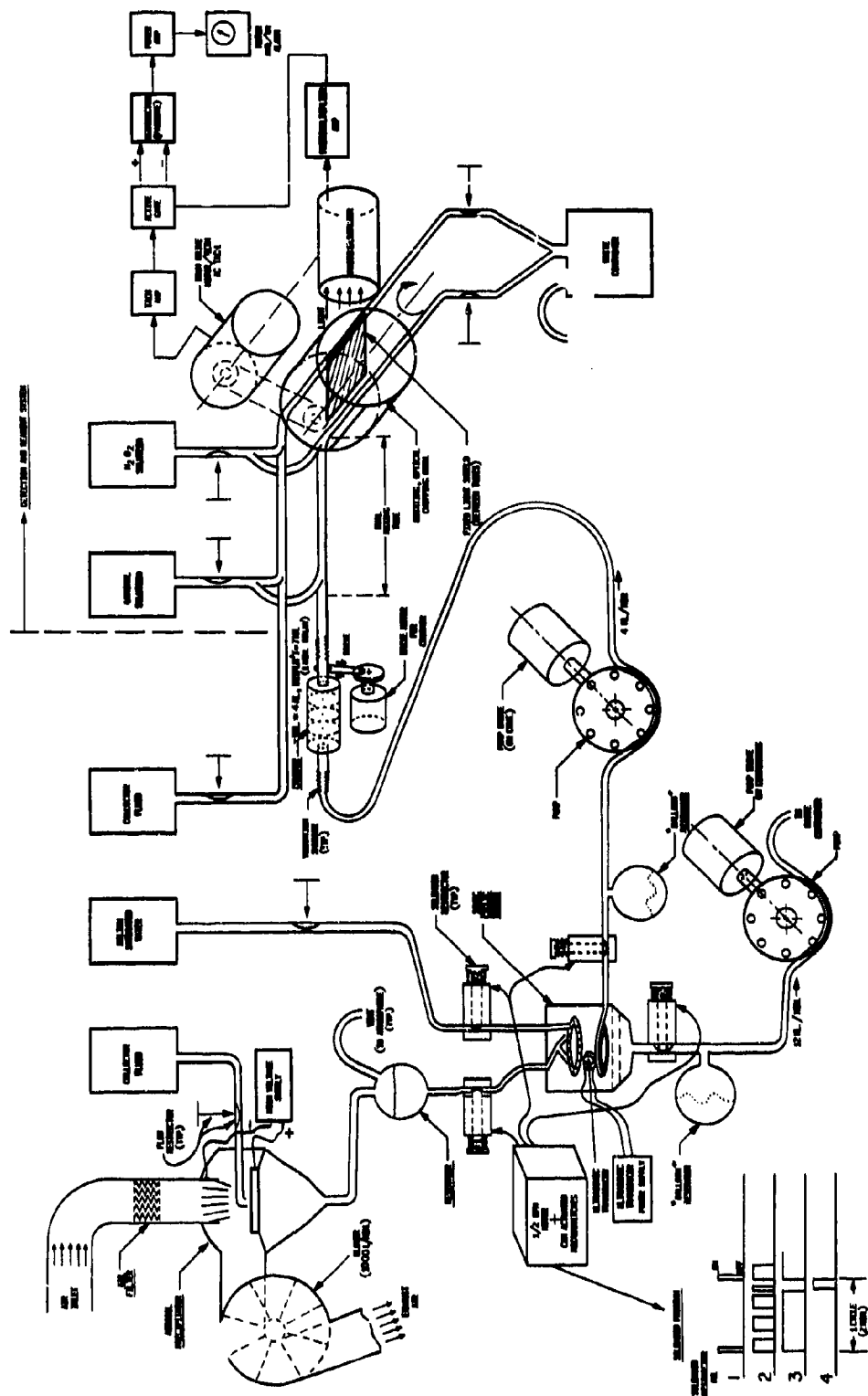


Figure 4-26. Chemiluminescence Detection System

- e. Reactions - Parallel reaction tubes are specified, one for the sample stream, and one for a control stream of unused collector fluid. The reagent solutions of (1) basic luminol, and (2) H_2O_2 are introduced into each stream. However, at an early stage in the experimental program, aerosol-type containers which would reduce the number of pumps required as well as provide an economical and an ultraclean method of packaging should be evaluated as a means of dispensing the reagents.
- f. System Readout - Continuous comparison of light emission from the two reaction tubes corrects for the reference reaction of luminol and H_2O_2 . Two reaction tubes are positioned inside on optically-chopping rotating drum, turning relatively slowly at approximately 10 to 15 rps. An end-window photomultiplier views both reaction tubes on a shared-time basis. Optical chopping of the light by the rotating drum allows the use of AC circuit techniques, eliminating the DC drift currently present in the existent detector. Secondly, optical chopping with a rotating drum makes possible the time sharing of one photomultiplier to monitor the light level from two separate reaction tubes. Without time sharing, two photomultipliers per system would have been required. Following its generation, the photomultiplier signal is amplified and separated into two signals, one for the controlled channel and one for the sampling channel. Next, a linear subtraction is performed and, finally, the difference is monitored with an alarm system.

4.1.4.1.4 CONCLUSIONS

The demonstrated level of detection of the CDS is of the order of 10^3 to 10^4 B. globigii (vegetative) per minute at a concentration level of 10^4 /ml (Petroff-Hausser count). To meet the program objectives of 1 bacterium per liter of air, further improvement in CDS sensitivity would be required if the 1000 l/min PEEP were used as a collector (i.e., 1×10^4 bacteria/5 ml liquid volume = 2×10^3 /ml). The other alternative would be use of a concentrator to increase the concentration of the bacterial suspension from the PEEP effluent about tenfold. The results of the process variables study indicate that further improvement in sensitivity can be achieved by use of disrupted bacteria (French cell) and a reactor cell design which insures more rapid and efficient mixing. Without modification, the detector can also be used for detection of virus carrier with a demonstrated sensitivity of 3×10^{-4} Hela cells (static system) or 10^{-9} g egg solids/ml (corresponding to 3×10^3 1 μ egg particles).

Preliminary studies on the contribution of background to chemiluminescence indicated that the blank luminescence is increased by less than 20 percent on going from filtered to unfiltered air. Nebulized B. globigii can be detected at a level of 10^4 /ml or 10^4 to 10^5 per minute. Additional studies are required to establish more clearly the multi-agent capability of this instrument, particularly in the presence of diverse types of atmosphere.

4.1.4.1.5 REFERENCES

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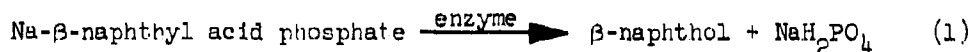
4.1.4.2 ALKALINE PHOSPHATASE

4.1.4.2.1 SUMMARY

The present level of detection of a continuous fluorimetric assay system based on Na β -naphthyl acid phosphate as substrate is 10^4 to 10^5 B. globigii (veg)/ml (10^2 to 10^3 bacteria in a 4 μ l viewing cell) within a 3-minute reaction time. Other organisms which were found to exhibit detectable phosphatase activity at a level of 10^4 to 10^6 bacteria per ml in a 5-minute reaction time include E. coli, S. marcescens, Leucothrix sp., S. cerevisiae, and P. fluorescens. Spores (10^5 to 10^6 /ml) of B. globigii and B. cereus also exhibit phosphatase activity without cell disruption.

4.1.4.2.2 INTRODUCTION

An enzymatic reaction involving alkaline phosphatase in which a fluorescent product is formed has been investigated at Space-General because of the inherently high sensitivity of fluorimetry and the ubiquitous nature of alkaline phosphatase. This enzyme, important in cell metabolism of spores and vegetative cells of many pathogens and non-pathogens, is a nonspecific phosphomonoesterase which hydrolyzes singly esterified phosphoryl groups, with activity optima at alkaline pH. Sodium β -naphthyl acid phosphate, which is converted into the fluorescent β -naphthol on hydrolysis, forms the basis for the detection scheme at Space-General, thus



The method is an adaptation of one reported by Greenberg⁽¹⁾. Other substrates have been examined, but have been discarded either for lack of stability (high autohydrolysis rate), or insufficient sensitivity. The results supplied below indicate that the required detection sensitivity of 10^4 bacteria/ml is attainable with the present system. The use of substrates which produce fluors which are water insoluble, thus permitting detection of phosphatase activity within a single organism, is also discussed. The status of the work in each of these areas is described briefly below.

4.1.4.2.3 STATUS

4.1.4.2.3.1 Na- β -NAPHTHYL ACID PHOSPHATE SYSTEM

Static System - Process variable were first evaluated in a static system^{*}. Unless otherwise indicated, the procedure consisted of adding an aqueous bacterial suspension (B. globigii (veg) or E. coli, 16 hour cultures, in sterile water) to a reagent solution containing sodium β -naphthyl acid phosphate and magnesium acetate in Tris buffer (pH 10.4, 0.1M), reacting for 5 minutes and then comparing the fluorescence with a control containing all the components except the bacteria. An evaluation of process variables (Table 4-11) indicated the following:

- a. Temperature: Reaction rate increases with temperature up to 57°C. (Subsequent studies using a flow system with a 3-minute reaction time indicated an increase in reaction rate up to 70°C.) The optimum procedure is to react at elevated temperatures and cool to ambient prior to measuring fluorescence.
- b. pH: Reaction carried out with reagents buffered at pH 8.4, 9.4, and 10.4 with Tris buffer showed a maximum rate at pH 10.4. Greenberg, working with the pure enzyme, reports pH 10.4 as optimum.
- c. Reagent Concentration: The optimum concentration of reagent solution appears to be at 4.1 mg sodium β -naphthyl acid phosphate and 215 mg magnesium acetate per 100 ml of Tris buffer (0.1M, pH 10.4). Increasing the substrate concentration further only serves to increase the fluorescence of the blank without increasing the reaction rate. The substrate shows some degradation (hydrolysis) on storing the solution 24 hours at 10°C. As a remedy, hydrolysis product (β -naphthol) can be removed readily by ether extraction or the solution can be prepared fresh daily.
- d. Suspension Medium for Bacteria: No significant difference in reaction rate was observed using bacterial suspensions of E. coli in sterile distilled water, 0.85 percent NaCl or

* In the static system activation of the β -naphthol was at 350 m μ (Corning 7-60) and fluorescence at 410 m μ (Filtraflex 410). Measurements were made using a Photovolt Fluorometer (Model 540) and a Photovolt Varicord Amplifier Recorder Model 43). Viewing cells were either of 1 ml or 25 μ l volume.

Table 4-11

EFFECT OF PROCESS VARIABLES ON ALKALINE
PHOSPHATASE ACTIVITY (STATIC SYSTEM)

Variable	Number of Bacteria	Reaction Conditions	Fluorescence Ratio Sample/Control	
			Uncooled*	Cooled*
Temperature	1 x 10 ⁸ <u>E. coli</u> (1 ml cell)	10 min reaction at 18°C	1.23/1	-
		28°C	1.51/1	-
		38°C	1.97/1	-
		48°C	2.69/1	3.00/1
		57°C	3.10/1	3.59/1
		5 min reaction at 48°C	-	1.73/1
		57°C		1.88/1
pH	1 x 10 ⁸ <u>E. coli</u> (1 ml cell)	5 min reaction at 48°C at pH 8.4	-	1.45/1
		pH 9.4	-	1.26/1
		pH 10.4 (Tris buffer)		1.96/1
Magnesium acetate concentration	1 x 10 ⁸ <u>E. coli</u> (1 ml cell)	5 min 51°C normal (Mg ⁺⁺)	-	2.10/1
		2 x normal	-	1.99/1
		1/2 normal	-	1.81/1
Bacterial suspension media	1 x 10 ⁸ <u>E. coli</u> 1 x 10 ⁸ <u>E. coli</u> 9 x 10 ⁷ <u>E. coli</u> (1 ml cell)	5 min at 51°C in dist. H ₂ O	-	1.49/1
		in 0.85% NaCl	-	1.39/1
		0.1M phosphate buffer (pH 7.25)	-	1.43/1

* Uncooled means fluorescence measurements made at reaction temperature, cooled means samples precooled 15 sec. at 0°C prior to making measurement. All measurements made on cooled samples (and pH 10.4) unless otherwise indicated.

Table 4-11 (Continued)

EFFECT OF PROCESS VARIABLES ON ALKALINE
PHOSPHATASE ACTIVITY (STATIC SYSTEM)

Variable	Number of Bacteria	Reaction Conditions	Fluorescence Ratio Sample/Control	
			Uncooled*	Cooled*
Sensitivity	1×10^8 <u>E. coli</u>	5 min at 52°C	-	1.43/1
	6×10^7			1.25/1
	3×10^7			1.14/1
	1×10^7 (1 ml cell)			1.08/1
	4×10^6 <u>E. coli</u> (25 μ l cell)	5 min at 50°C		1.09/1
	1×10^6 <u>B. globiggi</u> 6×10^5 (veg.) (1 ml cell)	5 min at 55°C	-	1.86/1 1.71/1
	6×10^4 <u>B. globiggi</u> 3×10^4 (veg.) (25 μ l cell)	5 min at 55°C	-	2.06/1 1.47/1
	2×10^7 BG (spores) 9×10^6 (1 ml cell)	5 min at 51°C	-	2.15/1 1.51/1

0.1M phosphate buffer (pH 7.25). Consequently in all the runs, B. globigii or E. coli (16 hour cultures, TGE media) suspensions in sterile distilled water were used.

- e. Sensitivity: Best results obtained with B. globigii with a static system in a 25 μ l cell and a reaction time of 5 minutes are indicated below:

	Fluorescence Ratio (Sample/Blank)
6×10^4 BG (3% spores)	2.1/1
3×10^4 BG (3% spores)	1.5/1

- f. Spores and Other Microorganisms: Phosphatase activity could also be detected in spores of B. globigii (9×10^6 /ml) and B. cereus (6×10^4 /ml. see Table 4-12) without sonicating rupture. Extension of this study to other available microorganisms (Table 4-12) indicated that the following also had detectable phosphatase activity at a level of 10^4 to 10^6 bacteria in a 5-minute reaction time: E. coli, S. marcescens, B. cereus, Leucothrix sp., and S. cerevisiae. A negative response was obtained with 1×10^6 Chromobacterium violaceum.

- g. Photographic Record: In an attempt to achieve further increases in sensitivity, photographic film (Polaroid 3000) was used in place of the photomultiplier tube to record the intensity of the fluorescence produced in the enzymatic reaction. The Gelman EEL scanner was used to measure the photographic density of the film. A comparison of the results obtained with the photomultiplier tube and the photographic film is shown below:

<u>Bacteria</u>	<u>Photomultiplier*</u> Fluorescence Ratio (Sample/Blank)	<u>Photographic Film*</u> Density Ratio (Sample/Blank)
1.3×10^6 <u>B. globigii</u>	2.02	1.63
6×10^5 <u>B. globigii</u>	1.51	1.30

The results show no improvement in sensitivity for the photographic method (which records the integrated light output over a 5-minute period) compared to that obtained with a photomultiplier tube.

*Reaction conditions: 1 ml reaction volume; 5 min at 60°C, 15 sec at 0°C, 5 min at ambient; shutter was open and sample exposed to film during final 5 min.

Table 4-12

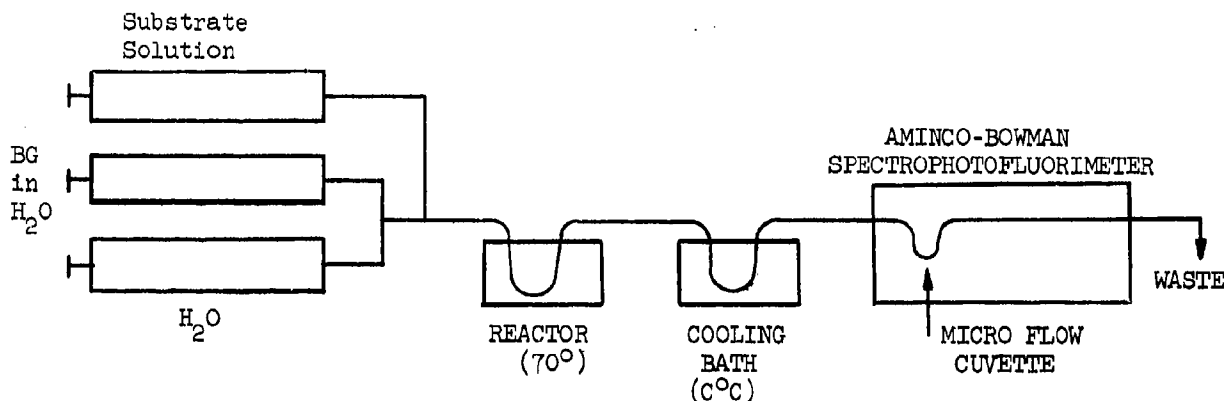
ALKALINE PHOSPHATASE ACTIVITY OF
SEVERAL MICROORGANISMS

<u>Organism</u>	<u>Number of Bacteria</u>	<u>Fluorescence Ratio*</u> <u>Sample/Control</u>	
		<u>1 ml. Volume</u>	<u>0.025 ml. Volume</u>
<u>Pseudomonas fluorescens</u>	2×10^7	4.14/1	-
	5×10^5	2.21/1	-
	9×10^3	insign. (1/1)	
<u>Serratia marcescens</u>	5×10^7	1.70/1	-
	1×10^6	-	1.15/1
<u>Leucothrix sp.</u>	3×10^6	1.38/1	-
	4×10^4	1.11/1	-
<u>Saccharomyces cerevisiae</u>	9×10^5	1.33/1	-
	2×10^4	insign.	-
<u>Bacillus cereus</u> (Spores)	3×10^6	1.28/1	-
	6×10^4	-	1.13/1
<u>Chromobacterium violaceum</u>	4×10^7	insign.	-
	1×10^6	-	insign.

* 5 minute reaction time at 50°C followed by 15 second immersion at 0°C prior to taking reading; primary filter UG1, secondary 410 Filtraflex.

- h. Background Interference: Background was simulated by an aqueous suspension of Arizona road dust of the same optical density as BG suspensions normally used. Addition to phosphate reagent showed no significant increase in fluorescence after 5 minutes over that of a control (containing reagent only).

Flow System - The results shown below for B. globigii (veg) were obtained with an Aminco-Bowman Spectrophotofluorimeter modified for use with a micro flow cuvette of 4 μ l viewing volume. A schematic of the equipment is shown below.



A suspension of B. globigii in sterile water is reacted with substrate solution for 3 minutes at 70°C and cooled to ambient before passing into the micro flow cuvette where the fluorescence of the β -naphthol is measured (excitation is at 350 m μ and fluorescence at 410 m μ). Data obtained are shown below:

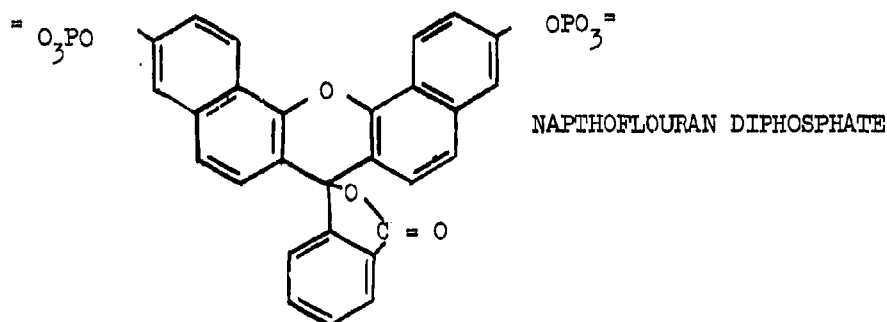
<u>B. globigii/ml</u> <u>(in reactor)</u>	<u>Total B. globigii</u> <u>in 4 μl cuvette</u>	<u>Scale Reading</u> <u>(Arbitrary Units)</u>		<u>Fluorescence</u> <u>Ratio</u> <u>Sample/Blank</u>
		<u>Sample</u>	<u>Blank</u>	
5 x 10 ⁵	2 x 10 ³	70	45	1.56/1
8 x 10 ⁴	3 x 10 ²	61	55	1.11/1

Although total flow rates of 0.9 ml/min were used above, flow rates can be as low as desired, being dictated only by the 3 minutes required for reaction,

and the holdup volume of the system. Based on the present sensitivity of 10^4 to 10^5 bacteria per ml, and a practical flow rate of 0.1 ml/min, sensitivities of 10^3 to 10^4 /min are presently attainable. However, it is evident that a concentration step would be required to bring the organisms to the 10^5 /ml concentration level. The results shown in the table above were obtained with whole cells of *B. globigii*; no improvement in activity was noted with disrupted cells, suggesting that diffusion of substrate through the cell wall was not rate-determining. Further work confirmed that the substrate and the β -naphthol produced on hydrolysis can diffuse readily through the cell wall.

4.1.4.2.3.2 ALTERNATIVE SUBSTRATES

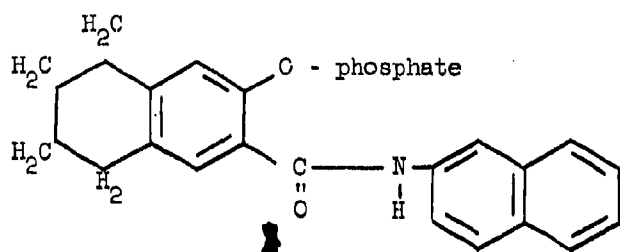
Other substrates which possess higher quantum yields of fluorescence, such as 3-o-methyl fluorescein phosphate and naphthofluoran diphosphate, have also been under investigation at Space-General. The former substrate was found to have an objectionably high spontaneous rate of hydrolysis (in absence of bacteria), even at pH 8, with the result that blank readings were high and irreproducible. Naphthofluoran disphosphate, the naphthalene analogue of fluorescein shown below, was originally developed by Lederberg⁽²⁾ and is extremely stable (spontaneous hydrolysis rate less than 0.00035 percent/hr.).



Although the diphosphate has as yet not been synthesized in our laboratories, the hydrolysis product, 6, 6'-dihydroxy-naphthofluoran, has been, and its detection sensitivity established at approximately 1×10^{-9} Molar with an Aminco-Bowman Spectrophotometer (excitation at 595 m μ , emission at 650 m μ). Based on a reported hydrolysis rate at ambient temperature of 2×10^{-3} μ M of

dihydroxynaphthofluoran per liter per hour at a B. globigii concentration of 6×10^4 /ml, it would take approximately 1/2 hour for detection at this bacterial concentration⁽²⁾. Conceivably, use of higher reaction temperatures might reduce the detection time to 5 minutes at the 10^4 /ml level. Another characteristic noted for the dihydroxynaphthofluoran was its very limited solubility in the aqueous buffered reaction medium, suggesting its use for assay of phosphatase activity within a single cell. Further exploration of this system is desirable.

The ultimate in sensitivity might be achieved by use of substrates which produce water-insoluble fluors on hydrolysis, thus permitting detection of phosphatase activity within a single organism. An example of such a substrate, shown below, was recently developed by Burstone⁽³⁾.



5, 6, 7, 8, β -tetralol
carboxylic acid - β -
naphthylamide phosphate

The naphthol released on hydrolysis by alkaline phosphatase has less than 1 percent of the solubility of β -naphthol. Whereas the enzymatically released naphthol is a fluorochrome, the phosphate ester exhibits negligible fluorescence. This substrate has been synthesized and a major problem encountered in a cursory examination of this material is its very limited solubility in aqueous systems. Although soluble in an aqueous-dimethylformamide mixture, the optimum ratio has yet to be determined.

4.1.4.2.4 CONCLUSIONS

It has been demonstrated that a continuous fluorimetric assay procedure utilizing sodium β -naphthyl acid phosphate as the substrate is capable of detecting 10^4 to 10^5 B. globigii/ml (or 10^2 to 10^3 organisms in a 4 μ l flow cuvette) within a 3-minute reaction time. Spores of B. globigii and B. cereus also produced a response without prior disruption being required. The

multi agent capability of this system has also been demonstrated in several other available microorganisms.

It may be concluded that the inherent simplicity of the Na- β -naphthyl acid phosphate system and the ability to detect spores by this method make it a very attractive candidate for breadboarding, provided the sensitivity can be improved by one order of magnitude (to 10^3 to 10^4 /ml). Further evaluation of naphthofluoran disphosphate and the disodium salt of the phosphate ester of tetralol carboxylic acid β -naphthylamide as substrates for monitoring the alkaline phosphatase activity in single cells is also warranted.

4.1.4.2.5 REFERENCES

- (1) Greenberg, L.J., Biochem. and Biophys. Res. Comm. 9: 430, 1962.
- (2) Lederberg, J. et al, Sanford University School of Medicine, Technical Report IRL-1010, July 1, 1964, NASA Grant NSG 81-10.
- (3) Burstone, M.S., in Histochemistry and Cytochemistry, edited by R. Weymann, MacMillan Co., N.Y. (1963) p. 351.

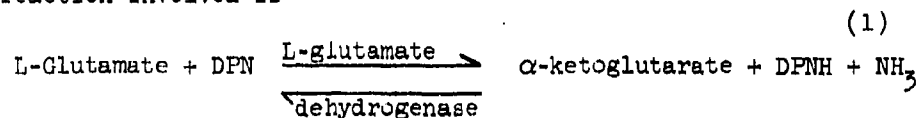
4.1.4.3 L-GLUTAMATE DEHYDROGENASE

4.1.4.3.1 SUMMARY

The minimum number of bacteria detectable by the L-glutamate dehydrogenase reaction is about 10^6 BG in a 2-ml reaction volume. Increase of sensitivity by use of microvolumes is precluded by the low intensity of the DPNH fluorescence produced, and further work does not appear warranted.

4.1.4.3.2 INTRODUCTION

An important enzyme by which many bacteria (pathogen and non-pathogen alike) utilize glutamate in their metabolism is L-glutamate dehydrogenase. The reaction involved is



and the formation of the fluorescent DPNH was utilized as a basis for a detection scheme.

4.1.4.3.3 STATUS

The minimum numbers of bacteria detectable by their L-glutamate dehydrogenase activity was found to be approximately 10^6 B. globigii/ml in a 5-minute reaction time.

The fluorescence produced on reacting potassium glutamate with DPNH in the presence of bacteria for 5 minutes at ambient temperature is shown below. Fluorescence measurements were made at a pH 9.7 (glycine-NaOH buffer) and a reaction volume of 2 ml in a Photovolt Fluorimeter. Primary and secondary filters with transmission at 340 and 456 mμ, respectively* were employed. A control, containing all of the components except the bacteria and run concurrently with the sample, served as a blank.

*Primary is Corning 7-60, secondary is Filtraflex K peaking at 460 mμ.

<u>No. of Bacteria</u>	<u>Fluorescence (Arbitrary Units)</u>		
	<u>Sample</u>	<u>Control</u>	<u>Difference</u>
2×10^6 <u>B. globigii</u> (Containing 0.02% Spores)	92	74	18
	94	74	20

A study of several important variables of this reaction is summarized briefly below:

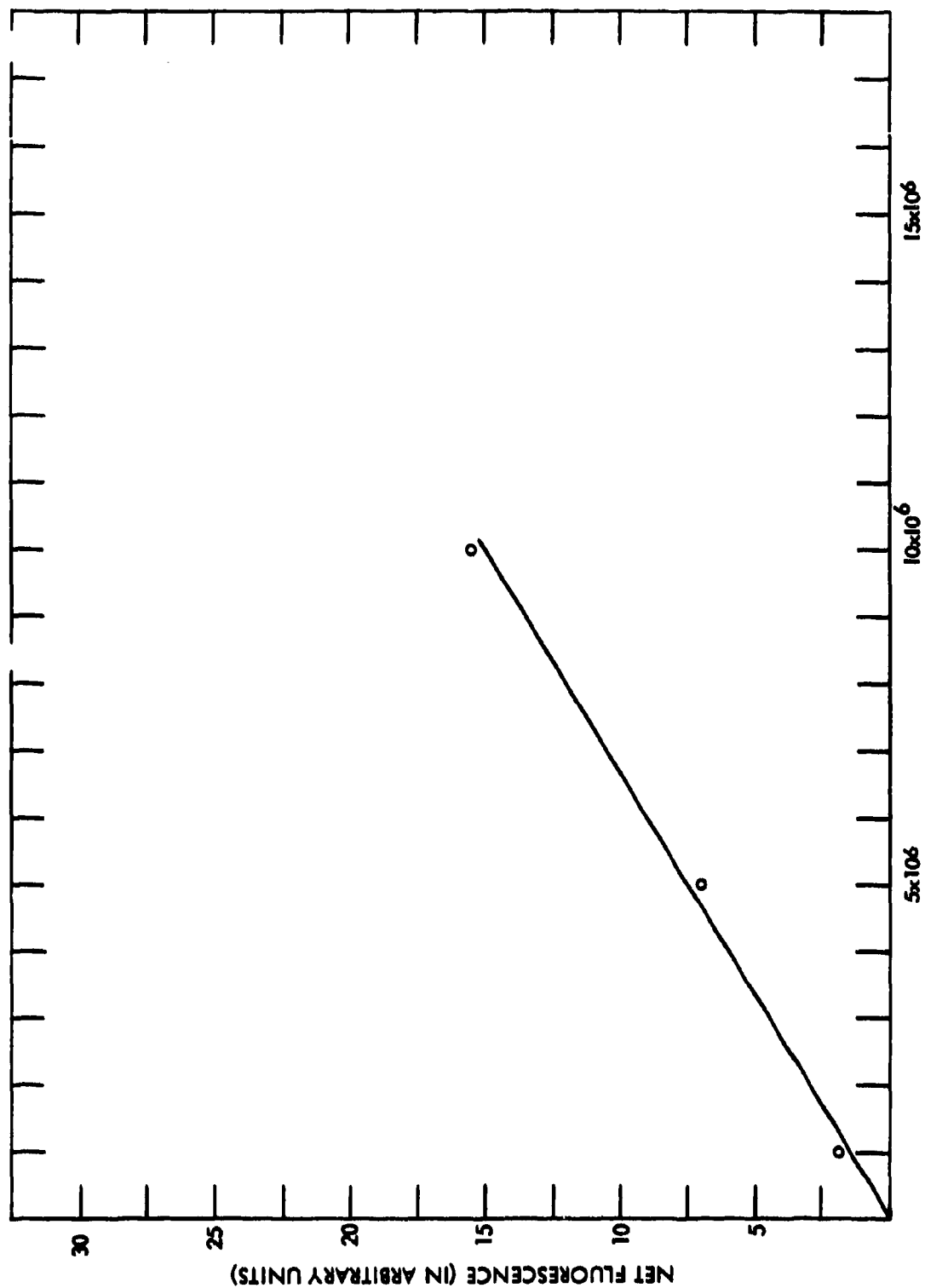
BG Spores - BG spores were found to exhibit less than 10 percent the activity of BG vegetative cells.

BG Concentration - A study of the effect of B. globigii (vegetative) concentration on the amount of fluorescence produced in 5 minutes at ambient temperature is shown in Figure 4-27. The results indicate that, within the range of interest, the net fluorescence (sample minus blank) is approximately linear with concentration and the minimum detectable amount is of the order of 10^6 BG/ml.

Reaction Temperature - Since DPNH fluorescence is reported to increase 20 percent on decreasing the temperature by 10°C , it was of interest to determine the effect of running the reaction at an elevated temperature and then cooling prior to measuring the fluorescence. The results shown below indicate no advantage in increasing the reaction temperature.

<u>Reaction Conditions</u>	<u>Temperature of Fluorescence Measurements, $^\circ\text{C}$</u>	<u>Fluorescence in Arbitrary Units</u>
5 min at 24°C	24	15.5
5 min at 42°C	24	12.7

Reproducibility - One of the problems associated with this reaction is lack of reproducibility in the enzymatic activity of BG suspensions prepared on different days. Thus, at a BG concentration of 2×10^6 cells, the net fluorescence may vary from 2 to 20 units in a 5-minute reaction period. Aside from the reduction in reactivity resulting from sporulation on standing, another variable is evidently contamination with traces of TGE culture medium which is known to fluoresce in the same spectral region as DPNH. Careful centrifugation and washing twice with distilled water or the use of a minimal culture medium containing basically inorganic components which show minimum



TOTAL NUMBER, *B. GLOBIGII* VEGETATIVE CELLS

Figure 4-27. Net Fluorescence of Reaction Mixture of Glutamate + DPN + *B. globigii*

fluorescence in the spectral region of interest has not completely resolved the problem. A contributing factor may be the inherent instability of the enzyme system itself. Some inactivation undoubtedly occurs with repeated washings. Treponemata cells have been reported to lose their L-glutamate dehydrogenase activity rapidly after harvesting. In contrast, sonic extracts appeared more stable⁽¹⁾.

Effect of Sonication on Fluorescence - Sonication of a BG suspension for 10 minutes at 20 kc was found to increase the net fluorescence formed in the enzymatic reaction from 5 to 20 units at a level of 2×10^6 BG (grown on a TGE medium). The enzymatic activity of this product decreased about half on storage for 3 days at about 10°C . A similar run made with BG grown on minimal media showed an increase in the net fluorescence from 6 to 11 units after sonication at a level of 6×10^5 bacteria.

Use of Microvolumes - A BG suspension which produced a net fluorescence of 18 units (from 74 to 92) at a level of 2×10^6 cells in a 2 ml reaction volume failed to show any significant difference in fluorescence over that of a control when compared using 25 μl cuvettes. The low quantum yield of fluorescence of DPNH (reported value 2 percent⁽²⁾) appears to preclude increasing the sensitivity of the method beyond 10^6 cells by going to smaller reaction volumes. The addition of ethanol or propanol to the reaction mixture, reported to increase intensity of DPNH fluorescence⁽³⁾, failed to increase the net fluorescence.

4.1.4.3.4 CONCLUSIONS

In view of the limiting sensitivity of 10^6 cells, further work on this system does not appear warranted.

4.1.4.3.5 REFERENCES

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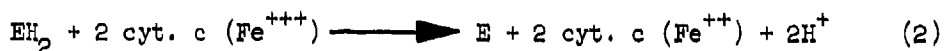
4.1.4.4 CYTOCHROME C REDUCTASE

4.1.4.4.1 SUMMARY

The existence of the DPNH-cytochrome c reductase enzyme in B. globigii (vegetative) was established by a spectrophotometric technique, and this system was then investigated for its ability to serve as a free radical initiator of luminol chemiluminescence. Although 4×10^{-8} g of this enzyme could be detected by the luminol reaction, the reductase is at least two orders of magnitude less effective (on a weight basis) than catalase as an initiator of chemiluminescence.

4.1.4.4.2 INTRODUCTION

Cytochrome c reductase, a flavin enzyme important in the respiratory sequence, catalyzes the reduction of mammalian cytochrome c by reduced pyridine nucleotide (DPNH or TPNH) with the overall stoichiometry being indicated by



Oxidation of the reduced enzyme in the second step is believed to involve formation of semi-quinone or free radical intermediates. This has been confirmed by Beinert and Sands⁽¹⁾, who showed by EPR that the free radical signals observed were due to re-oxidation of the enzyme, rather than oxidation or reduction of the substrate.

Recently, c-type bacterial cytochromes have been isolated from bacteria with absorption spectra similar to the spectrum of mammalian cytochrome c, but differing from the latter in their fluorescence, biochemical, and adsorptive properties. Although evidence indicates that the prosthetic group is the same, the difference in the isoelectric points suggests a difference in

the protein moiety. Similarly, cytochrome c reductase activity has also been reported for various bacteria (E. coli, S. faecalis, C. perfringens, P. denitrificans, etc.⁽²⁾). Demonstration of this activity was carried out with cell-free enzymes and mammalian cytochrome c, which is readily available. It is recognized that bacteria may show a preferential activity toward their own cytochromes and so the results obtained using mammalian cytochrome c in conjunction with microbial systems may not be quantitatively parallel. However, because of the commercial unavailability of microbial cytochromes and the difficulty attendant in their separation, many investigators have used mammalian cytochrome c as a first approximation in studying cytochrome c reductase activity in bacteria.

The cytochrome c reductase system was investigated with the hope of adapting it to the luminol reaction since the latter is known to chemiluminesce in the presence of free radicals (without need of H_2O_2). The sensitivities attainable by this method would then be compared with that of the conventional catalase-luminol- H_2O_2 system.

4.1.4.4.3 STATUS

Initial evidence for the existence of cytochrome c reductase activity in B. globigii was obtained spectrophotometrically (DK-2) by following the change in optical density at 550 mμ on addition of the bacterial suspension to a DPNH-cytochrome c reaction mixture. In the results shown in Figure 4-28, the upper curve was obtained with pure enzyme, the lower curve with approximately 2×10^7 B. globigii (vegetative, Petroff-Hausser count) in a 3-ml cuvette. The reaction is quite rapid, being virtually complete in about 3 minutes at ambient temperature. No attempt was made at this stage to optimize reaction conditions, either by investigating the use of other co-factors (TPNH or menadione) or the use of cell extracts, both of which are known to influence sensitivity for other bacteria.

Prior to adapting this system to the luminol reaction, a check was also made of the effect of adding cytochrome c and cytochrome reductase individually to luminol both with and without further addition of H_2O_2 . No DPNH was used in these preliminary experiments. The results obtained are tabulated below:

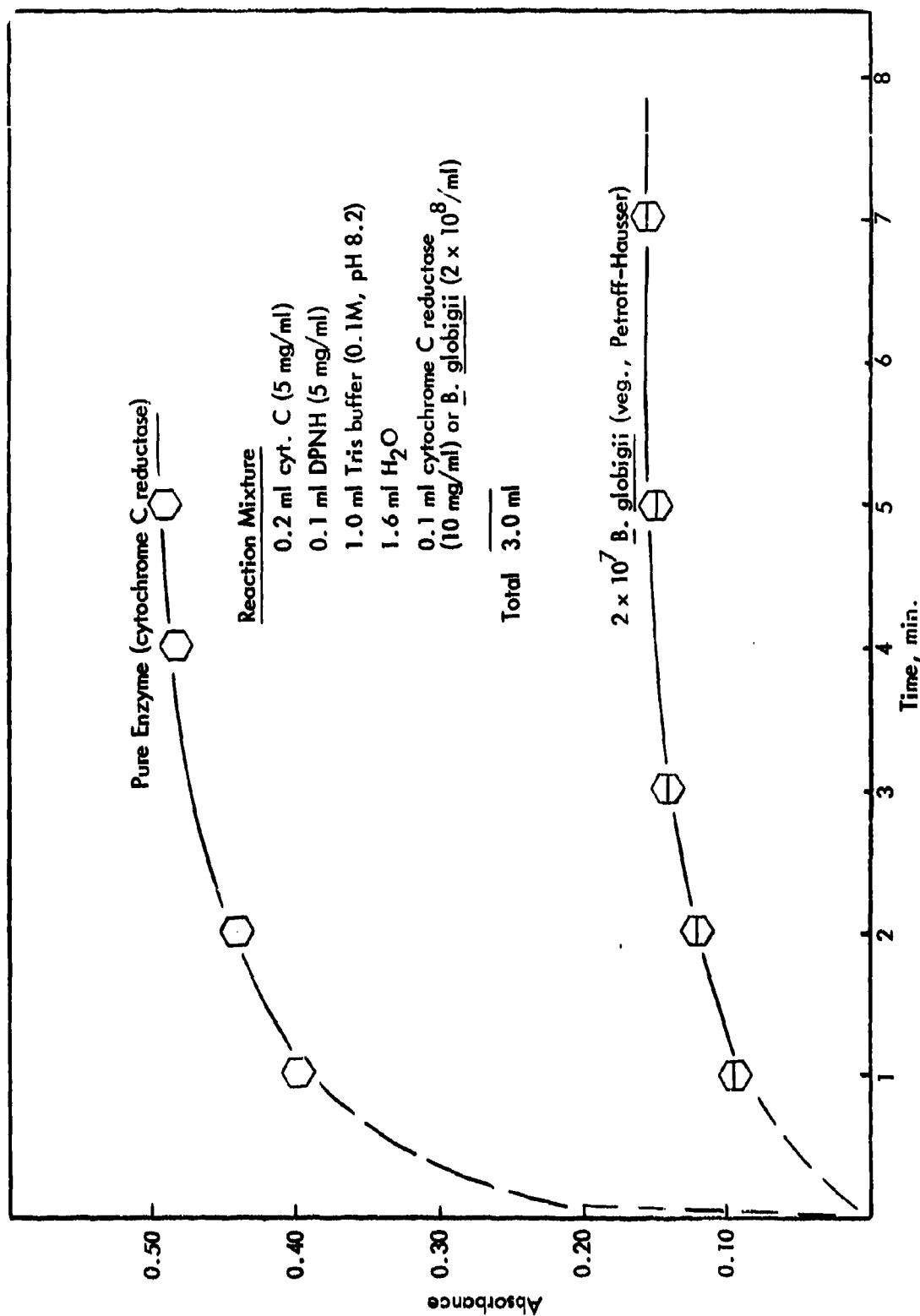


Figure 4-23. DPNH-Cytochrome-c Reductase Activity (Beckman DK-2 at 550 mμ.)

Sample/Blank Luminescence Ratio

	<u>Without H_2O_2</u>	<u>With H_2O_2</u>
Cytochrome c	2.5 mv/ ~ 1 mv = 3/1 (1×10^{-7} g)	120 mv/25 mv = 5/1 (1×10^{-7} g)
Cytochrome c reductase	4 mv/ ~ 1 mv = 4/1 (3×10^{-7} g)	60 mv/25 mv = 2/1 (4×10^{-8} g)

The data indicate that both cytochrome c as well as the reductase produce chemiluminescence either with or without further addition of H_2O_2 . The light intensity in the presence of H_2O_2 , however, is about 50 times greater and the sample/blank luminescence ratio somewhat higher. A more sensitive recorder which would permit more accurate reading of the low light levels in the case of the samples without H_2O_2 would be needed to establish this with certainty.

In adapting this system to the luminol reaction, DPNH and cytochrome c were added to the luminol prior to injecting the reductase enzyme. No significant increase in signal over and above that obtained for each of the components themselves was observed, indicating no synergistic effect due to formation of free radicals. The high alkalinity of the luminol (pH 12.4 instead of 8.2 which is optimum for the reductase reaction) may have been a factor. Anaerobic conditions may also be required to ensure reduction of the enzyme by the DPNH (reaction (1)).

The results of these experiments may be summarized as follows:

- a. The observation that cytochrome c will produce chemiluminescence with luminol is to be expected inasmuch as this material is a hemoprotein. Earlier work by Dr. Neufeld at Fort Detrick indicated that the luminol reaction is a sensitive detector for iron porphyrins in general.
- b. The observation that cytochrome c reductase causes luminescence (with or without H_2O_2) is perhaps surprising in that this flavoenzyme contains nonheme iron; α - tocopherol has been identified in solubilized beef heart DPN-cytochrome c reductase. However, there is no information of bound metals in purified bacterial reductases.

4.1.4.4.4 CONCLUSIONS

It is evident that since c-type cytochromes as well as the reductase are known to be present in many microorganisms, the luminol- H_2O_2 reaction as it is now being used is being initiated by these components as well as catalase. However, the contribution of the former is expected to be small in view of the fact that, on a weight basis, catalase (detection sensitivity is 1×10^{-10} g) is at least 2 orders of magnitude more effective as a initiator of chemiluminescence. These preliminary results would suggest that the cytochrome reductase system does not have the required sensitivity when used in conjunction with luminol. Other flavoprotein enzymes known to undergo oxidation-reduction reactions (i.e., lipoyl dehydrogenase), producing free radical intermediates and thus capable of initiating chemiluminescence, may prove to be more effective in this regard.

4.1.4.4.5 REFERENCES

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- (2) Gunsalus, I.C., and Stanier, R.Y., The Bacteria, Academic Press, N.Y., 1961, Vol II, p. 328.

4.1.4.5 TRANSAMINASES

4.1.4.5.1 SUMMARY

A fluorimetric assay technique for glutamate-oxalacetate transaminase activity has been found to be sufficiently sensitive to detect 2×10^4 ml B. globigii or 80 bacteria in a 4 μ l volume. Further development appears justified.

4.1.4.5.2 INTRODUCTION

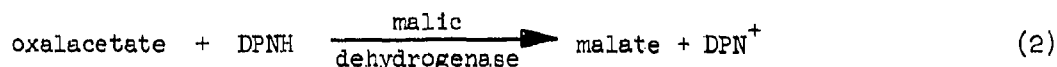
The enzyme-catalyzed transamination reaction involves the reversible transfer of an amino group from an α -amino acid to an α -keto acid, as for example



In view of the fact that bacteria are rapidly growing organisms and must be capable of extremely rapid protein synthesis, the widespread distribution of transaminases in most microorganisms is understandable. Bacteria generally contain several transaminases, each with some substrate specificity. However, with the exception of B. anthracis and B. subtilis which require D-glutamate, almost all will react with L-glutamate, as indicated above⁽¹⁻⁴⁾. The existence of the glutamate-oxalacetate transaminase (GOT) in rickettsiae has also been confirmed⁽⁵⁾. Preliminary data obtained at Space-General using a fluorimetric method for assay of GOT in B. globigii indicated that the required sensitivity is attainable. The status of work to date is presented below.

4.1.4.5.3 STATUS

The fluorimetric procedure of Laursen and Esperson⁽⁶⁾ was found to be sufficiently sensitive to detect 10^4 B. globigii per ml. The method consists of coupling the transamination shown by reaction (1) to malic dehydrogenase as shown below



Oxalacetate formed in reaction (1) is determined enzymatically by adding the specific enzyme malic dehydrogenase and DPNH. The DPN⁺ that is formed is assayed fluorimetrically by forming a condensation product with methyl ethyl ketone (MEK), which produces a strong stable fluorescence on treatment with alkali. Fluorescence of the final product was measured by excitation at 360 mμ using an Aminco-Bowman spectrophotofluorimeter. A control similarly processed, containing all the reagents except the bacteria, served as the blank in each instance. Results obtained using 3 ml cuvettes are tabulated below (DL-aspartate used):

Final Concentration <u>B. globigii/ml</u>		<u>Scale Reading</u>		Fluorescence Ratio	
<u>Petroff-Hausser</u>	<u>Viable</u>	<u>Sample</u>	<u>Blank</u>	<u>Sample/Blank</u>	<u>Condition</u>
3 x 10 ⁶	6 x 10 ⁵	47	26	1.8/1	10 min at ambient temp.
3 x 10 ⁴	6 x 10 ³	35	28	1.3/1	
3 x 10 ⁶	2 x 10 ⁶	42	16	2.6/1	10 min at ambient temp.
3 x 10 ⁴	2 x 10 ⁴	15	10	1.5/1	
3 x 10 ⁶	2 x 10 ⁶	51	15	3.3/1	10 min 35°C; conc. of DPNH and malic dehy- drogenase doubled
3 x 10 ⁴	2 x 10 ⁴	28	15	1.9/1	
2 x 10 ⁴	8 x 10 ³	19	13	1.5/1	10 min at ambient temp.; conc. DPNH and malic dehydrogenase doubled
2 x 10 ⁴	8 x 10 ³	16	11	1.5/1	8 min at ambient temp.; conc. DPNH and malic dehydrogenase doubled

In a study of the effect of substrate (i.e., α-ketoglutarate and DL-aspartate) and DPNH concentrations, those suggested by Laursen and [unclear] appeared to be optimal. Substituting D-aspartate for DL-aspartate, however, raised the sample/blank ratio by about 30 percent. The use of fresh malic dehydrogenase reagent (prepared daily) appears to be critical.

The above data indicate that B. globigii can be detected at a level of 2×10^4 /ml by their transaminase activity. Taking the bacteria at this same concentration, but using a 4 μ l viewing volume, the sample/blank fluorescence ratio was 2.2/1 and 1.7/1 in two separate experiments. From the standpoint of reliability, ratios of this size are unmistakable evidence of a positive signal.

This indicates that as few as 80 bacteria (total amount in a 4 μ l volume) can be detected. Further improvement in sensitivity can undoubtedly be achieved by a systematic study of process variables (pH, temp., cofactors such as pyridoxal or pyridoxamine phosphate), and use of disrupted bacteria. Preliminary results in the SGC laboratory indicate that transaminase activity may be associated with viability since complete loss of this enzyme activity in B. globigii occurred on heating (boiling) or exposure to ultraviolet for 1/2 hour. It has been reported, however, that loss of viability of a rickettsiae suspension induced by freezing and thawing left the transaminase activity unaffected⁽⁵⁾. High orders of glutamate-oxalacetate transaminase activity have been reported for E. coli, S. dysenteriae, S. typhosus, B. subtilis, P. pyocaneus, P. fluorescens, A. vindandii, S. aureus, S. albus, C. welchii, S. hemolyticus, S. viridans and Pneumonas Type I.

4.1.4.5.4 CONCLUSIONS

A preliminary examination of the fluorometric method of Laursen and Esperson indicates it is sensitive enough to detect 10^4 BG/ml by their transaminase activity. Further improvements in sensitivity might be achieved through a more systematic examination of process variables. Therefore further development is justified.

This reaction in somewhat modified form has recently been adapted for analyzing for GOT activity in serum⁽⁷⁾. The procedure has been automated using a Technicon Autoanalyzer which is capable of analyzing forty samples of serum per hour for GOT activity. The method employs reactions (1) and (2) above; however the transaminase activity is monitored by measuring the decrease in native fluorescence of DPNH with time as opposed to complexing the DPN⁺ formed with MEK. The latter method is expected to be more sensitive owing to

the higher intensity of the DPN-MEK fluor compared to native DPNH fluorescence. Use of the Autoanalyzer may provide a convenient method for the continuous monitoring of GOT activity in bacteria.

4.1.4.5.5 REFERENCES

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- (6) Bulletin N-53P "Serum Glutamic-Oxalacetic Transaminase (SGOT) Via Fluorometer" Technicon Instruments Corp., Chauncey, N.Y.

4.1.4.6 FERMENTATION MATRIX

4.1.4.6.1 SUMMARY

A fermentation matrix shows the capabilities of 24 pathogenic and non-pathogenic bacteria for fermenting 25 substrates. Its purpose is to distinguish pathogens via their characteristic reaction patterns. A mathematical analysis of the concept showed that it might distinguish single organisms readily, but is distinctly less attractive for distinguishing one class or mixed group from another (e.g., pathogens in general from non-pathogens). Hence, it is not now recommended for further development.

Concurrent experimental work demonstrated that fermentation is measurable with adequate sensitivity through changes in pH. In the model reaction of E. coli with glucose, bacterial concentrations of 3×10^4 to 10^6 per ml appeared detectable in test volumes of 200 μ l. The threshold of detectability lies at responses of a few-thousandths units of pH per minute. Further improvements in sensitivity might be realized.

4.1.4.6.2 INTRODUCTION

A detection method based on pH changes induced by bacteria was proposed for study by the Beckman laboratories. The objective is to detect pathogenic bacteria on the basis of their capability for selective fermentation of different substrates. Since a characteristic group of substrates is metabolized by each species, a "fermentation matrix" of bacteria versus substrates may be constructed which is potentially capable of detecting the pathogenic members by means of their reaction patterns.

The initial problem in testing this concept is compilation of a sufficiently complete matrix, including both pathogenic and non-pathogenic species. For purposes of research, this need not be the ultimate matrix, but can be a model, possibly including pathogen simulants. Mathematical analysis must be applied to select a subset of reactions which is sufficient for distinguishing pathogens from non-pathogens. It is also necessary to show that this indication will not be confused by the introduction of a new organism with new reaction patterns, or by combinations of species.

Experimentally, it is necessary to demonstrate that measurements of pH changes are sufficiently rapid, sensitive, and selective. Information in the literature should be considered, and supplemented by experiment with the most advanced apparatus available.

4.1.4.6.3 STATUS

Preparation of Matrix - A preliminary fermentation matrix to be studied as a model was assembled. It is presented in Table 4-13. It includes a list of 12 representative bacteria known to be pathogenic for human beings. This was supplemented, as far as possible, with literature data on non-pathogenic airborne organisms. It must be noted that identification of these contaminants is not well documented and the spectrum of carbohydrate metabolism is very incomplete. The missing data raise concern that non-pathogens might give false alarms, unless their acid-producing patterns are clearly defined. The substrates include 21 hydroxy and carbohydrate compounds. Data are also provided on nitrate reduction and litmus milk reactions.

The need for further recasting of the preliminary matrix is evident. Adaptive reactions and redundant information should be eliminated. It should also be helpful to supply additional experimental data on the metabolic patterns of some species. In addition, further information is needed on rate of acid production as a function of species and of growth phase.

Theoretical Evaluation - An analysis of the usefulness of the fermentation matrix method was performed by Professor S. W. Golomb. The complete text is included in a Comprehensive Report⁽¹⁾. From a mathematical standpoint, this treatment examines the problem of distinguishing one or more organisms in the model matrix. Experimental difficulties are disregarded, and each + or - in the table is assumed to represent an attainable result.

Theoretically, as few as $K_1 = \log_2 N$ tests may suffice to distinguish among N organisms, when they occur singly. Actually, in spite of the lack of information (zeros) in many cells of the matrix, the general level of distinguishability of individual organisms is high. However, if the responses of two organisms may become superimposed, a larger number of tests is required. If it

EXPANDED FERMENTATION MATRIX

1. $\text{H}_2\text{O} + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + \text{OH}^-$ Acid-Base
 2. $\text{H}_2\text{O} + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + \text{OH}^-$ Acid-Base
 3. $\text{H}_2\text{O} + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + \text{OH}^-$ Acid-Base

EXPANDED FERMENTATION MATRIX

[illegible]

becomes reasonable to expect many different organisms in the same sample, it becomes less and less practical to resolve the various alternatives by the fermentation matrix method. In this case purely specific methods (e.g., specific antibody methods) are recommended to identify the sample components on a one-by-one basis.

Fermentation Studies - To examine the basic experimental premise, the fermentation of glucose to lactic acid by E. coli was considered. A literature search indicated that 10^{-14} to 10^{-13} ml of O_2 per second is consumed by a single E. coli cell when glucose is metabolized. On the assumption that the fermentation produces 1.3 moles of acid per mole of O_2 consumed, the rate of change of pH can be calculated from the ionization constant of the acid. Thus a cell concentration of 10^4 bacteria/ml will change an initial pH of 7.0 (in an unbuffered medium) to 6.992 in 1 minute.* This is larger than the minimal detectable change employing standard commercial pH instrumentation. Therefore sensitivity of this reaction appears adequate, especially considering the possibility of use of more advanced equipment.

This reaction was tested repeatedly, with encouraging results. A thermostatic blood pH electrode assembly of 200- μ l was used to contain the reaction mixture of bacteria newly mixed into a 1 percent glucose nutrient medium. The electrode was connected to a Beckman Research pH meter capable of detecting changes of a few thousandths of one pH unit.

Under standard conditions the level of detectability was in the region of 10^5 to 10^6 bacteria per ml. At these cell concentrations, pH changes of from 10^{-3} to 10^{-2} units per minute have been consistently observed at 37.5° or 38°C. Figure 4-29 below shows the dependence of response on concentrations, besides illustrating the extent of scatter encountered.

Tests showed that the nutrient medium (containing NH_4Cl , $NaCl$ and $MgSO_4$ dissolved in dilute buffer) is buffered sufficiently to produce some adverse effect in the pH response. It is likely that this effect can be decreased without impairing the stability of the system. Other variations of the standard technique have included reduction of test volume to 50-70 μ l and variations in temperature of the reaction. Temperature-response curves indicate that, although the optimum temperature for a sustained fermentation (5-minute reaction

* A calculation of the pH change reported earlier⁽²⁾ is in error and should be corrected.

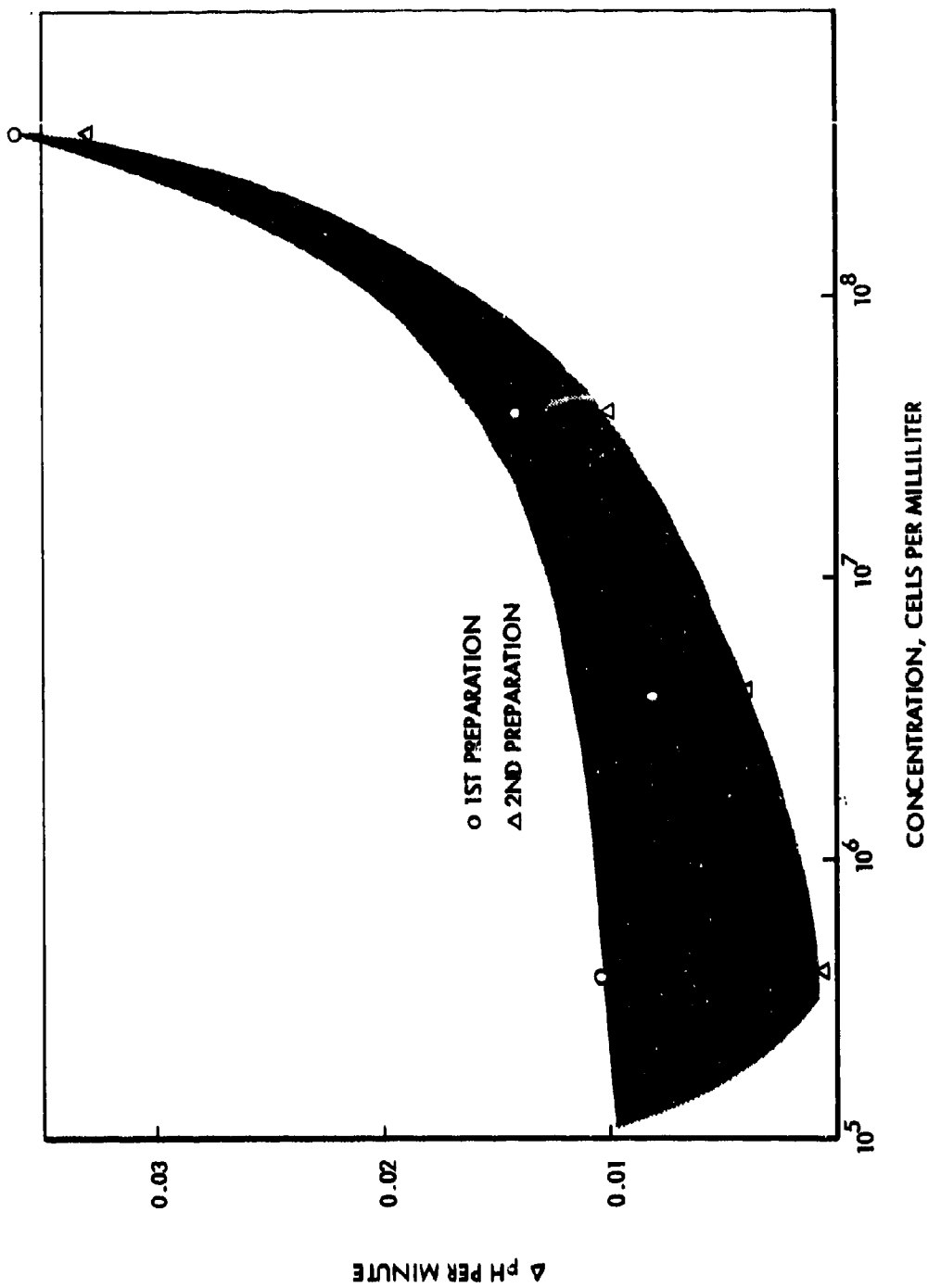


Figure 4-29. Rate of pH Change in 1 Percent Glucose Nutrient Medium as Function of E. coli Concentration

time) is approximately 38°C, the initial rate (1-minute reaction time) at 45°C is nearly 3 times the 5-minute rate of 38°C. In addition, some qualitative experiments demonstrated sensitive response in a miniaturized flow system for detecting fermentation.

4.1.4.6.4 CONCLUSIONS

It is logical first to evaluate the fermentation matrix concept from the standpoint of pattern recognition, disregarding experimental feasibility. The evidence here suggests that the method may be useful in distinguishing individual organisms. However, the actual requirement is for a multi-agent capability to detect any of several pathogens against a background containing some non-pathogenic organisms. The need to distinguish many organisms, either alternatively or in groups, means that many responses may be superimposed. Even if a test pattern can be constructed, it will be very complex.

On the other hand, the evidence of experimental feasibility, although promising, is confined to a single test system.

On the whole, the effort required for further development of this concept appeared not to be justified, and work was terminated.

4.1.4.6.5 REFERENCES

- (1) Fifth Comprehensive Report, SGC 382R-7 Research on BW Detection, October, 1964 - March, 1965 pp. 3-159 to 3-164.
- (2) Fourth Comprehensive Report, SGC 382R-6 Research on BW Detection, April - September, 1964, pp. 3-96, 3-97.

4.1.4.7 C^{14} -LABELLED GLUCOSE

4.1.4.7.1 SUMMARY

A detection scheme based on the metabolic production by bacteria of $C^{14}O_2$ from C^{14} -labelled glucose has been used to detect as few as 5×10^4 B. globigii in a 10-minute reaction time. However, indications of appreciable activation lags with some bacteria led to termination of work on this principle.

4.1.4.7.2 INTRODUCTION

A detection scheme based on the metabolic production of $C^{14}O_2$ by bacteria from a radioactive substrate possesses several inherent advantages over devices which depend on production of a fluorescent or chemiluminescent species or on a pH change. Aside from the potential high sensitivity of the method, the background problem is minimal with this approach. Thus, although there are particulates in the atmosphere which are weak beta emitters, these would not interfere if they appeared in the reaction mixture, since the evolved $C^{14}O_2$ generated by the bacteria could be counted in the gas phase downstream from the reaction chamber. Non-specific absorption would not be a problem with this technique. An investigation of this approach was made using uniformly labelled glucose as the source for $C^{14}O_2$. The results obtained are described below.

4.1.4.7.3 STATUS

The scheme based on the metabolic production of radioactive CO_2 has been used to detect as few as 5×10^4 bacteria in a reaction time of 10 minutes.

The culture medium used for metabolism by the bacteria to generate the labelled $C^{14}O_2$ consisted of the conventional TCE broth (10 ml) containing 10 μ c of D-glucose- $U-C^{14}$ (uniformly labelled) as the only glucose source. The experimental procedure consisted of adding the bacterial suspension (0.5 ml) to 0.5 ml of labelled medium in a stoppered container and maintaining the reaction mixture at about $37^\circ C (\pm 2^\circ C)$ for 10 minutes. The mixture was then acidified to

drive off the $C^{14}O_2$ (0.5 ml of 1:10 H_2SO_4) and the $C^{14}O_2$ was absorbed in aqueous alkali (0.5 ml of 0.5 N NaOH). An aliquot of the latter was then evaporated to dryness on a planchet and a count made with a Baird gas-flow proportional counter. A control was run using sterile water in place of the bacterial suspension. The results are summarized below.

<u>No. of Bacteria</u>	<u>Sample</u>	<u>Counts/min</u>	
		<u>Control</u>	
3.7×10^6 <u>B. globigii</u>	71, 94, 83, 94, 90	28, 24, 25, 26, 30	
(2.8×10^2 spores)	84, 80, 84, 82, 76	32, 33, 27, 36, 35	
	(av = 84)	(av = 30)	

An alternate procedure involved measuring the loss in radioactivity of the reaction mixture at the conclusion of the run. An aliquot was removed from the reaction mixture and evaporated to dryness on a planchet prior to counting*. The results of several such runs are shown below.

<u>No. of Bacteria</u>	<u>Counts/min, Average*</u>		
	<u>Sample</u>	<u>Control</u>	<u>Difference</u>
8×10^4 <u>B. globigii</u> (5×10^2 spores)	5222	5625	403
5×10^4 <u>B. globigii</u> (1×10^2 spores)	5419	5728	309
1.7×10^7 <u>E. coli</u>	3797	6201	2404

*Average of 4 or more counts

The ostensibly greater sensitivity shown by the alternate procedure is probably due to the following consideration. The counting efficiency of C^{14} activity can be very low in evaporated samples due to absorption of the low-energy beta particles by extraneous solids in the reaction mixture (i.e., NaOH or TGE medium). In the alternate procedure involving evaporation of the reaction mixture, the decrease due to absorption will be the same for both the sample and control. However, in the first procedure described (evaporation of NaOH solution) the decrease due to absorption will appear disproportional since the decrease will show up only in the NaOH solution containing the $C^{14}O_2$ and not

*In this procedure, diluted HCl (1:10) rather than H_2SO_4 was used to prevent degradation of the labelled glucose on evaporating to dryness.

in the control which contains no radioactive component at all. The net difference (between sample and control) will then be less in the latter case.

The effect of process variables was investigated by the first procedure described above (evaporation of NaOH solution) with the results shown in Table 4-14. Although less sensitive than the alternate procedure of counting the activity of residual labelled glucose, this method simulated the technique which might be used in a continuous device (i.e., counting the $C^{14}O_2$ in the gas phase).

Even though 16-hour cultures were used throughout and the organisms were in most cases run immediately after harvesting, the data indicate considerable variation in activity between different batches of B. globigii. The variability may be due to a difference in the amount of metabolites still remaining in the harvested bacteria. Since each of the variables shown was studied on a single day using the same single batch of bacteria, the conclusions drawn below would be considered valid.

<u>Variable</u>	<u>Conclusion</u>	<u>Applicable Runs</u>
1. Type and pH of buffer	Optimum activity is with a phosphate buffer at pH 7.3.	219-7
2. Temperature	Maximum activity is at about 37°C.	264-30
3. Reaction time	Amount of $C^{14}O_2$ generated increases approximately linearly with time; significant evolution after 5-minute reaction time.	264-28
4. Equilibration time	The amount of $C^{14}O_2$ recovered increases about two-fold on increasing the equilibration time from 15 minutes to 1 hour.	264-30
5. Media composition	Water-glucose is superior to TGE-glucose.	277-2
6. Nitrogen atmosphere	$C^{14}O_2$ evolution is still evident when reaction is conducted under nitrogen atmosphere; reaction to be repeated to ensure trace amounts of oxygen absent by evacuating reaction mixture and passing N_2 over heated copper.	264-33

Table 4-14

EFFECT OF PROCESS VARIABLES ON $C^{14}O_2$ EVOLUTION*

Run No.	Variable Studied	Reaction Medium	No. of <i>B. globigii</i>	Results			
				Radiation Counts/Min			
				Sample	Control	Difference	
264-27	Type buffer	TGE-glucose	4.5×10^6 (.025% spores)	Phosphate	246	20	226
				Tris	215	25	190
				None	218	23	195
219-7	pH (phosphate buffer)	TGE-glucose	2.5×10^6 (.025% spores)	pH 6.3	258	18	240
				pH 7.3	333	(25)**	308
				pH 8.3	299	21	278
264-30	Temperature (1 hour equilibration)	TGE-glucose	8×10^6 (.13% spores)	25°C	696	25	671
				37°C	1269	24	1245
				45°C	1278	23	1255
264-28	Reaction time	TGE-glucose	4.5×10^6 (.025% spores)	5 min	81	24	57
				10 min	140	20	120
				30 min	505	23	482
264-30	Equilibration time	TGE-glucose	8×10^6 (.13% spores)	15 min	610	25	585
				60 min	1269	24	1245
277-2	Medium composition	TGE-glucose vs Water-glucose	2.2×10^7 (3.3% spores)	TGE-glucose	922	28	904
				Water-glucose	1503	28	1475
264-33	Nitrogen Atm.***	TGE-glucose	2×10^6 (.014% spores)	Nitrogen	2076	2395	319
219-9	BG spores	TGE-glucose	6×10^6 spores	Vegetative (control)	119	21	98
				BG spores	25	21	4
264-33	Sonicated spores	TGE-glucose	9×10^5 spores	Sonicated	2374	2400	26
277-2	Sonicated BG (veg.)	TGE-glucose	2.2×10^7 (3.3% spores)	sonicated BG	849	18	831
				Unsonicated BG	922	18	904
277-2 277-4	Refrigerator storage	TGE-glucose Water-glucose	2.2×10^7 (3.3% spores)	Fresh BG			
				TGE-gl.	922	18	904
				Water-gl.	1503	28	1475
				3-Day Old BG			
				TGE-gl.	485	29	456
				Water-gl.	1108	32	1076

* Phosphate buffer pH 7.3, 10 min reaction time, 15 min equilibration in air atmosphere - unless otherwise indicated.

** Control lasty figure indicated represents an average value.

*** Count made on dried aliquot from residual TGE-glucose reaction mixture.

<u>Variable</u>	<u>Conclusion</u>	<u>Applicable Runs</u>
7. Spores	BG spores show no activity.	219-9
8. Sonicated spores	Sonicated BG spores show no activity.	264-33
9. Sonicated BG vegetative	No increase in activity is obtained over unsonicated BG vegetative.	277-2
10. Refrigerator storage	Re-run of BG stored for 48 hours at $\sim 10^{\circ}\text{C}$ indicated decrease in activity of about 25 to 50 percent.	Compare 277-2

4.1.4.7.4 CONCLUSIONS

The sensitivity of this method appears to be of the order of 10^4 to 10^5 B. globigii (resting cells). However, it was indicated that some of the bacteria of interest might be relatively inactive during the resting phase and there may be an appreciable lag before activation occurred. Consequently, further work on this system was discontinued.

4.1.4.8 NH_3 DETECTION BY ELECTRON CAPTURE

4.1.4.8.1 SUMMARY

Although possessing a high sensitivity (10^2 bacteria) the use of electron capture for detecting trace amounts of NH_3 by complexing it with an acid suffers from lack of reproducibility owing to the ease with which the latter is hydrolyzed or absorbed on various surfaces.

4.1.4.8.2 INTRODUCTION

The feasibility of using an electron capture detector for the indirect determination of NH_3 formed by an enzymatic reaction or by pyrolysis of bacteria was investigated. Although insensitive toward NH_3 , this detector is sensitive to materials which have a high affinity for electrons, such as those containing halogens (i.e., BF_3). NH_3 is known to react rapidly with BF_3 to form a volatile solid adduct $\text{NH}_3 \cdot \text{BF}_3$ which could then be metered into the electron capture detector and its signal determined. Excess BF_3 can be removed by fractionation. The drop in ion current which results from the presence of $\text{NH}_3 \cdot \text{BF}_3$ vapor is a function of the concentration of halogen present. Another alternative is to react the NH_3 with a measured excess of BF_3 whose signal has been previously determined, condense out the $\text{NH}_3 \cdot \text{BF}_3$ solid complex, and then measure the signal of the recovered excess BF_3 . The results obtained with boron halides and other halogenated acids are described below.

4.4.1.8.3 STATUS

It was established that although this method is inherently more sensitive than other devices currently being used to detect NH_3 (i.e., the Mine Safety Appliance pyrolysis device), full utilization of the sensitivity of the electron capture detection could not be achieved because of the problems of hydrolysis and adsorption of the complexing agent, and poisoning of the detector with continued use. The state of the art achieved with this system is described below. Work was carried on at both Space-General and Beckman, the former using an Aerograph Model 600-6 E.C. detector from Wilkens Instruments Co. (Calif.), while a detector from Ionics Research, Inc. (Texas), was used by Beckman for these measurements.

Since data were not available on the sensitivity of the electron capture toward the specific complexing agents of interest, these were determined with the following results:

Boron trifluoride (BF_3) - Injection of a 1 μl (gas) sample (0.93 ppm BF_3 in N_2 or 5×10^{-9} g $\text{BF}_3/\text{cc N}_2$) into a nitrogen gas carrier flowing at 100 cc/min produced the response shown in Figure 4-30. Sample response was 1.8×10^{-10} amp. Noise level was 5×10^{-12} amp. Blank obtained by injecting 1 μl of N_2 gas is shown for comparison. It may be calculated that the sensitivity or concentration of BF_3 as seen by the detector lies somewhere between 9×10^{-14} and 2×10^{-13} g $\text{BF}_3/\text{cc N}_2$. On the basis of a 1:1 complexing ability of BF_3 with NH_3 , this would correspond to 2×10^{-14} to 5×10^{-14} g $\text{NH}_3/\text{cc N}_2$, respectively. (The total sample required to produce the signal shown in Figure 4-30 was actually 3×10^{-12} g BF_3 or 8×10^{-13} g NH_3 . Assuming that on pyrolysis of a single bacterium about 1 percent of its dry weight is converted into NH_3 , this would mean a detectability threshold of about 80 bacteria by this technique⁽¹⁾).

An examination of whether NH_3 could be detected by sublimation of the $\text{NH}_3 \cdot \text{BF}_3$ adduct directly into the E.C. detector proved unsuccessful owing to the low volatility of this solid (v.p. < 0.1 mm at ambient temperature). The solid could not be detected even when its solutions in methyl alcohol were injected into the detector.

A problem was encountered with the alternate approach (i.e., reacting NH_3 formed with a measured amount of a boron halide (BF_3 or BCl_3) or other acid whose signal has been predetermined by the electron capture detector, followed by condensing out the solid, and measuring the decrease in signal of the unreacted boron halide). This problem was the lack of reproducibility, resulting from hydrolysis and/or adsorption of the boron halide (BF_3 or BCl_3) on various surfaces. The problem could not be resolved even by presaturating the vessel with the boron halide prior to making a run.

Chloracetic Acids: The use of the less hydrolyzable mono- and trichloroacetic acids as substitutes for the boron halides was investigated. The sensitivity toward trichloroacetic acid (TCA) was found to be 4×10^{-11} moles TCA, corresponding to 7×10^{-10} g of NH_3 or about 1/1000 the sensitivity of BF_3 .

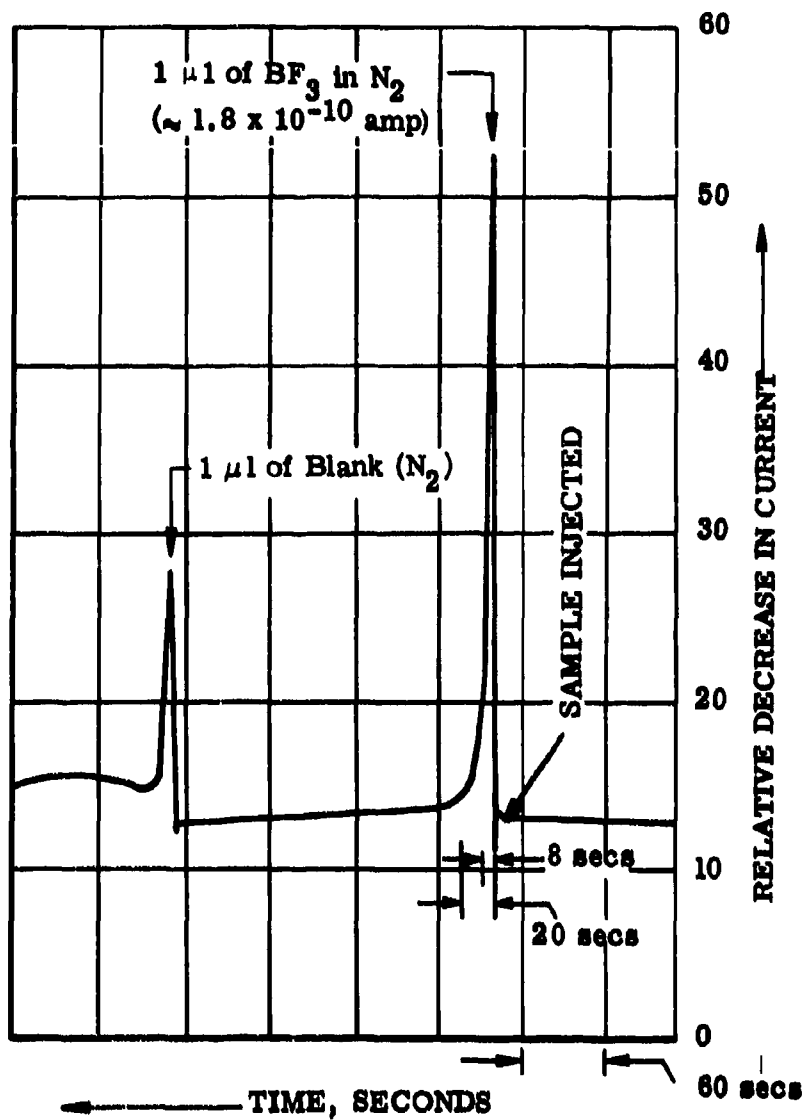


Figure 4-30. Gas Chromatogram of BF_3 , Detected by Electron Capture

Sensitivities obtained with monochloroacetic acid (MCA) ranged from 9×10^{-11} g/sec to 3×10^{-10} g (equivalent to 5×10^{-11} g NH_3 to 1.6×10^{-11} g NH_3).*

Several attempts to detect the reduction in MCA signal following complexing with NH_3 proved unsuccessful. Inconsistencies in repeated determination were probably due to adsorption onto available surfaces, and are indicative of the type of problems encountered in sampling at these low vapor concentrations. One of the other problems encountered with both of these halogenated acids is the apparent poisoning of the detector, which occurs with sustained flows (1 minute or longer). At least a 5-minute purge with N_2 is required to return to baseline zero.

4.1.4.8.4 CONCLUSIONS

The use of electron capture for detecting trace amounts of NH_3 by complexing it with an acid possesses a high sensitivity (approximately 10^2 bacteria). However, it suffers from lack of reproducibility, owing to the ease with which the acid is hydrolyzed or adsorbed on various surfaces. Saturation of the detector in continuous operation is another shortcoming with these complexing agents, requiring an appreciable purging period to return the signal to baseline zero. Both of these major problems would have to be resolved before the potentially high sensitivity afforded by the method can be utilized.

4.1.4.8.5 REFERENCES

- (1) Mine Safety Appliance reports on experimentally determined 0.94 percent conversion NH_3 from B.G. spores, Fourth Quarterly Progress Report 1/1/62-3/31/62 p. 11.

* 10^{-11} g NH_3 corresponds to the amount that would be generated on pyrolysis of 10^3 bacteria.

4.1.4.9 CHEMILUMINESCENT DETECTION SYSTEM BASED ON HEMIN TAGGING

4.1.4.9.1 SUMMARY

The sensitivity of chemiluminescent detection utilizing a hemin-tagged antibody conjugate was found to be approximately 10^5 bacteria (BG spores). Direct staining of agent with hemin chloride, however, permitted detection of either 10^4 BG spores or 10^4 LD₅₀ viral (NDV) particles in static and flow systems. Hemin tagging of embryonated egg, B. globigii (vegetative) or S. marcescens increased the respective chemiluminescent signals from luminol-H₂O₂ by about two- or three-fold (in a static system).

4.1.4.9.2 INTRODUCTION

The observation that certain iron complexes, particularly iron porphyrins, are effective activators of luminol chemiluminescence might be utilized for extending the range of the chemiluminescent detector to spores and viruses, in addition to vegetative bacteria and virus carrier. This might be achieved by tagging either an antibody or the agent directly with an iron porphyrin. Even with vegetative bacteria and virus carrier, the further tagging with the iron porphyrin was found to enhance the innate chemiluminescence several-fold. Hemin chloride was selected as the tagging agent in both applications because of its unique effectiveness in initiating luminol chemiluminescence.

The sensitivity that is attainable using hemin as a tag for luminol chemiluminescence can be calculated using the detection limit of 10^{-12} g hemin/ml⁽¹⁾. This would correspond to 10^8 molecules of hemin/ml. Assuming a multiplicity of attachment of 10^3 antibodies per bacterium, with 10 hemin molecules per antibody, this would mean a total of 10^4 molecules per bacterium. The detection limit would then correspond to 10^4 bacteria/ml.

The general procedure used entails treating the organism, virus, or carrier with hemin chloride or with the hemin tagged antibody conjugate, removing the unattached tag, and treating the stained agent with luminol and H₂O₂ to produce chemiluminescence. The intensity of the luminescence is a linear function of the hemin concentration.

The initial studies were concerned with evaluation of a Fe-tagged antibody conjugate since it does have the advantage of specificity. Direct staining of the agent (organism, virus, or carrier) was utilized when it was found that specificity was destroyed on forming the hemin-tagged antibody conjugate. A summary of the work in each of these areas is presented below.

4.1.4.9.3 STATUS

4.1.4.9.3.1 HEMIN-TAGGED BG ANTIBODY

It is well known that in alkaline solution hemin and hematin will combine readily with organic bases (i.e., imidazoles, histidine, pyridine) to give hemichromes⁽²⁻⁶⁾. Coordination of the nitrogen of the base to the iron in the porphyrin ring is believed to be involved. Direct reaction of hemin with B. globigii antisera was attempted in the belief that conjugation of a similar sort would occur through an amino group in the protein and the iron in the hemin. One additional advantage of this type of conjugation is that it is usually reversible, with dissociation taking place on acidification or at high alkalinity. Dissociation may be desirable after the tagged bacteria have been filtered on tape and washed to remove excess tagging agent. The hemin could then be released from the bacteria by an acid or alkaline rinse and luminol chemiluminescence could be triggered in a liquid flow system.

The procedure used in forming the conjugate was to react a hemin solution (1.5×10^{-3} M in 0.5 M bicarbonate buffer, pH 9.0*) with goat anti-BG globulin over night at 4°C in a molar ratio (hemin: antibody) of 2.6:1. The mixture was then subjected to gel filtration through a Sephadex column (G25 coarse) and the absorption spectra (Beckman DK-2A) of serially collected 2-ml fractions taken. One green, somewhat diffuse band moved rapidly through the column while another green band remained relatively stationary at the top. The collected band appeared to be the conjugated globulin (Figure 4-31 through 4-33) and the other apparently excess hemin. The spectra of the collected portions from the 2.6:1 molar reaction ratio maintained a fairly constant peak height ratio (hemin peak ~ 390 mμ, globulin peak ~ 280 mμ) of about 1:3.7 (avg) (see below) indicating that a stoichiometric species was formed and collected.

*Hemin shows negligible solubility in water at pH 7.

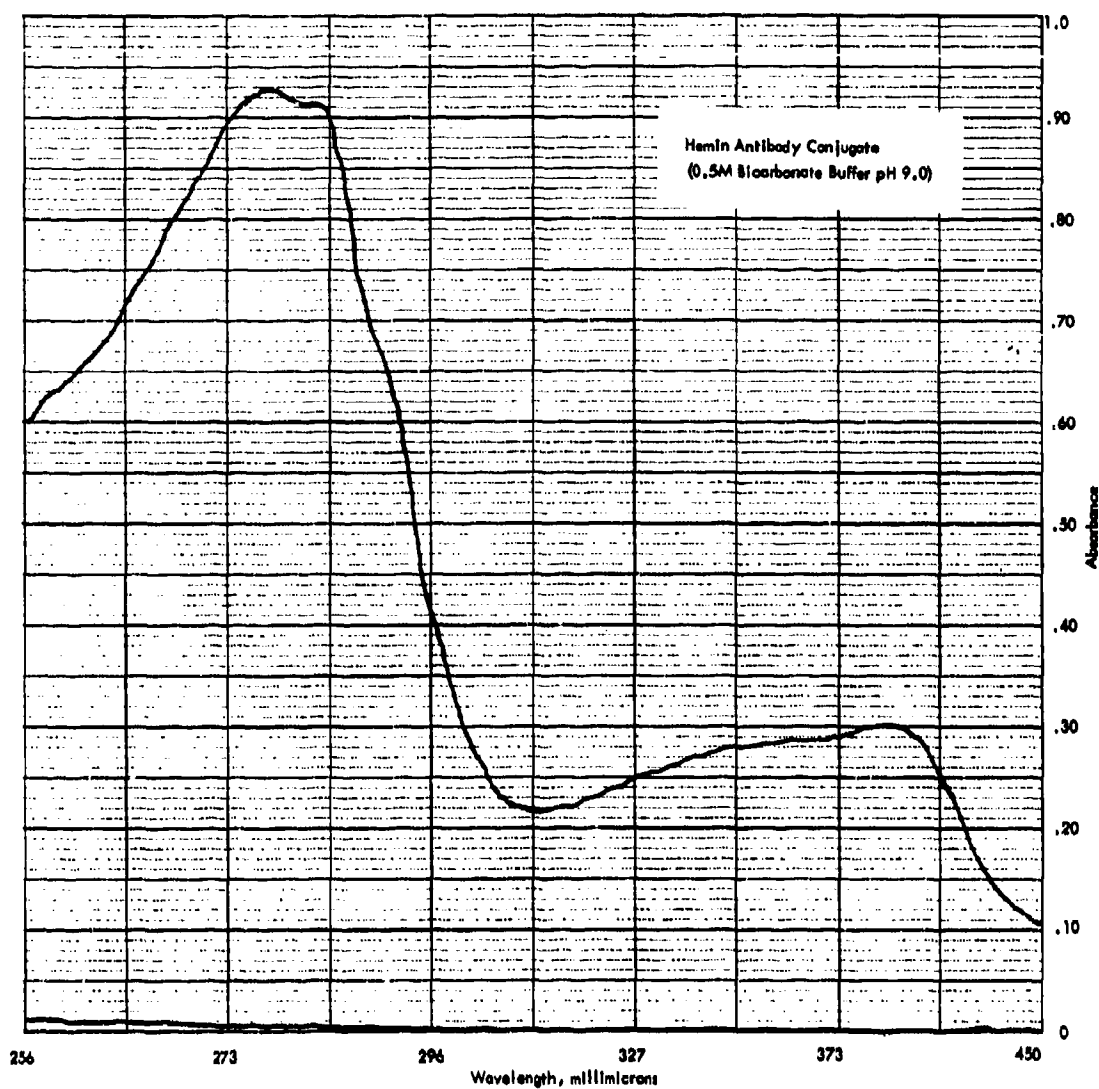


Figure 4-31. Absorbance of Hemin Antibody Conjugate

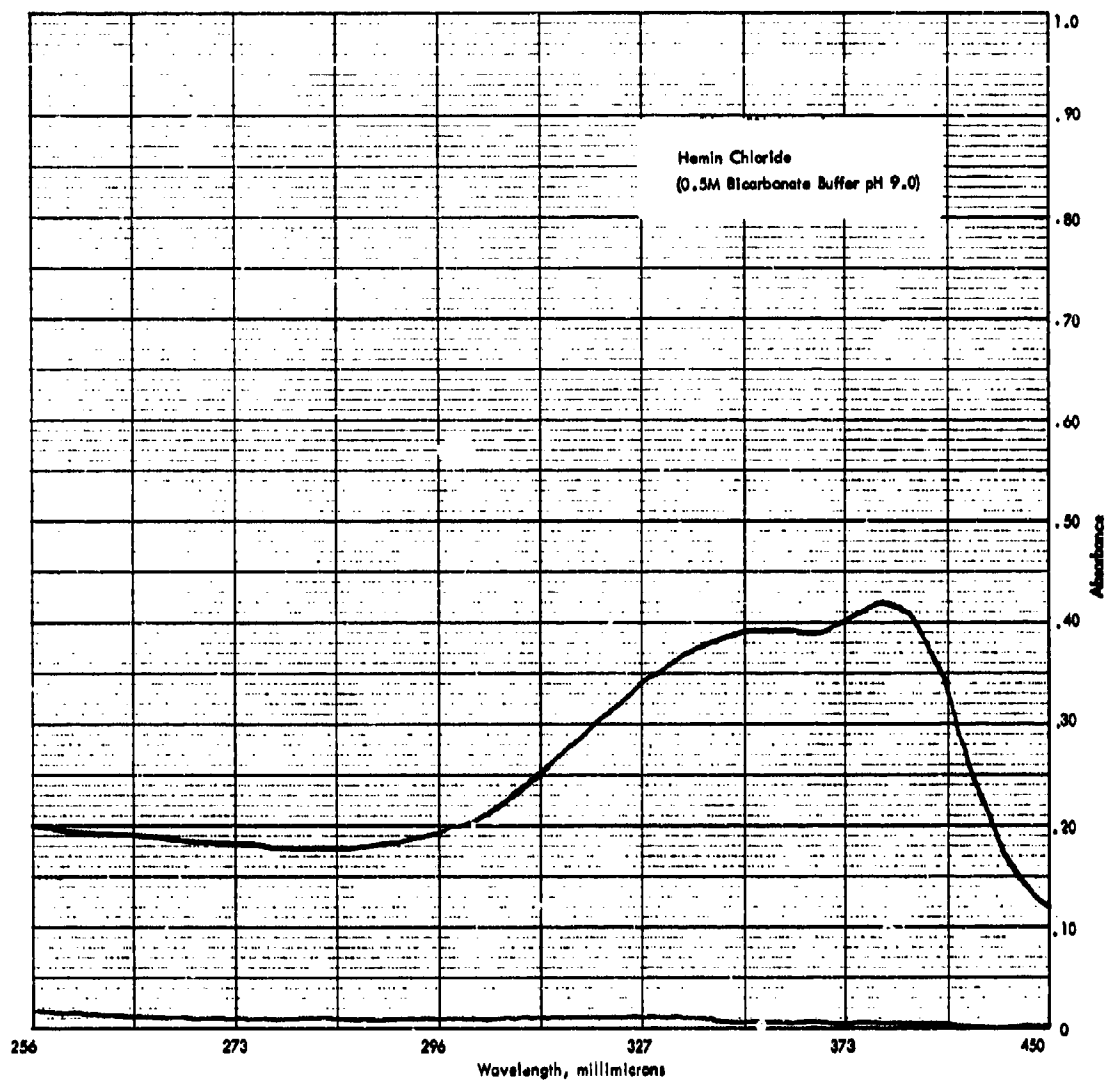


Figure 4-32. Absorbance of Hemin Chloride

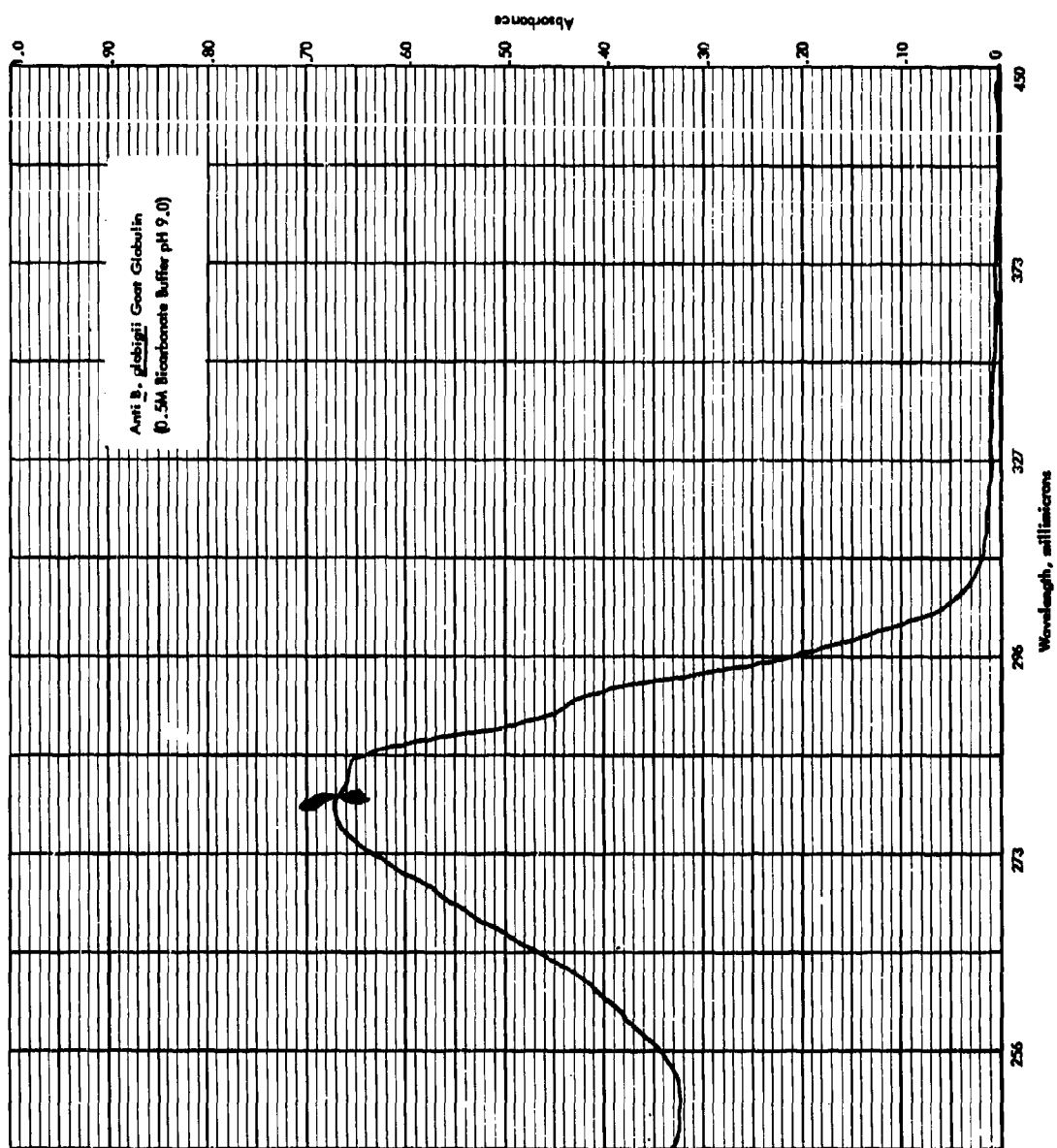


Figure 4-33. Absorbance of Anti *B. globigii* Goat Globulin

Peak-Height Ratios
Absorption Spectra of Collected Fractions
Hemin-Antibody Conjugate
2.6:1 Hemin:Globulin Molar Ratio

<u>Fraction</u>	<u>Peak Height Ratios</u> <u>(380 mμ:280 mμ hemin:globulin)</u>
#3	1:3.6
#4	1:3.0
#5	1:3.1
#6	1:3.3
#7	1:4.0
#8	1:4.0
#9	1:4.3
Avg	1:3.7

Attempted preparations of the conjugate in which hemin was considerably in excess (hemin: antibody of 26:1 and 260:1) gave somewhat erratic peak height ratios (of 1:1 to 1:16) with the hemin peak disproportionately large. The nature of the species formed under these conditions is not known.

Conjugation of goat anti-BG globulin with hemin chloride was repeated at a pH of 8.2 in phosphate buffer. The reaction was run exactly analogously to that at pH 9.0, except that the much lesser solubility of hemin at the lower pH required the conjugation to be run on a larger scale with subsequent reconcentration by ultrafiltration prior to Sephadex filtration. The eluate of the concentrate from the Sephadex column produced fractions with average peak height ratios of 1:4.1. Similarly, a ratio of 1:4.3 was obtained for the peak heights of another preparation of the conjugate (at pH 9.6) in which rabbit rather than goat BG antibody was used. In this preparation, a chemical analysis (nitrogen by micro-Kjeldahl, iron by colorimetric method) of a conjugate fraction indicated 0.044 percent nitrogen and 0.0007 percent Fe. This represents a combining ratio of approximately 7 moles hemin to 1 mole γ -globulin*. However, the molar ratio of hemin/protein calculated on the basis of the known absorbancies of hemin and globulin at their respective wavelengths

* Mol. Wt. hemin is 652 and 160,000 for γ -globulins; actual molar ratio is calculated to be 7.25/1.

gives a value of 2/1 for a peak height ratio. The presence of an unstable impurity* in hemin (absorbing at 267 m μ) was in evidence and may be responsible, at least in part, for this observed discrepancy in the molar combining ratios of hemin and protein.

4.1.4.9.3.2 STAINING WITH HEMIN CONJUGATE

A specified number of bacteria were filtered on tape, treated with hemin conjugate for 5 minutes at ambient temperature, and then washed with Difco buffer (pH 7.2) to remove excess conjugate. The tape containing the tagged bacteria was then immersed in luminol (pH 12.4)** to dissociate the complex and bring into solution the hemin moiety responsible for triggering luminol chemiluminescence. An aliquot was taken from the luminol solution for test by reacting with H₂O₂ solution. Variables studied included (a) relative staining efficiencies of hemin chloride versus hemin-tagged antibody and (b) preparation of bacteria. That is, BG vegetative cells processed as described above, except that the tagging step with hemin antibody was omitted, were compared with BG vegetative cells added directly to the luminol reagent mixture. This comparison would reveal if complete recovery of bacterial catalase is achieved by the Millipore route.

Run No.	Procedure	Bacteria	Treatment	Sample/Blank Luminescence Ratio
1	Millipore	3 x 10 ⁵ BG spores	None	1.3/1
2	"	"	Hemin Chloride	3/1
3	"	"	Hemin BG antibody	8/1
4	Suspension	3 x 10 ⁵ BG vegetative	None	2/1
5	Millipore	"	"	1.4/1, 1.7/1
6	"	"	Hemin Chloride	4/1
7	"	"	Hemin BG antibody	9/1, 11/1
8	Suspension	3 x 10 ⁵ SM	None	3/1
9	Millipore	"	"	1.7/1
10	"	"	Hemin Chloride	3/1
11	"	"	Hemin BG antibody	8/1

* Obtained by concentration.

** Reagent mixture A used (see Section 4.1.4.1).

- a. A comparison of runs 4 and 5, 8 and 9 indicates that some loss in activity is encountered by the Millipore procedure. This may be due to inactivation of the enzyme in processing or to failure to recover all the hemin by alkaline (pH 12.4) extraction.
- b. A comparison of runs 2 and 3, 6 and 7, 10 and 11 indicates that the hemin conjugate ostensibly stains more effectively than hemin chloride.
- c. The observation that SM also picks up the tag (although slightly less effectively than BG) may indicate either that conjugation of the antibody with hemin has altered its specificity, that the non-specific adsorption of the conjugate to the tape is accountable, or that the conjugate solution contains an insoluble component which is retained on the filter.

The effect of centrifugation in removing this postulated insoluble component is shown below. Two series were run, the difference between them being that the conjugate, previously stored at 10°C, was centrifuged (30 minutes at 8000 rpm at 4°C) just prior to staining for one of the series, while the other was not. The results follow.

<u>Bacteria</u>	<u>Treatment</u>	<u>Sample/Blank Luminescence Ratio</u>	
		<u>Uncentrifuged Conjugate</u>	<u>Centrifuged Conjugate</u>
3 x 10 ⁵ BG Spores	Hemin-BG antibody	8/1	5/1
None	Hemin-BG antibody	4/1	3/1
3 x 10 ⁵ BG Spores	None	1.6/1	1.6/1
3 x 10 ⁴ BG Spores	Hemin-BG antibody	7/1	4/1
None	Hemin-BG antibody	4/1	3/1

The data indicate that centrifugation has lowered the staining efficiency ostensibly by removing some insoluble hemin fraction. (DK-2 adsorption spectra of centrifuged and uncentrifuged conjugate appeared identical, however.) Non-specific adsorption of the hemin-tagged antibody is also apparent from the fact that the tape without bacteria treated with conjugate and washed still exhibits chemiluminescence when treated subsequently with luminol and H₂O₂. In summary, the data indicate that the sensitivity of detection by this method is of the order of 10⁵ bacteria and that approximately half of the observed signal is due to non-specific adsorption by the Millipore tape.

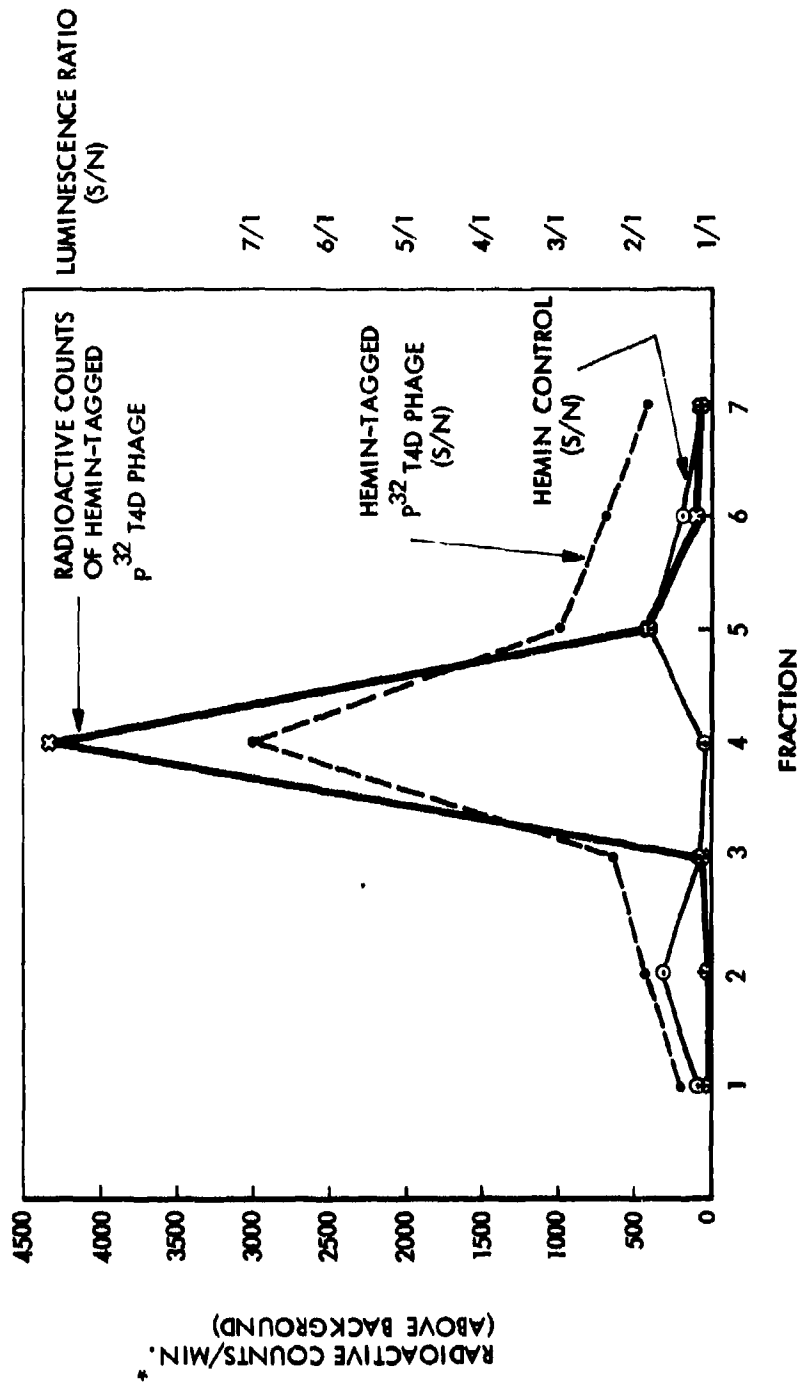
In comparing the technique of staining B. globigii with hemin-tagged BG antibody with that of direct staining with hemin chloride itself, it appeared that the latter technique would be simpler and provide the same threshold of detection of about 10^5 BG spores, (about 10^5 BG spores, 5-minute staining time). Specificity intended to be achieved by use of the antibody approach has not been realized, perhaps due to structural alteration of the antibody on conjugation with hemin. Based on these considerations, the emphasis was shifted to the investigation of direct staining of agent with hemin chloride.

4.1.4.9.3.3 DIRECT STAINING WITH HEMIN CHLORIDE

As with the iron-porphyrin conjugate, the method of tagging consists of treating the organism or (virus) carrier with hemin chloride, removing the excess hemin, and treating the stained agent with luminol and H_2O_2 to produce chemiluminescence. The intensity of luminescence is a linear function of the hemin concentration. The multi-agent capability of hemin tagging has been demonstrated for several bacteria (B. globigii, spores and vegetative; S. marcescens), virus carrier (embryonated egg and HeLa cells), and virus (NDV, T2 and T4 phage). The hemin-tagged agent produces the same rapid response (in seconds) as bacterial catalase on reaction with the luminol- H_2O_2 system. Some of the data obtained under static and flow (chemiluminescent detector breadboard) conditions are shown below:

<u>System</u>	<u>Agent</u>	<u>Sample</u>	<u>Luminescence Ratio (or Signal)</u>
Static	<u>B. globigii</u> spores	4×10^4 BG spores + hemin	4.6/1
		Hemin control (no spores)	1.3/1
Flow	<u>B. globigii</u> spores	1×10^4 hemin-tagged BG spores/ml*	6.7 (volts)
		Hemin control	1 (volt)
Static	NDV in allantoic fluid	$\sim 10^4$ LD ₅₀ hemin-tagged virus	6/1
		Untagged virus (control)	1/1
		Hemin (control)	1.2/1
Static	NDV in Hanks medium	$\sim 10^5$ LD ₅₀ hemin-tagged virus	7/1
		(13-day) Allantoic fluid + Hemin control	1/1

*Luminol flow rate 0.36 ml/min; U-tube reactor used in detector.



* ADJUSTED TO 10 μ l ALIQUOT

Figure 4-34. Sephadex Filtration of Hemin-Tagged P^{32} T₄ Phage

<u>System</u>	<u>Agent</u>	<u>Sample</u>	<u>Luminescence Ratio (or Signal)</u>
Flow	NDV in allantoic fluid	$\sim 10^4$ LD ₅₀ hemin-tagged virus/ml	1600 (mv)
		Allantoic fluid + Hemin (control)	30 (mv)
Static	T4 phage	3×10^7 PFU-hemin tagged phage	7/1
		Hemin control (no phage)	1.1/1

Two different techniques were actually used for tagging bacteria and viruses: (1) Bacteria were filtered on Millipore tape, treated with hemin in bicarbonate buffer (pH 9) for 5 minutes at ambient, washed to remove excess hemin, treated with alkaline luminol (pH 12.4) to solubilize the hemin tag, and the luminol extract was reacted with H_2O_2 to produce chemiluminescence. (2) A different technique had to be employed for separating the excess hemin from the stained virus. After treating the virus suspension with hemin in bicarbonate buffer, the mixture was centrifuged and washed to remove most of the hemin**, then filtered through Sephadex gel to remove the last traces of hemin. (Liquid partition may provide an effective substitute for the centrifugation step. Preliminary experiments on the partitioning efficiency of PVA-dextran solutions toward hemin indicated a distribution ratio of about 5/1 in favor of the PVA phase.)

Striking evidence that the agent is indeed being tagged with hemin and that the stained agent can be separated effectively by the Sephadex treatment is shown by Figure 4-34. The results shown plotted were obtained on a single sample of P^{32} T4 phage which had been tagged with hemin and subjected to the centrifugation and Sephadex treatment. Each of the fractions eluting from the Sephadex was then checked for radioactive count and luminol chemiluminescence (S/N). A hemin control (no T4 phage) is shown for comparison. It is evident that a fairly sharp separation occurs with both the maximum radioactivity and luminescence showing up in Fraction 4. Recovery efficiencies of hemin stained P^{32} T4 phage and hemin tagged BG spores through a Sephadex column were found to

* Luminol flow rate of 0.36 ml/min; bulb-type reactor used in detector.

** Hemin tagged virus forms a dark pellet on centrifuging which is visible.

be 83 and 93 percent, respectively. With 6 and 10 ml Sephadex columns, the holdup time required for complete elution of the hemin tagged agent was 5 to 8 minutes. The hemin-tagged virus which appears in Fraction 4 was diluted further and an aliquot taken for treatment with luminol- H_2O_2 in the conventional manner. Controls were also run using untagged virus, hemin alone, and allantoin fluid treated with hemin chloride, where applicable. The actual number or NDV particles in a test sample was determined from the experimental observations that $1 \text{ HA} = 4 \times 10^5 \text{ LD}_{50} \text{ NDV}$, and that hemin-tagging does not affect the hemagglutinating properties of the virus.

The results shown in the table above indicate that 10^4 LD_{50} viral (NDV) particles or 10^4 BG spores can be readily detected by direct tagging with hemin chloride. Although 3×10^7 PFU of $T4^*$ phage do absorb hemin sufficiently to permit detection over a hemin control, it was found that 1×10^6 PFU of $T4$ could not be detected. The higher efficiency of hemin adsorption exhibited by NDV (10^4 LD_{50} units) compared to $T4$ phage may be due to its higher lipid content or to the fact that dead or non-infectious NDV particles (not appearing in the LD_{50} count) are also contributing to hemin adsorption. Lipoproteins have been reported to have a strong affinity for protoporphyrins^(7,8). $T4$ phage are virtually free of lipids. It was also demonstrated by the data below that hemin tagging of embryonated egg, B. globigii (vegetative) or S. marcescens increased the respective chemiluminescent signals by about two- or three-fold (in a static system).

	Luminescence Ratio**
Hemin Treated Embryonated Egg	50/1
Embryonated Egg (control)	8/1
Hemin (control)	8/1
3×10^5 <u>B. globigii</u> (veg)	1.6/1
Hemin treated BG	4/1
3×10^5 <u>S. marcescens</u>	1.7/1
Hemin treated SM	3/1

* Radioactive P^{32} $T4$ phage used for hemin tagging to facilitate counting of phage in sample.

** Reagent mixture A used (see Section 4.1.4.1).

4.1.4.9.4 CONCLUSIONS

The feasibility of utilizing direct tagging with hemin chloride as a means of detecting virus and (BG) spores by luminol-chemiluminescence at the desired sensitivity has been demonstrated. Hemin tagging also serves to increase by several-fold the innate capacity of vegetative bacteria and (virus) carrier to give a chemiluminescent signal with luminol- H_2O_2 .

4.1.4.9.5 REFERENCES

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4.1.4.10 SIALORESPONSIN RELEASE AS AN EARLY DETECTION SCHEME OF VIRUS INFECTION

4.1.4.10.1 SUMMARY

Approximately 4000 hemagglutinin units of Newcastle disease virus (corresponding to approximately 10^8 LD₅₀ infectious NDV particles) was detectable within 15 minutes after infection. A colorimetric assay method for neuraminic acid was utilized. Higher sensitivities for this method are cited by another investigator (Bogoch) for influenza PR8. A C¹⁴ tracer assay method is discussed which is capable of detecting 0.01 to 0.1 HA unit.

4.1.4.10.2 INTRODUCTION

It was first shown by Bogoch⁽¹⁻⁴⁾ that sialoresponsin, a glycosidically linked sialic acid, appears in the chorioallantoic fluid during the first few minutes of influenza PR8 virus infection in vitro as well as in vivo. The almost instantaneous release of sialoresponsin by the host cell is believed to represent the earliest defense mechanism of the host against the presence of virus in the extracellular fluid. This substance(s) is distinguished from free neuraminic (sialic) acid, as liberated by neuraminidase (sialidase), since it is bound (presumably through its aldehyde group in a glycosidic linkage), and is distinguished from interferon by its content of neuraminic acid and by the time of its appearance. Since sialoresponsin is a material which is produced very early in infection, 5 to 15 minutes after contact with the host, monitoring of this material should provide a useful early and quantitative method for the detection of microbial pathogens.

For convenience, a colorimetric assay technique was used by Bogoch for defining the basic parameters related to sialoresponsin production. Thus, an S-shaped dose-response curve was observed for the quantity of sialoresponsin produced in vitro with increasing titers of influenza PR8 virus, the yield of sialoresponsin being always larger in vivo than in vitro. Prior heat treatment of influenza PR8 virus at temperatures up to 100°C for 30 minutes did not appear to inactivate influenza PR8 virus in terms of its ability to produce

sialoresponsin. Specific antiserum neutralization of virus did not inhibit sialoresponsin production. Chorioallantoic membrane per se was not sufficient for the production of sialoresponsin. Preliminary experiments indicated that cytoplasmic particles in chorioallantoic fluid may be involved in the production of sialoresponsin. The presence of added free sialic acid in vitro increased the amount of sialoresponsin produced. Based on these initial findings by Bogoch, a study of the applicability of this phenomenon to another myxovirus, Newcastle Disease Virus (NDV), was initiated at Space-General.

4.1.4.10.3 STATUS

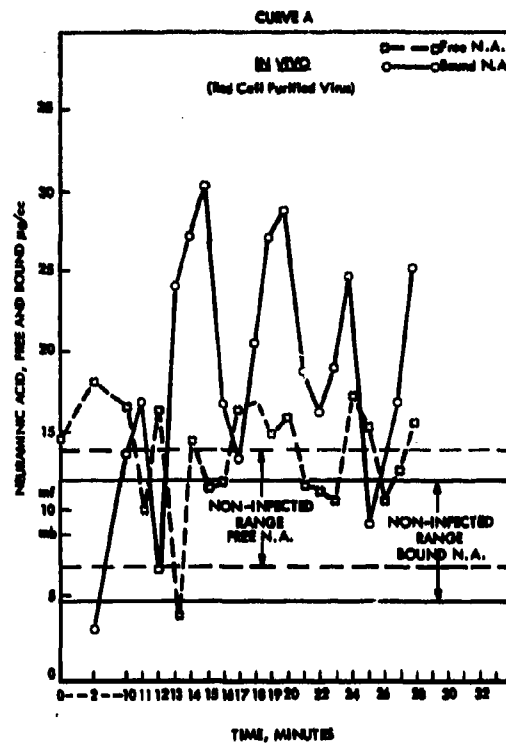
The assay of sialoresponsin is based on determining the bound neuraminic acid hydrolytically liberated from glycosidic binding. This parameter is obtained by subtracting the free neuraminic acid (due to virus neuraminidase action) from the total neuraminic acid (obtained by hydrolysis with 0.1N sulfuric acid at 80°C for 1 hour.) The method of Waravdekar and Saslow⁽⁵⁾ as modified by Warren⁽⁶⁾, known to be highly quantitative and specific for free neuraminic acid, was used for analysis. In this method the liberated neuraminic acid is oxidized with sodium periodate in concentrated phosphoric acid, and the oxidation product is coupled with thiobarbituric acid to form a chromophore. The latter is then extracted with cyclohexanone and its absorption is measured at 549 mμ (peak adsorption of neuraminic acid chromophore).

In an actual determination, the chorioallantoic cavities of intact 11-day-old chick embryos were inoculated with 0.2 ml of a PR8 strain of influenza virus with an HA titer of about 10,000 units per ml. The eggs were then sealed with collodion, inverted several times to mix the fluid, and maintained at 37°C until time for sampling. The eggs were sampled by removing 1.5 to 2.5 cc of allantoic fluid and analyzing for total neuraminic acid and free neuraminic acid. Appropriate controls which may consist of uninjected eggs, eggs injected with normal saline, and eggs injected with normal uninfected chorioallantoic fluid are sampled at the same time intervals as for virus-injected eggs.

The results obtained by Dr. Bogoch with influenza PR8* may be compared with those obtained with NDV at Space-General. Bogoch found that the total neuraminic acid (NA) (bound + free) begins to show significant increases above a control within 15 minutes after virus infection. This increase in the total NA is due not only to the presence of more free neuraminic acid, expected due to previous neuraminidase action, but to the presence of greater amounts of bound neuraminic acid. The results of Dr. Bogoch shown plotted in Figure 4-35⁽⁷⁾ indicate that the concentration of the bound NA shows an initial increase above the mean value for controls (non-infected eggs) 10 minutes after infection by virus. Free NA also shows periodic increases over the control. This is to be expected due to the action of viral neuraminidase which cleaves free neuraminic acid from sialoresponsin as the latter appears in the fluid, with a resultant increase in the level of free NA. It is noteworthy that the free NA does not accumulate. It has been suggested by Bogoch that the free NA is either hydrolysed by an as yet unidentified neuraminic acid hydrolase or it must be transported from the fluid into the chorioallantoic membrane, perhaps by the same system that is involved in transport of virus from the fluid into the membrane ("eclipse phase"⁽⁸⁾).

Two sets of data obtained at Space-General with NDV are shown below. The first set was obtained on injection of 0.2 cc NDV suspension (2048 hemmagglutinin units per 0.2 cc), the second set by injection of 0.2 cc NDV containing twice the concentration (i.e., 4096 hemagglutinin units per 0.2 cc). Based on the experimental observation in our laboratories that one hemagglutinin unit equals 4×10^5 LD₅₀ NDV units, this would mean that approximately 8×10^8 and 1.6×10^9 LD₅₀ NDV units respectively, were used in each set to infect each of the eggs. Since there is approximately 6 to 8 ml of allantoic fluid in an egg, this would correspond to an approximate virus concentration of 10^8 LD₅₀ NDV units/ml of allantoic fluid.

* The results reported for influenza PR8 were performed by Dr. Samuel Bogoch, et al., at the Foundation for Research on the Nervous System, and Division of Psychiatry, Boston University School of Medicine.



Legend for Curve A - The Production of Sialorespsin in Vivo.

The amount of sialorespsin (in microgram/cc of bound neuraminic acid) produced with red cell purified influenza PR8 virus, 0.2 cc of virus per egg, HA titer 10,240 HA units/cc, injected into characteristic 50s of 14 day old eggs at time 0. min indicates the mean for bound neuraminic acid/cc and of the mean for free neuraminic acid/cc, in non-infected eggs. The limits of this range for these normal values are shown by horizontal solid and broken lines.

Figure 4-35. The Production of Sialorespsin in vivo

Effect of NDV Infection on
Chorioallantoic Fluid

Experiment A-2048 hemagglutinin units NDV in 0.2 cc injection

Concentration $\mu\text{g/ml}$ allantoic fluid

Time After Injection (min)	Infected			Uninfected (control)*		
	Total NA	Free NA	Bound NA	Total NA	Free NA	Bound NA
0	14	14	0	14	14	0
5	17	7.2	9.8	14	7.2	6.8
15	15	9.6	5.4	12	4.8	8.4

Experiment B-4096 hemagglutinin units NDV in 0.2 cc injection

0	7.2	2.4	4.8	7.2	2.4	4.8
5	18	11	7.2	**	7.2	-
15	20	14	6.0	11	2.4	8.4
25	4.8	0	4.8	3.6	0	3.6

A comparison of the two sets of data indicate that in both sets the concentration of total neuraminic acid is higher for the infected egg compared to that of the control. While this is not the case for the bound NA in the second set, the fact that the free NA concentration is indeed higher for the infected egg suggests that viral neuraminidase had cleaved free neuraminic acid from the sialoresponsin during processing of the sample. A modification suggested by Bogoch⁽⁷⁾ to inactivate the neuraminidase and minimize this effect entails a chloroform extraction on the allantoic fluid as it is removed from the egg prior to periodate oxidation.

* Control injection of 0.2 cc uninfected allantoic fluid
** Sample lost

Although additional tests are required to establish the magnitude of this effect, these preliminary results suggest that NDV behaves in a similar manner to influenza PR8 virus with respect to production of sialoresponsin in vivo. Approximately 2000 and 4000 hemagglutinin units of influenza PR8 and NDV, respectively, are required to produce a really significant signal over that of a control by this colorimetric method. This was previously estimated to be approximately 10^8 LD₅₀ infectious NDV particles based on the experimentally determined relation one hemagglutinin unit = 4×10^5 LD₅₀ NDV. The number of influenza viral particles in 1 HA unit is believed to be several orders of magnitude less, being variously reported as 1 to 100; thus 2000 HA influenza PR8 = 2×10^3 to 2×10^5 influenza PR8 particles.

Aside from the complexity of the method, the colorimetric procedure for neuraminic acid possesses marginal sensitivity, at best. The observation by Bogoch, that the presence of extra free sialic acid leads to the increased synthesis of sialoresponsin, suggests that the addition of C¹⁴-labelled free sialic acid to a medium in which sialoresponsin is being produced may provide a means of incorporating synthetically a C¹⁴ label into the sialic acid portion of the sialoresponsin molecule. This is based on the assumption that the added free sialic acid is utilized in production of sialoresponsin. The next step would involve separation of the tagged sialoresponsin from excess free sialic acid prior to measuring the radioactivity of the former. Some initial success has been achieved in separating free sialic acid from sialoresponsin on a Dowex-1-formate column⁽¹⁰⁾. An estimation of the ultimate sensitivity of the radiotracer method can be made based on the following considerations. C¹⁴-labelled glucose of specific activity of 180 millicuries per millimole is commercially available. Since one mole of glucose is incorporated into one mole of sialic acid, a specific activity of 180 mc/mole for sialic acid is possible. Since about 2000 HA units of PR8 influenza virus will form a maximum of 15 µg of bound neuraminic acid, the latter amount would correspond to 8.7×10^{-3} mc of C¹⁴. This corresponds to 1.9×10^7 dpm. For a liquid scintillation counter, 90 percent efficient, this is equal to 1.7×10^7 cpm; for a Geiger-counter chromatogram scanner, 10 percent efficient, 1.9×10^6 cpm. Assuming that 100 cpm is needed for a distinct signal, one should be able to detect 0.01 HA units with the liquid scintillation counter and 0.1 HA units with the chromatogram scanner.

4.1.4.10.4 CONCLUSIONS

Monitoring of sialoresponsin production appears to offer promise as a detection scheme for early viral infection (within 15 minutes after infection). All the work to date has been done with two myxoviruses, influenza PR8 and NDV. Additional work is needed with other virus groups (arboviruses) to determine whether this is a limited or general phenomenon. If the latter is shown to be true, a micro-assay technique for sialoresponsin would have to be developed with greater sensitivity than the marginal thiobarbituric acid (colorimetric) method currently being used.

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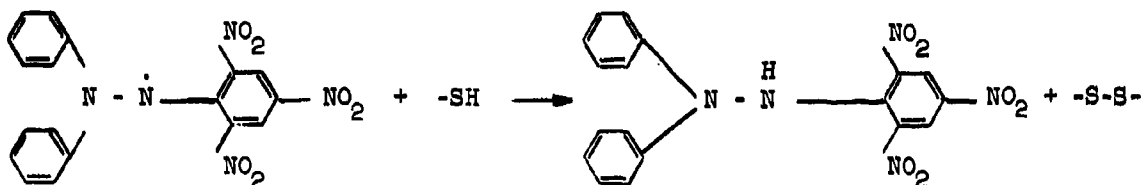
4.1.4.11 QUENCHING OF LUMINESCENCE

4.1.4.11.1 SUMMARY

Sulfhydryl groups in bacteria can decrease chemiluminescence of a luminol-diphenyl picrylhydrazyl system. This principle was used to detect 10^4 bacteria. Objection has been raised that this system lacks specificity, insofar as components other than sulfhydryl groups may cause quenching of the DPPH-luminol chemiluminescence. These efforts were terminated.

4.1.4.11.2 INTRODUCTION

It was observed in these laboratories that 2, 2-diphenyl-1-picrylhydrazyl (DPPH), a stable organic radical, will cause luminol to chemiluminesce, the intensity of luminescence being a function of the free radical concentration. This free radical is also known to react quantitatively with sulfhydryl groups ($-SH$)^(1,2), in accordance with the following:



Thus, the introduction of microorganisms, with their attendant $-SH$ groups on the surface, should inactivate some of the DPPH with a resulting decrease in the intensity of luminescence.

4.1.4.11.3 STATUS

An evaluation of this concept was made using the photomultiplier arrangement (static) described previously in connection with the bacterial porphyrin-luminol system. The procedure consists of reacting a known concentration of DPPH with a bacterial suspension for 3 minutes at ambient temperature. An aliquot is withdrawn with a hypodermic syringe and injected rapidly

into a reaction tube containing the luminol*. The intensity of the light produced is compared with that from a control mixture containing no bacteria. Important variables are order of addition of ingredients and rate of mixing. Greater light intensities are achieved by rapid addition of a mixture of DPPH and bacteria to the luminol rather than the reverse. The sensitivity of the method depends on the limit of detection of the DPPH. Under present conditions, 0.8×10^{-11} mols of DPPH produce detectable chemiluminescence. Blanks are run concurrently with the sample because significant decomposition of the DPPH solution (1:1 ethanol-water) occurs even on standing for 24 hours at about 10°C. Thus the intensity of the chemiluminescence produced by 1.5×10^{-11} mols DPPH decreased to about 1/6 of its value under these conditions. For maximum sensitivity, runs are made at the threshold of detection of the DPPH at the maximum gain setting of the instrument. In a sample run, 50 µl of suspended B. globigii (1.3×10^8 viable vegetative cells/ml) are mixed with 400 µl DPPH solution (1.34×10^{-9} mols DPPH per ml 1:1 ethanol-water) and permitted to react for 3 minutes at ambient temperature. An aliquot is withdrawn (40 µl containing 6×10^5 B. globigii) and injected into 0.25 ml luminol (0.2 mg luminol per ml in 0.1N NaOH) and the intensity of the light emission recorded. A blank is run with 50 µl sterile water and 400 µl DPPH solution from which a 40 µl aliquot is withdrawn and injected into the luminol. The light intensity dropped from 8 units to 2 units for the sample containing the bacteria. Similar tests with E. coli indicated a lower sensitivity.

The sensitivity of the method, which is limited by the sensitivity of the photomultiplier tube to detect the emitted light, was increased by pre-immersion of the tube (RCA 1P21) in liquid nitrogen for at least 8 hours prior to use⁽³⁾. The instrumentation used for measuring the light intensity was identical to that used for the luminol bacterial detection. A comparison of the results obtained is tabulated below and a record of one comparison is plotted in Figure 4-36. The figures in the last column below represent the ratio of the light intensities of the DPPH + luminol mixture and of DPPH + luminol + bacteria.

* Luminol did not contain EDTA



Figure 4-36. Light Traces for DPH-Iuminol Reaction (Static System)

	Number of Bacteria (BG)	Luminescence Ratio, Without Bacteria/With Bacteria
Previous Results	6×10^5	8/2
Current Results	3×10^5	24/~ 0 (Figure 4-36)
	5×10^4	10/3
	3×10^4	8/4

The data indicate that, at the level of 3×10^4 B. globigii (viable, vegetative), the light intensity is reduced by about 50 percent. Sonication, and the use of protein denaturants (i.e., guanidine hydrochloride⁽³⁾), might expose an even greater concentration of sulfhydryl groups and improve sensitivity.

4.1.4.11.4 CONCLUSIONS

Although possessing a demonstrated sensitivity of the order of 10^4 bacteria, an objection has been raised that this system lacks specificity insofar as atmospheric components other than sulfhydryl groups may cause quenching of the DPPH-luminol chemiluminescence. Consequently, work on this system was discontinued.

4.1.4.11.5 REFERENCES

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4.1.4.12 FLUORESCENCE QUENCHING OF EOSIN Y BY PROTEIN

4.1.4.12.1 SUMMARY

A method which involves quenching of Eosin Y fluorescence by protein was used to detect 10^5 bacteria/ml. It was not studied further because of inherent disadvantages.

4.1.4.12.2 DISCUSSION

At the proper pH, protein is known to quench the fluorescence of certain dyes. The method utilizing Eosin Y as the fluorescent dye, has been successfully used for measuring μ quantities of protein-nitrogen in partially purified proteins and protein in rat tissue⁽¹⁾. Based on the reported sensitivity, 10^4 bacteria should be readily detectable by this method.

The procedure, modified for use with bacteria, consists of extracting the protein by aqueous caustic, neutralizing with acid, centrifuging, and then adding an aliquot of the clear supernatant to a phosphate-buffered solution of Eosin Y (pH 3.2). The fluorescence is measured after 10 minutes with a Photovolt Fluorimeter using primary and secondary interference filters (Filtraflex-B) at 517 and 540 μ , respectively. A control similarly processed without bacteria serves as a blank. The results of several runs using a 2 ml reaction volume are shown below.

Number of Viable (veg) <i>B. globigii</i> /ml	Fluorescence, Arbitrary Units			
	Control (C)	Sample (S)	C-S	% Decrease
5.0×10^4	461	400	61	13
5.0×10^4	739	666	73	10
3.5×10^5	469	412	57	12

Variations in the fluorescence of the control are related to the freshness of Eosin Y solution. The controls were run at the same time as the sample. The data indicate that a bacterial concentration (viable, vegetative BG) of approximately 10^5 /ml will produce a decrease in Eosin Y fluorescence of about 12 per cent. In the last run shown, an attempt was made to measure the difference in fluorescence of the control and sample using an 0.025 μ l cuvette

instead of the 2 ml normally used. The results shown below indicate that although the percentage decrease in fluorescence is of the expected magnitude, the absolute difference is almost insignificant. However, since signal amplification can be utilized, this does not present a problem.

Number of Viable Bacteria in 25 μ l volume	Fluorescence, Arbitrary Units			
	Control (C)	Sample (S)	C-S	% Decrease
9×10^3	16	14.5	1.5	9.4

4.1.4.12.3 CONCLUSIONS

The data indicate that although a total of 10^4 B. globigii (vegetative, viable) would be required to produce a detectable signal, the actual concentration required would be of the order of 10^4 to 10^5 /ml. While a more systematic study of process variables might produce the required sensitivity, an inherent disadvantage of the method is its complexity and long processing time required (minimum of 35 minutes). Consequently, further work on this method was suspended in favor of other techniques which appeared more promising.

4.1.4.12.4 REFERENCES

- (1) Kiraka, T. and Glick, D., Anal. Biochem. 5: 497-504, 1963.

4.1.4.13 PHOSPHORESCENCE OF PROTEINS

4.1.4.13.1 SUMMARY

Phosphorescence of proteins excited by ultraviolet light at liquid nitrogen temperature was used for detection of 10^6 B. globigii (viable, vegetative). Further studies are needed to determine effects of impurities on background, sensitivity, and multi-agent capability of this principle.

4.1.4.13.2 DISCUSSION

It has been reported that many proteins (egg albumin, γ_2 -globulin, etc.) and protein-containing materials (i.e., E. coli) will emit blue phosphorescence under ultraviolet excitation at low temperatures (77 to 193°K)⁽¹⁾. There are two components to the phosphorescence, one involving an exponential decay (mean lifetime about 3 seconds) and the other of much longer duration. The former is attributed to tyrosine, tryptophane, and possibly phenylalanine. It is pH-dependent, insensitive to temperature, and the excited phosphorescent state is the lowest-lying triplet level. The protein phosphorescence of long duration which accompanies the exponential decay phosphorescence, and which is attributed to certain forms of the aromatic amino acids, is also pH-dependent, highly sensitive to temperature, and is believed related to the denaturation of proteins by UV light. Besides providing a very sensitive detection scheme for proteins (10^{-9} g of tyrosine give a discernable phosphorescence), the possible relation between protein phosphorescence of long duration and protein denaturation may provide a test for viability. With this method non-bacterial matter should provide less of a background problem since there are not many materials which will exhibit the phosphorescence characteristics of protein (protein phosphorescence appears at about 400 to 480 m μ , depending on pH).

In an evaluation of this concept, samples of albumin (solid and in solution) and B. globigii contained in sealed capillary tubes (soft glass) were irradiated for 1 minute with a Pen-Ray Quartz UV Lamp* while immersed in liquid nitrogen. Phosphorescence detectable by the naked eye was observed in each case. The results are summarized below.

Solid egg albumin (3×10^{-4} g) - Bright phosphorescence lasts ~10 to 20 seconds depending on whether sample is removed or kept immersed in liquid N₂ after irradiation.

* Ultra Violet Products, Inc., San Gabriel, California

Aqueous solution ovalbumin - Bright phosphorescence lasts ~ 20-25 seconds
(4×10^{-6} g ovalbumin
/2 μ l solution)

Aqueous solution ovalbumin - Faint phosphorescence lasts ~ 10-20 seconds
(6×10^{-7} g ovalbumin
/3 μ l solution)

B. globigii suspension - Faint phosphorescence lasts ~ 2-4 seconds
(Compacted by centri-
fugation, 10^6 bacteria)

4.1.4.13.3 CONCLUSIONS

This preliminary data would indicate a sensitivity of the order of 10^6 bacteria (viable BG vegetative) provided that the observed phosphorescence was not due to trace contamination by nutrient or agar. Higher sensitivities might be achieved by use of an all-quartz container for the bacteria to permit passage of the 254 m μ Hg line. The latter was recently used for initiating phosphorescence on paper of L-tyrosine, phenylalanine and other aromatic ring structures at liquid nitrogen temperature⁽²⁾. Further studies would be required to establish the sensitivity and multiagent capability of this method.

4.1.4.13.4 REFERENCES

- (1) Debye, P. and Edwards, J.O., Science, 116: 143, 1952.
- (2) Gordon, M.P. and South, D., J of Chrom. 10: 513, 1963.

4.1.5 RADIOTAGGED ANTIBODY METHODS

4.1.5.1 SUMMARY

Antibody preparations have been successfully labelled with S^{35} -sulfanilic acid. Initial experimental data have been obtained on some of the properties of these preparations, their reaction with specific antigen, and level of non-specific adsorption to a microporous Millipore filter tape. It was determined that ~ 180 antibody molecules attach per Bacillus globigii spore per minute and that there were at least an average of 41,000 antigenic sites per organism. Preliminary experiments showed as little as 0.014 percent non-specific adsorption of antibodies to a microporous filter tape. It was estimated that at least 10^4 B. globigii could be detected with S^{35} -labelled antibody within a 5-minute period at a 2 to 1 signal-to-noise ratio.

4.1.5.2 INTRODUCTION

Detection methods utilizing an isotope labelled antibody are based on reaction of the agent to be detected (the antigen) with labelled antibody, effective separation of unreacted antibody from the antigen-antibody complexes, and measurement of the radiation emitted from the complexes.

The present status of knowledge based on literature studies and experimental data received from in-house research indicates that the use of isotope labelled antibody can result in an extremely sensitive, specific, rapid, and reliable detection method which can be readily adaptable to automation. Furthermore, it would appear that atmospheric background radiation will probably not result in any significant interference⁽¹⁾.

The use of S^{35} sulfanilic acid as a tag for antibody preparations provides this detection method with a simple and convenient labelling process. The long-half-life isotope allows for storage for reasonable time, and high specific activity can be obtained. Other factors which favor the use of S^{35} sulfanilic acid are that S^{35} , being a weak beta emitter, may have less effect on alteration of antibody reactivity, and that its use in an automated system will require much less shielding than will use of a γ -emitting isotope.

Although other isotopes were considered, S^{35} sulfanilic acid seems at the present time to be the most feasible tag for this system.

4.1.5.3 STATUS

A theoretical estimation of the detection sensitivity of this method was calculated. This estimation was based on the theoretical values for the number of S^{35} atoms obtainable per antibody and the number of antibodies which can be attached per organism within a given period of time. Experimental data obtained by Dr. J. Garvey (personal communication) suggested that at least 40 S^{35} atoms, as the diazonium salt of sulfanilic acid, could be obtained per antibody without significant alteration in the properties of the antibody. However, practical considerations on the daily radioactivity requirements for a continuous detection system would appear to limit this value to between 10 and 20 S^{35} atoms. Estimates of the number of antibody molecules attainable per organism have ranged from 5000 to over 100,000. Recent experimental evidence indicates that it would not be unreasonable to assume that between 5000 and 10,000 antibodies containing 10 S^{35} atoms per molecule can be obtained per organism within 5 minutes. If 5000 antibody molecules were attached per bacterium, 2,800 dpm would result from 10^4 bacteria. If a liquid scintillation counting system were employed, over 90 percent of the dpm would be counted. It would therefore appear that 10^4 organisms can be theoretically detected by this method.

Experimental work thus far has been mainly concerned with preparation of S^{35} -labelled antibody and obtaining some initial data on some of its properties, its reaction with specific antigen, and level of non-specific adsorption (NSA) to a Millipore filter membrane.

After two previous unsuccessful attempts by International Chemical and Nuclear Corporation to prepare an S^{35} -labelled, anti-Bacillus globigii gamma globulin⁽²⁾, an antibody preparation was successfully labelled at SGC using procedures similar to those of Campbell, et al⁽³⁾. In this procedure, S^{35} sulfanilic acid is prepared from $H_2 S^{35}O_4$ and diazotized and coupled to the tyrosyl and histidyl groups of the antibody globulin. The labelled preparation is purified by dialysis against borate buffered saline (pH 7.8).

For the present experiments, antibody was labelled only with a trace amount of S^{35} isotope to establish more readily the basic procedures. A final preparation was obtained in which there was an average of 120 total S atoms and 0.002 S^{35} atoms per antibody molecule.

Preliminary studies were conducted to determine the kinetics of reaction of labelled antibody with B. globigii spores and to obtain an estimate of the number of antigenic sites per organism. In these studies $\sim 9 \times 10^8$ bact/ml were reacted with one or several different concentrations of antibody at room temperature. After specified periods of time, the spores were removed from reaction by centrifugation, washed 3X in buffer, and radioactivity was measured on dried aliquots with a gas-flow proportional counter. Controls were run in an identical manner with Serratia marcescens to determine the amount of antibody which was adsorbed non-specifically or was occluded in the spore pellet. These values were subtracted from those obtained with the BG spores. Figure 4-37 represents the increase in multiplicity of attachment of antibodies per BG spore with time when a spore concentration of 9.1×10^8 /ml was reacted with ~ 0.7 mg/ml of labelled gamma globulin containing B. globigii antibody.

It can be concluded from these data that the rate of adsorption of antibody molecules to B. globigii spores is 180 antibodies/spore/min and that there are at least 41,000 reactive antigenic sites per B. globigii spore. It is hoped that the rate of reaction can be significantly increased by decreasing the total number of sulfur atoms per antibody molecule and by the use of higher titered antisera.

The following table represents the multiplicity of attachment versus time when the antibody concentration was varied.

ATTACHMENT OF ANTIBODIES TO B. GLOBIGII SPORES

Globulin Concentration (μ g/ml)	730	73	7.3
Reaction Time (min)	Number of Antibody Molecules/Spore		
15	4200	880	160
30	8000	2300	560

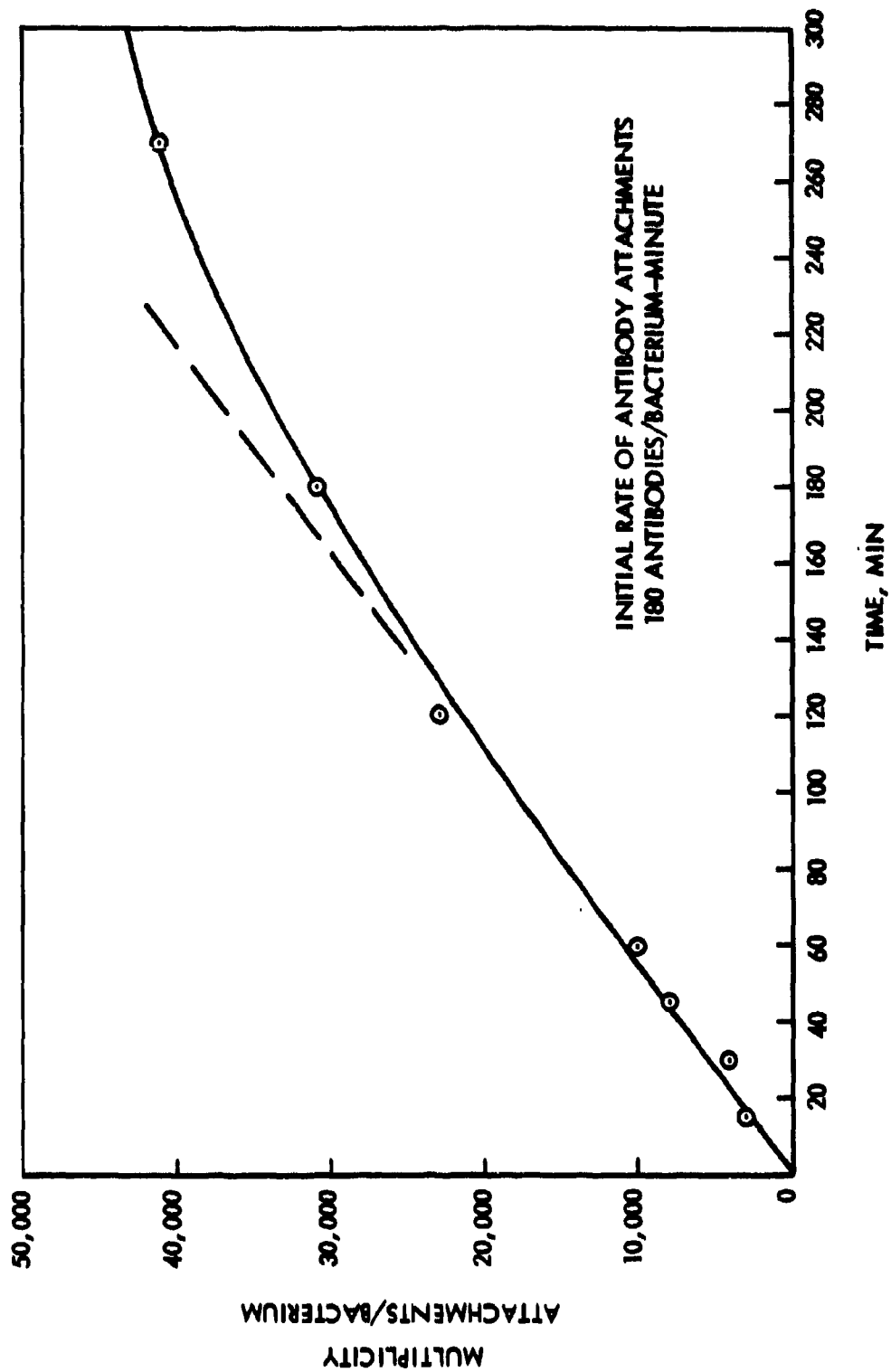


Figure 4-37. Multiplicity of Antibody Attachment per
B. globigii Spore versus Time

These results indicate that the rate of attachment and multiplicity are not proportional to the first power of the antibody concentration.

Initial experiments were also conducted to determine the amount of non-specific adsorption (NSA) of labelled antibody to a 0.45μ Millipore filter membrane. In these studies 20 ml of phosphate buffered saline (PBS) (pH = 7.2) containing 0.1 percent gelatin was first passed through a 0.45μ Millipore filter, followed by 50 ml of the same buffer containing 0.5 ml of a 1/100 dilution of labelled antibody. The filter was washed with 40 ml of the buffer solution and radioactivity on the filter determined. It was calculated that 15 percent of the total globulin was retained on the filter.

The above experiment was repeated with some alterations in procedure. A borate buffered saline (pH 7.6) containing 0.02 percent of Tween 80 was used instead of the PBS-gelatin media and the globulin was prefiltered prior to use. Pretreatment was accomplished by passing 5 ml of a 1/100 dilution of globulin (0.8 mg/ml) through a Millipore filter which was previously pretreated with 20 ml of a borate buffered saline-Tween 80 solution. NSA in this instance was 0.014 percent.

The above data afford some preliminary estimates on the sensitivity of the method. As indicated from Figure 4-37, 540 antibodies can be experimentally obtained per BG spore in about 3 minutes. With 10 S^{35} atoms per antibody molecule, 10^4 organisms would emit ~ 300 cpm. Normal background plus that due to NSA for unattached antibody (0.014 percent) would be about 150 cpm. A 2 to 1 signal-to-noise ratio thus appears to be experimentally obtainable. At a counting efficiency of 50 percent it would appear that it is presently experimentally possible to detect 10^4 BG spores. It is expected that the number of antibodies obtainable per organism can be increased by about tenfold by improvement in the labelled antibody preparation.

Initial experiments were conducted with presently available radioactive detection instrumentation constructed at SGC to demonstrate the general feasibility of continuously detecting labelled antibody-organism complexes on porous tape. In these experiments BG spores were reacted with S^{35} labelled antibody (prepared as indicated above), washed by centrifugation, and filtered.

through a moving porous Millipore filter tape (0.45 μ). A preparation containing 2.5×10^9 BG spores with $\sim 10,000$ antibodies per spore and 0.002 S^{35} atoms per antibody resulted in a significantly higher signal ($\approx 12X$) than that obtained from a similar preparation containing S. marcescens.

4.1.5.4 CONCLUSIONS

It has been experimentally demonstrated that an S^{35} -tagged antibody preparation can probably be used to detect at least 10^4 BG spores. This level of sensitivity, along with advantages in specificity, multi-agent capability, virus detection, and relatively low background interference from atmospheric sources, would appear to rank this method as one of the better ones for BW detection. It is recommended that experimental efforts should be continued on the development and application of this method for automated BW detection.

Improvement in detection sensitivity is dependent on improvement of reaction rate and decreased NSA of antibody to the porous tape. Improvement in reaction rate will be accomplished by use of higher titered and purer antibody preparations with optimum S^{35} / antibody ratios. Reduction of NSA to porous tape may be accomplished by appropriate alteration in the antibody preparation, filter properties, and filtration procedures, along with the use of instrumentation now under development, such as the sonicator-washer or liquid partition separator, for removal of uncomplexed antibody prior to filtration.

4.1.5.5 REFERENCES

- (1) U. S. Dept. of Health, Education and Welfare, "The Air Pollution Measurements of the National Air Sampling Network, Analyses of Suspended Particles, 1955-1961".
- (2) Status Reports 19 and 20, Research Program on BW Detection.
- (3) Campbell, D. H., Garvey, J. S., Cremer, N. E. and Susdorf, D. H., "Methods of Immunology", W. A. Benjamin, N. Y., 1963.

4.1.6 NUCLEIC ACID METHODS

4.1.6.1 SUMMARY

It has been shown that both RNA and DNA can be efficiently concentrated from dilute solutions of 0.1M salt by single passage of the solutions through 0.1 ml beds of either strong or weak base anion exchangers at flow rates of 15 ml/minute. The beds can be washed with 1M NaCl without removing significant quantities of adsorbed nucleic acid, but treatment with dilute base readily elutes the nucleic acid from the polyamine exchangers, Amberlite IR-45 and CG-4B. Some preliminary evidence of uptake of Ca^{45} dependent on amount of nucleic acid adsorbed to Amberlite CG-4B has been obtained. The estimated detection sensitivity of the technique described is at least one bacterium per liter of air in 5 minutes or less. A modification which could increase the sensitivity, possibly to the point of detection of single virus particles, is discussed as well.

4.1.6.2 INTRODUCTION

Since nucleic acids are unique to biological organisms, a device for rapid, specific quantitation of minute amounts of nucleic acid would provide a good estimate of biologically derived material. Microorganisms, because of their smaller size, can be readily separated from other nucleic acid-containing material likely to occur in an aerosol sample. Similarly, the difference in size permits separation of bacteria and viruses. Subsequent measurement of the nucleic acid level of a given sample would provide a good estimate of microbiological content. Since all microorganisms contain nucleic acids, a detection system based on these would have excellent multi-agent capability.

The polyanionic character and large molecular size of nucleic acids permits their rapid, specific, and quantitative isolation on anion exchange resins. Passage of a dilute aqueous solution of nucleic acid through an anion exchange resin effectively concentrates the nucleic acid on the charged surfaces of the resin particles. Being polyanionic macromolecules, nucleic acids are bound practically irreversibly to these large insoluble particles and the entire system is readily freed from extraneous material such as protein, dust, etc.

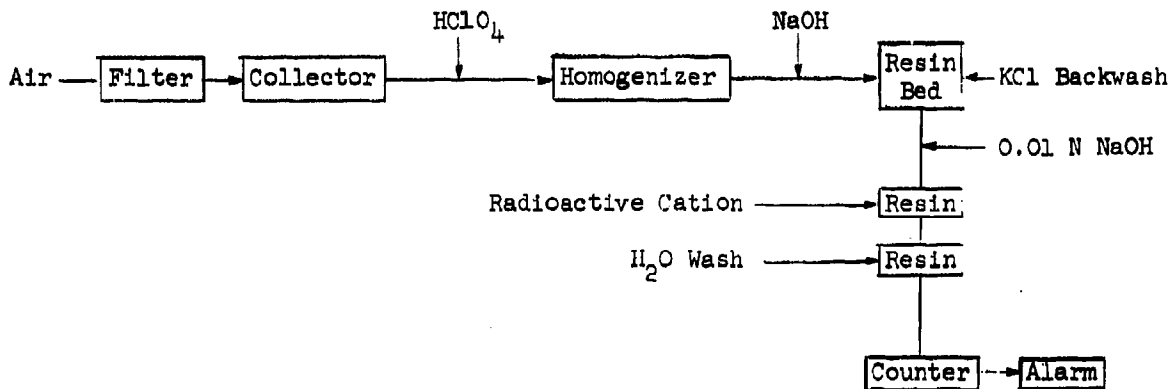
Soluble material that is not polyanionic has a much lower affinity for the resin, and filtered dust has a particle size several orders of magnitude smaller than the resin beads. Both can, therefore, be easily removed by backwashing a resin bed with concentrated salt solutions.

Since the isolation procedure described is specific for nucleic acids, their subsequent detection can be based on speed, sensitivity, and simplicity rather than selectivity. The simplest method to detect nucleic acid bound to a resin would be to exchange a radioactive cation into those portions of the chain not bound to the resin. In the case of strong base resins such as Dowex I, the functional group is a quaternary amine which maintains a positive charge under all conditions of pH. It is, therefore, unlikely that there would be many unbound portions of the chain available for cation exchange. Weak base anion exchangers of the polyamine type, however, are deprotonated under mildly alkaline conditions, but take up anions as effectively as quaternary amines at pH values below 7. They can, therefore, be used to isolate nucleic acid under mildly acidic conditions, and then deprotonated to leave the nucleic acid bound by secondary forces only. Under these conditions, the nucleic acids would function as cation exchangers due to the primary phosphate dissociations and should readily exchange monovalent for divalent cations. The quantity of cation bound should be a function of the amount of nucleic acid present and use of radioactive cation would enable estimation of nucleic acid by a short direct count of the resin bed.

Use of a radioactive cation such as Ca^{45} , with high specific activity, at a binding ratio of one Ca^{45} per ten nucleic acid phosphates, should permit detection of differences of 10^{-9} grams of nucleic acid (10^4 bacteria). A modification which would greatly increase this sensitivity would be use, as the radioactive cation of a P^{32} -labelled colloidal suspension of a quaternary amine resin such as Dowex I. Being polyvalent, this should bind very effectively to nucleic acids held on a partially deprotonated polyamine resin. The unbound colloidal resin should be readily removable from the main resin bed without loss of P^{32} . Since a Dowex I particle 1μ in diameter is potentially capable of binding 10^8 molecules of P^{32} , each of these particles would produce a strong

signal. At a binding ratio of one particle per nucleic acid molecule it would be possible to detect individual viruses.

A detection system utilizing these principles could be envisioned as follows:



System Characteristics - Filtration removes particles $< 5\mu$ followed by collection (LVAS) at 10,000 l air/min into 20 to 30 ml/min Triton X-100 solution. Microorganisms are disrupted with HClO_4 at $\sim 60^\circ\text{C}$ followed by neutralization to $\text{pH} \sim 3$. Solution is passed through polyamine resin bed at 20 to 30 ml/min, and bed is backwashed with 1M KCl, then treated as indicated. Sensitivity is estimated at < 1 bacterium per liter of air with carrier-free radioactive cation.

Time Delays

Collection, homogenization, adsorption to resin	1-1/2 min
Treatments of resin	1-1/2 min
Counting	<u>2 min</u>
	5 min

4.1.6.3 STATUS

4.1.6.3.1 NUCLEIC ACID ADSORPTION

Some of the principles cited have been tested under conditions expected to apply in a detection system such as that outlined. The investigations

were carried out at Space-General Corporation utilizing commercially available sperm DNA and yeast RNA. In all cases, 100 micrograms (γ) of nucleic acid were dissolved in 5 ml of 0.1 N NaCl or NaClO₄ and forced through small beds of resin from a 10cc syringe at flow rates of approximately 15 ml per minute. Nucleic acid was measured by ultraviolet adsorbance at 260 m μ , that bound to the resin being estimated by difference in absorbance resulting from passage of a solution through a bed of resin. The volume of all wash solutions was 5 ml and all flow rates were approximately 15 ml per minute.

NUCLEIC ACID (γ)

<u>Resin</u>	<u>Bound to Resin</u>		<u>Removed by 1MNaCl</u>		<u>Percent Removed by Base</u>	
	<u>RNA</u>	<u>DNA</u>	<u>RNA</u>	<u>DNA</u>	<u>RNA</u>	<u>DNA</u>
Dowex I (200-400#, 0.1 ml bed)	55	90	< 2	< 2	-	-
Amberlite, CG-4B (200-400#, 0.05 ml bed)	72 73	96 100	< 2 < 2	< 2 < 2	15* 99***	2* 80***
Amberlite IR-45 (20-50#, 0.1 ml bed)	28	26	< 2	< 2	70****	50****

*0.025N NaOH, **5% NH₄OH, ***0.02N NaOH

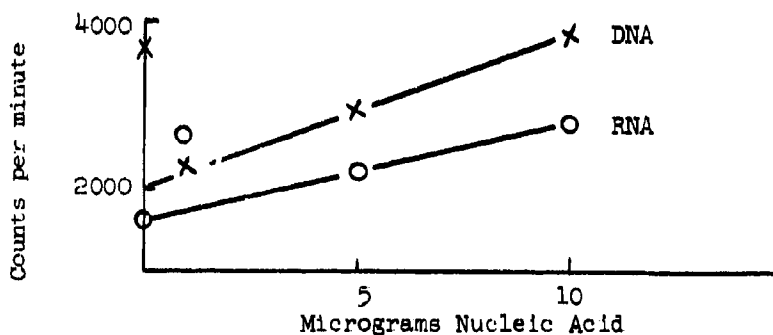
Higher concentrations of nucleic acid were bound less effectively, but lower concentrations were taken up quantitatively by Dowex I or Amberlite CG-4B as far as could be determined from UV absorbance. The results tabulated for Dowex I and Amberlite CG-4B were consistently reproducible. Those for Amberlite IR-45 are the result of a single measurement, but are consistent with the data obtained for the other two resins when the larger particle size of the IR-45 is taken into consideration. Since kinetic, rather than equilibrium, conditions must apply at the high flow rates employed, it is to be expected that the larger particles have a lower apparent capacity for nucleic acid. The tenfold difference in diameter between 200-400 and 20-50 mesh spheres results in a 100 fold difference in surface area. The eightfold decrease in apparent capacity found with IR-45 seems small in view of these differences and implies that quantitative uptake of small amounts (< 0.1 γ) of nucleic acid would be as efficient with the 20-50 as with the 200-400 mesh material.

The arguments invoked to explain the decreased binding of nucleic acids by IR-45 can also be used to explain the greater ease of elution of nucleic acids from this resin than from CG-4B. However, the difference in composition of the two resins (IR-45, polystyrene amine; CG-4B, phenolic amine) could account for some of the differences noted above.

It is evident from the tabulated data that DNA is generally bound more effectively than RNA, indicating that large molecular size is necessary for efficient uptake from dilute, fast flowing solutions of 0.1M salt. The fact that RNA, a polyanionic macromolecule, has some tendency to pass through the resin beds suggests that proteins and other soluble materials that are not polyanionic would not be held by the resin under these conditions. The ability of adsorbed nucleic acid to withstand washing with molar salt enhances the specificity of the technique, permitting backwash of a resin bed to remove insoluble debris and elute any residual protein or extraneous anions that could interfere in the subsequent determination of nucleic acid.

4.1.6.3.2 Ca^{45} UPTAKE

Some preliminary evidence of Ca^{45} uptake by adsorbed nucleic acid has been obtained with the weak base anion exchanger, Amberlite CG-4B. The nucleic acids were adsorbed on a 0.05 ml bed of resin from 5 ml 0.1M NaClO_4 at pH 3. After the usual wash cycles of water and 1M NaCl , the beds were washed with 2 ml 2 percent triethylamine followed by 2 ml H_2O . The Ca^{45} , in 0.025 ml 1M HCl (0.025 μ moles Ca^{45} or 7500 cpm), was placed directly on the bed and washed through with 5 ml H_2O . The resin was extruded onto planchetes and counted in a gas flow counter.



Ratios of nucleic acid phosphate to Ca^{45} of 6 for DNA and 10 for RNA were calculated from the slopes of the lines in the figure. The two points not on the lines were neglected. Though the data merely indicate a trend and the background values are high, the trend is in the right direction to encourage further investigation of this approach.

Several attempts were made to show binding of Ca^{45} to nucleic acids while adsorbed to Dowex I. These were uniformly unsuccessful, no detectable Ca^{45} remained on the resin in the presence or absence of as much as 5 micrograms of nucleic acid. This seems to indicate that there are few unbound portions of the nucleic acid chain available for cation exchange when adsorbed on a positively charged resin.

Preliminary experiments with unlabelled CdCl_2 and HgCl_2 have so far been inconclusive. It has been established that Hg^{++} is bound to both Dowex I and Amberlite CG-4B in both the presence and absence of bound nucleic acid. Neither elution of the bound Hg^{++} , with any treatment other than a brief water wash, nor saturation of the resins with Hg^{++} have yet been attempted. The presence of nucleic acid in solution did not interfere with the chemical determination of Hg^{++} , but it was not possible to measure the resin-bound Hg^{++} directly. Preliminary experiments with CdCl_2 have indicated that Cd^{++} is not bound by Dowex I in the absence of nucleic acid. Nucleic acid bound to Dowex I appeared to take up some Cd^{++} but the amounts involved were near the lower limit of resolution of the chemical assay employed. The interaction of Cd^{++} with nucleic acid bound to polyamine resins has not yet been investigated.

No attempt has been made to determine conditions for direct precipitation of sub-microgram quantities of nucleic acid with divalent cations.

The use of colloidal suspensions of strong base resins labelled with P^{32} in place of radioactive cations has not been attempted. The use of a fairly narrow range of particle sizes in the colloidal material is a prerequisite for reproducible quantitation of minute amounts of nucleic acid. The specially prepared suspensions of ion exchange resin available commercially (Bio-Rad Laboratories) with particle size ranges of 0.1 to 2 and 1 to 10 microns could probably be applied directly in the technique described in the earlier discussion.

4.1.6.4 CONCLUSION

The data accumulated to date demonstrate that nucleic acids can be rapidly and quantitatively isolated on anion exchange resins under conditions readily applicable to use in a detection device. When adsorbed to an exchange resin, they can be readily freed from extraneous material that is not polyanionic, making the isolation virtually specific for nucleic acid and eliminating background interference from non-microbiological materials. If polyamine exchangers are employed, the nucleic acids can be eluted with dilute base for subsequent enzymatic, immunological, or chemical characterization.

While quantitative detection of nucleic acid following isolation as described has not been demonstrated, some evidence of nucleic acid-dependent Ca^{45} binding has been obtained. Initial experiments have been carried out with Ca^{45} because of its ready availability, but Sr^{89} , with its more energetic β^- emission, would be more useful in a detection device.

In view of the ready elution of nucleic acid from polyamine resins, the conditions for cation binding by adsorbed nucleic acid need to be very carefully controlled. A more feasible procedure might be to elute the nucleic acid with dilute base and then precipitate it with a radioactive cation. In this event, Cd^{115} or Hg^{203} would lead to a higher degree of labelling because of their ability to coordinate with the nitrogenous bases as well as interact with the phosphate residues.

4.1.7 VIRUS DETECTION UTILIZING SYNTHETIC PARTICLES

4.1.7.1 SUMMARY

Several methods employing sensitized polystyrene latex particles were evaluated for detecting viruses. In the competitive inhibition principle, the sensitized beads were reacted either concurrently or sequentially with untagged virus (to be measured) and isotope-labelled virus. From 10^6 to 10^7 virus particles/ml could be detected by measurement of retained radioactivity.

Initial attempts to detect virus concentrated on beads with FITC-labelled antibody were unsuccessful, mainly because of the limited number of virus particles attainable per individual bead (1 to 2). A procedure utilizing radioisotope-labelled antibody could integrate counts from many particles, and give stronger signals.

4.1.7.2 INTRODUCTION

By reason of the small size and other unique properties, viruses require different approaches to their detection than do other organisms such as the bacteria. In many instances concentration of these organisms is required prior to detection. The use of sensitized polystyrene latex or other types of particles provides a method for selective concentration of viruses and virus carrier media which allows for subsequent specific immunological detection of these agents.

Several schemes utilizing antibody-coated particles were selected for further study and evaluation. These schemes were divided into three major groups: (1) procedures based on a competitive inhibition principle, (2) those methods utilizing tagged antigen and antibody, and (3) procedures based on an agglutination principle. The last is discussed under a separate section on Agglutination (Section 4.1.8).

The competitive inhibition principle is based on reaction of sensitized beads, either concurrently or sequentially, with untagged antigens and isotope-labelled antigens. Detection of antigen is based on a diminished uptake of the tagged antigen by the beads when in the presence of untagged antigens.

Radioactivity can be detected on the beads by passage of the reactants through a Millipore filter which retains the beads, and by determining the amount of radioactivity on the filter.

Virus or antigen can be specifically concentrated on sensitized particles and subsequently reacted with isotope- or fluorescent-labelled antibody. Detection of radioactivity or fluorescence can again be accomplished by measurement of the various emissions from deposits on a porous filter tape, following filtration of the sensitized particle-antigen-antibody complexes. Alternatively, in the above scheme, the various antigens could be first labelled with either a radiotracer such as I^{131} or fluorescent tracer such as Fluorescein isothiocyanate. Subsequent reaction with labelled antibody would thus be unnecessary.

These methods all have the advantage of specificity and multi-agent capability inherent in an immunological system and could be adapted to instrumentation which is being presently developed. The experiments conducted were thus directed mainly toward determining the sensitivity, rapidity, and general feasibility of detection of the various schemes presented above.

4.1.7.3 STATUS

Initial studies were conducted to determine the kinetics and general feasibility of concentrating virus on sensitized polystyrene latex particles. Data from these studies were utilized to determine the experimental plans for evaluating the various detection schemes.

4.1.7.3.1 REACTION OF VIRUS WITH SENSITIZED BEADS

In these experiments 0.8μ (Dow) polystyrene latex particles were sensitized with the globulin fraction of rabbit T₄D phage antisera of varying titers. Final preparations were obtained in which an estimated 20 to 35 percent of the bead surfaces were coated with globulin. Varying concentrations of this preparation were reacted with varying concentrations of P^{32} labelled phage. After specified periods of time, aliquots were removed from the reaction mixture, diluted in glycine saline buffer (pH 8.2) containing 0.1 percent Triton X 100,

and filtered through a 0.45 μ Millipore filter. Radioactivity on the filters was determined by a gas flow proportional counter. Beads coated in an identical manner with normal globulin were used as controls. The details of the method are presented in the Fourth and Fifth Comprehensive Reports^(1,2).

Initial results (see Figure 4-38) indicated that (1) the amount of reactivity of the sensitized latex particles with P^{32} -labelled phage was fairly reproducible, (2) the sensitized particles were stable over a period of at least 3 days (subsequent data indicated that the beads were stable for at least 2 to 4 weeks at 5°C), and (3) the amount of reaction appeared to be proportional to the concentration of phage. Subsequent data obtained on the kinetics of reaction were subjected to critical mathematical analysis.

Figure 4-39 is a plot of the log of the fraction of T4D phage which remains unadsorbed as a function of time. The linear nature of the plot indicates a first order dependence of adsorption on phage concentrations. The data of Runs 1 and 2 at 6.5×10^7 and 1.3×10^8 phage/ml are exactly coincident. The data of Runs 3 and 4 at 1.8×10^7 and 4.5×10^8 phage/ml are parallel and give the same rate constant. In Runs 1 and 2 antisera used for sensitization had a $K^{(3)}$ value of 77, and in Runs 3 and 4, the antisera used had a K value of 25. The shift between these two sets, which were obtained at phage concentrations which differed by a factor of 25, appears associated with a zero time measure.

Figures 4-40 and 4-41 show that the rate of adsorption is a linear function of the surface concentration. Figure 4-41 indicates the method by which the adsorption process may be made sufficiently rapid to become a negligible consideration in the operation of a breadboard. That is, high specific surface concentrations may be employed to make the adsorption reaction as rapid as desirable.

Table 4-15 indicates the concentrations, radioactivity levels in the original phage preparations, and rate constants of the various runs. The results indicate that adsorption rates depend on the titer of the T4D antiphage serum.

Table 4-16 summarizes the properties of the phage adsorption reaction on antibody-coated latex beads (Runs 1 and 2). These results indicate that

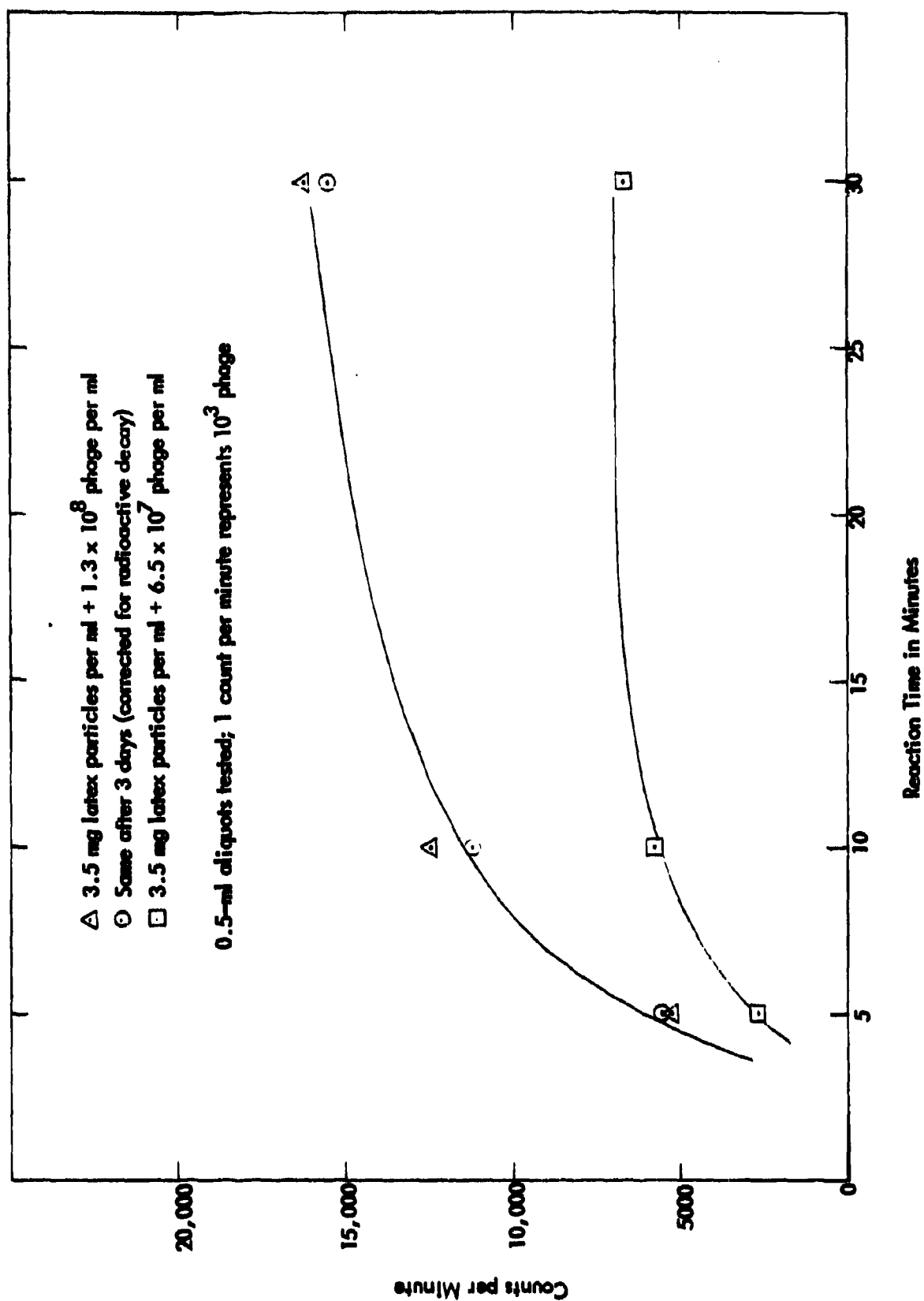
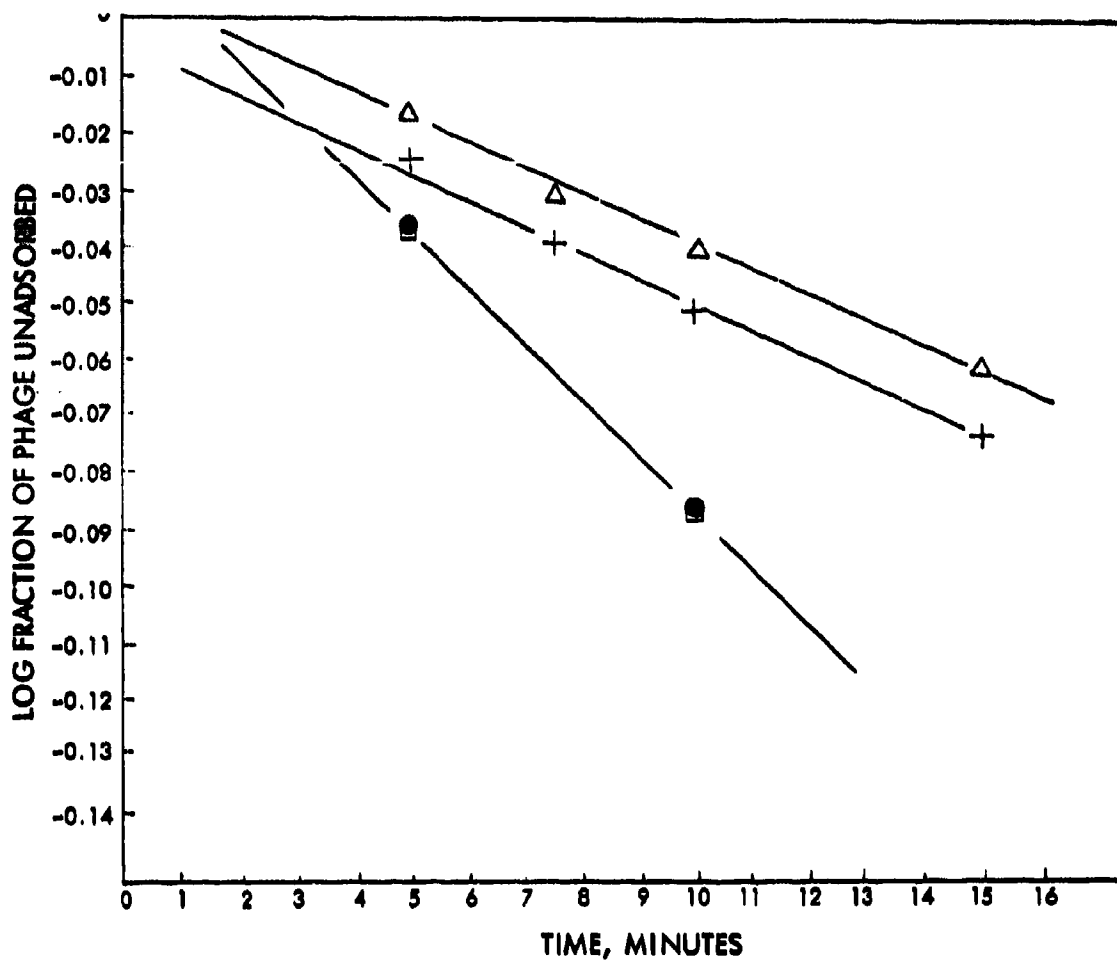


Figure 4-38. Reaction of P^{32} -Labelled Phage with Antibody-Covered Latex Spheres at 37°C



RUN 1 6.5×10^7 Phage/ml, Beads coated with Serum, $K = 77$
 RUN 2 1.3×10^8 Phage/ml, Beads coated with Serum, $K = 77$
 RUN 3 1.8×10^7 Phage/ml, Beads coated with Serum, $K = 25$
 RUN 4 4.5×10^8 Phage/ml, Beads coated with Serum, $K = 25$
 SEE TABLE FOR CONCENTRATION CONDITIONS

Figure 4-39. T4D Phage Adsorption on Antibody-Coated Latex Beads

The Effect of Bead Concentration on Rates of Adsorption

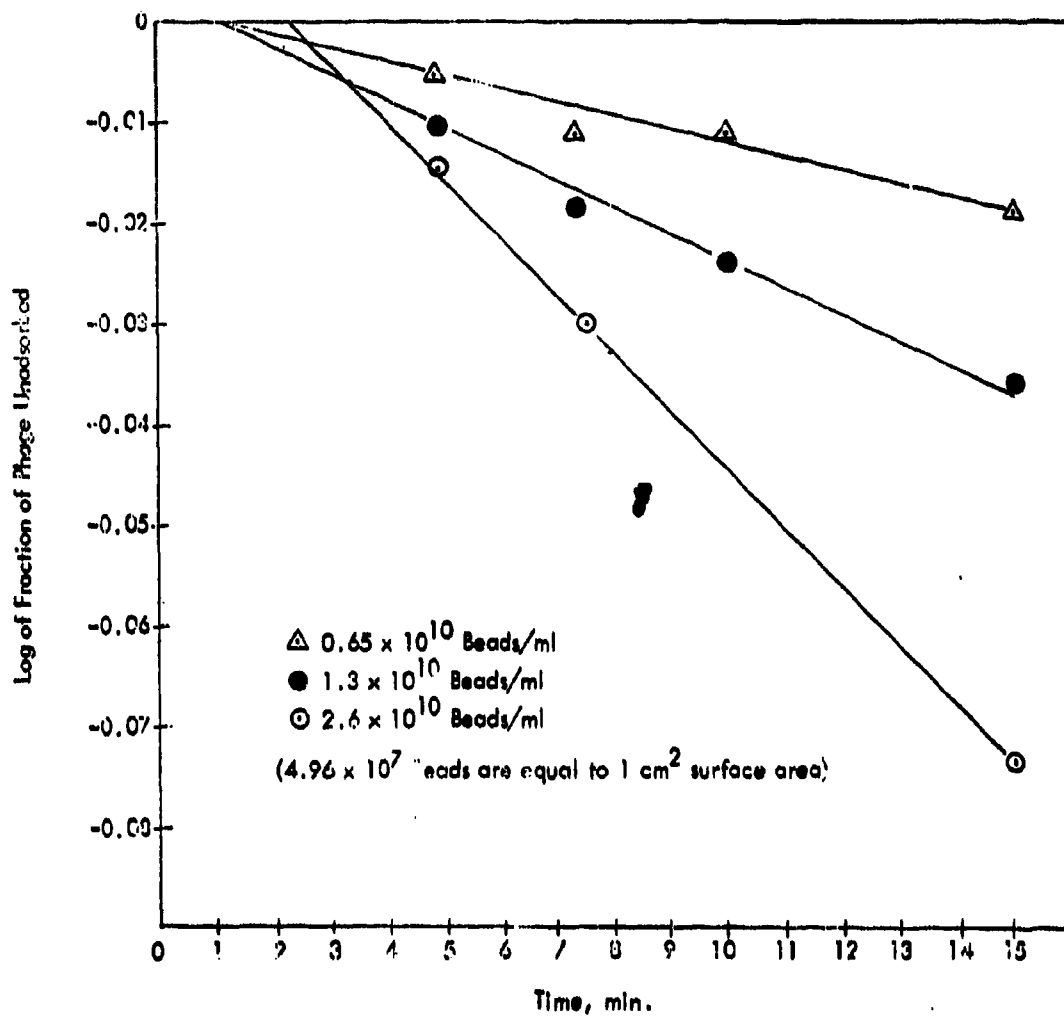


Figure 4-40. T4D Phage Adsorption on Anti-Serum Coated Beads

The Effect of Bead Concentration
on Rates of Adsorption

(1.34×10^{-2} mg are equal to 1 cm^2 surface area)

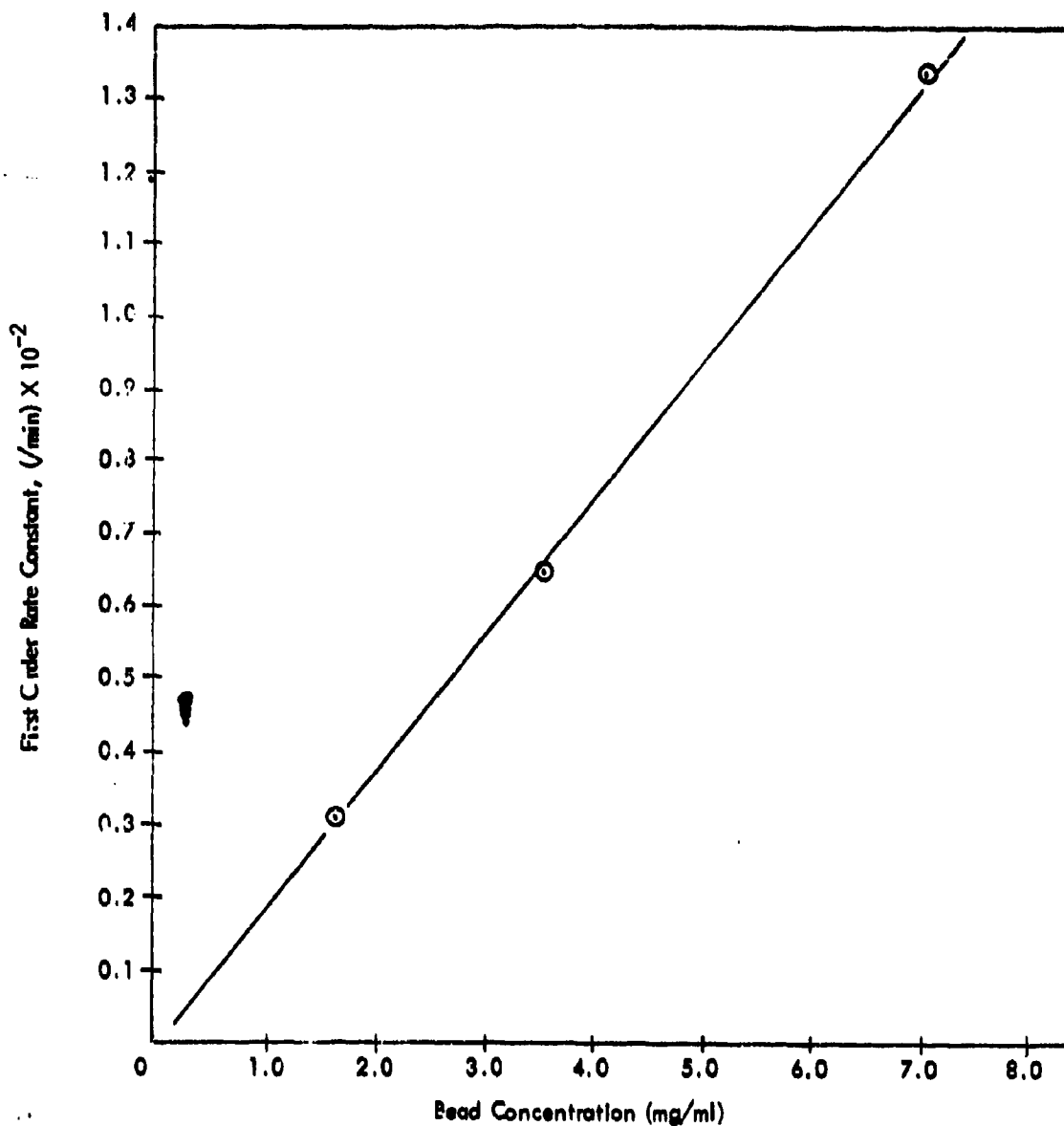


Figure 4-41. T4D Phage Adsorption on Anti-Serum
Coated Beads

Table 4-15
RATES OF T4D PHAGE ADSORPTION ON
POLYSTYRENE BEADS AT 37°C

Run	Bead Concentration (/ml)	Surface Area ($\frac{\text{cm}^2}{\text{ml}}$)	Phage Concentration (/ml)	First Order Rate Constant, k (/min)	Specific First Order Constant (ml/cm ² -min)	Rabbit Antiphage Serum K Values	Radioactivity (counts/phage-min)
1	1.3×10^{10}	261	6.5×10^7	2.3×10^{-2}	8.8×10^{-5}	77	1.0×10^{-3}
2	1.3×10^{10}	261	1.3×10^8	2.3×10^{-2}	8.8×10^{-5}	77	1.0×10^{-3}
3	2.6×10^{10}	522	1.8×10^7	1.1×10^{-2}	2.2×10^{-5}	25	2.3×10^{-3}
4	2.6×10^{10}	522	4.5×10^8	1.1×10^{-2}	2.2×10^{-5}	25	2.1×10^{-3}
5	2.6×10^{10}	522	1.1×10^8	1.3×10^{-2}	2.6×10^{-5}	25	0.83×10^{-3}
6	1.3×10^{10}	261	1.1×10^8	6.5×10^{-3}	2.5×10^{-5}	25	0.79×10^{-3}
7	0.65×10^{10}	131	1.1×10^8	3.2×10^{-3}	2.4×10^{-5}	25	0.72×10^{-3}

Table 4-16

T4D PHAGE ADSORPTION ON ANTIBODY-COATED LATEX BEADS

Bead Concentration $1.3 \times 10^{10}/\text{ml}$

Bead Surface Area $261 \text{ cm}^2/\text{ml}$

Radioactivity 1.0×10^{-3} counts/phage-min.

Rate of Adsorption (at 1.3×10^8 phage/ml)
 3.0×10^6 phage/min.

Rate Constant $2.3 \times 10^{-2}/\text{min.}$

Theoretical Phage Adsorption Limit/Bead 60
(on geometrical basis)

Fraction of Surface Covered by Phage at Equilibrium
 2.3×10^{-6}

Adsorption Coefficient 1.7×10^{-14} ml/phage

Phage/Bead Ratio 1/520

Estimated Reaction Efficiency by Collision 0.07%

it should be readily possible to increase the phage/bead ratio of 1/520 by use of higher titered and more highly purified antisera to sensitize the beads.

4.1.7.3.2 COMPETITIVE INHIBITION

Experiments were conducted to determine the sensitivity and feasibility of detection with this method. Effects of these variables on the sensitivity of detection were studied: the concentration of the reactants, the sequence in which the tagged and untagged phage were reacted with the beads, and the ionic environment. In general, the various materials and measurement procedures employed were similar to those used in Section 4.1.7.3.1 above.

Reaction of Beads Concurrently with Mixtures of Tagged and Untagged Phage - In these experiments, sensitized latex particles were reacted with labelled phage alone and this result was compared with the result obtained with mixtures of labelled and unlabelled phage. It was hoped that by varying the concentration of the reactants, the number of unlabelled phage required to decrease significantly the attachment of radioactive phage to the latex particles could be determined.

The following table indicates the degree of signal depression caused by the reaction of various concentrations of unlabelled phage with 1×10^6 labelled phage/ml and 0.0035 mg/ml of sensitized beads (10^5 antibody sites/ml):

Concentration of Added Cold Phage (#/ml)	Counts/Min.* on Filter When Only Hot Phage Reacted	Counts/Min. on Filter When Hot and Cold Phage Reacted	Depression of Signal %
10^6	166**	168	0
5×10^6	106***	95	10
10^7	274**	180	34

* Less normal background and background measurement from reaction with normal globulin coated beads.

** Approximately 1.7×10^{-9} μ c/phage.

*** Approximately 1.3×10^{-9} μ c/phage.

The signal depression is indicated as the percent of the difference between the experiment with hot phage alone compared to the signal obtained with the mixture of hot and cold phage. Tracer uptake by beads which had been coated with normal gamma globulin was utilized as the "background" measurement.

The data indicate that somewhere between 10^6 and 10^7 virus particles/ml can be detected by this method. No significant depression was detected when increased concentrations of beads were used. The data further indicate that the use of a significantly lower concentration of beads could also result in no measurable depression due to the decrease in reaction rate. Improvement of detection sensitivity could probably be increased somewhat by increasing the temperature of reaction, using phage containing the optimum amount of P^{32} label (10^{-8} μ c/phage), increasing the concentration of specific antibodies per bead and decreasing the background variation.

Experiments by Mandell⁽⁴⁾ and Jerne and Skovsted⁽⁵⁾ indicated perhaps that decreased ionic strength of the medium may increase the reaction rate between antibody and phage. They have demonstrated that the rate of inactivation of phage T4r by specific antisera may be increased by 1000 fold in a medium of low ionic strength, as compared to reaction in a 0.1 M salt solution. Experiments were conducted in this laboratory in which sensitized beads and phage were reacted in a 0.0005M glycine and 0.0005M phosphate buffer as well as in distilled H_2O . No increase in reaction rate could be demonstrated.

In some instances cold phage caused an increase in measurable radioactivity uptake by beads. This phenomenon occurred mainly when older labelled phage preparations (2 or more weeks of age) were used. It was felt that this phenomenon may be due to increased agglomeration of the labelled phage concentration and thus an increase in radioactivity uptake by the beads. A series of experiments were thus conducted in which the beads were reacted, alternatively first with unlabelled phage and then with labelled phage. It was hoped that this procedure would avoid agglomeration and also improve the sensitivity of the method.

Reaction of Beads with Untagged and Tagged Phage, Separately -

Experiments were conducted as above, using 10^6 labelled phage/ml, 10^7 unlabelled phage/ml and 0.0035 mg/ml of sensitized beads. In these experiments, however, the beads were reacted with unlabelled phage first and then with labelled phage.

Sensitized beads were reacted for 10 minutes with unlabelled phage and then in some instances either filtered or centrifuged to remove the excess untagged phage prior to reaction with labelled phage for the same period of time. The filtered beads were refiltered after reaction with labelled phage and the radioactivity retained on the filter membrane was measured. The centrifuged beads were recentrifuged following reaction with labelled phage and the supernatants measured for radioactivity. Only in the latter instance did the presence of untagged phage cause a decrease in the radioactivity on the beads. It appears that approximately a 40% decrease in radioactivity occurred. These results are similar to those obtained when labelled and unlabelled phage were reacted concurrently. Even though an increased sensitivity was not realized by the method, it may have advantages in that the radioactivity of the supernatant increases with retention of unlabelled phage by the beads, resulting in a positive signal. The variation between samples appears to be much less in these experiments than in those where beads are measured directly.

4.1.7.3.3 TAGGED ANTIGEN AND ANTIBODY

Polystyrene latex particles were sensitized with a high titered antiphage globulin by the best available methods. These were reacted with phage until saturation occurred. It was estimated that there was an average of 1 to 2 phage per bead. These phage particles were not detected after reaction with FITC conjugated antiphage globulin.

It became increasingly apparent from these and the previous studies that the sensitivity of detection of all methods utilizing sensitized beads may be limited by the reaction rates between the beads and specific antigen to be detected. This is especially true when considering those methods in which the fluorescence on an individual bead must be distinguished from background. One way to increase the reaction rate was to increase the concentration of specific antibody per particle. When the concentration of globulin on the surface of the beads was increased so that 50 percent of the surface was coated, phage uptake was decreased rather than increased, however.

One way of explaining the results is that the serum fraction used for coating the beads contained non-antibody protein which adsorbed more readily on the polystyrene than did the antibody globulin. It was felt that use of more highly purified globulin for sensitizing the beads would result in a greater concentration of specific antibody per bead. Initial experiments using particles coated with globulin fractionated by precipitation with 1/3 saturated NH_4SO_4 did not result in improved uptake. It was felt that further purification of antisera by other methods, such as ion exchange chromatography or specific adsorption and release at pH's of between 2.5 and 3.0 may be necessary.

Alterations in conditions of coating the particles such as pH of reaction mixture, temperature, and ionic strength also did not appear to improve uptake of antibody.

4.1.7.4 CONCLUSIONS

The reaction between virus and antibody-coated beads appears rapid and reproducible. Furthermore, storage at 5°C for at least 2 to 4 weeks did not appear to alter the capacity of the coated particles to react with viruses.

Detection of 10^6 to 10^7 virus particles/ml appears possible with methods based on the competitive inhibition principle. It would also appear, however, that this sensitivity level cannot be significantly improved, due to certain inherent requirements of the method. For instance, it was determined that the concentration of sensitized beads must be held to a minimum for the method to be workable. This imposes restrictions on the kinetics of reaction and thus the sensitivity of the method.

Sensitivity of methods based on detection of fluorescence on the individual particle appears limited by the concentration of specific antibody which can be obtained per particle. A concentration of specific antibody per bead which will allow uptake of sufficient virus for detection with FITC conjugated antibody has not as yet been experimentally obtainable.

The use of radiotracer methods for detecting virus concentrated on beads is not as restricted by the kinetic limitations as in the above methods, since the total emission from all of the particles can be collectively measured. It would thus appear that the use of sensitized particles, in conjunction with methods employing isotope-labelled antibodies, could result in an extremely sensitive and feasible method for the specific detection of viruses.

4.1.7.5 REFERENCES

- (1) Fourth Comprehensive Report, SGC 382R-6, Research Program on BW Detection, April 1964 - September 1964.
- (2) Fifth Comprehensive Report, SGC 382R-7, Research Program on BW Detection, October 1964 - March 1965.
- (3) Velocity Constant for Rate of Phage Inactivation by Antibody, Adams, M.H., Bacteriophages, Interscience Pub. Inc., N.Y.
- (4) Mandell, J.D., Thesis, California Institute of Technology, 1955.
- (5) Jerne, N.K. and Skovsted, L., Ann. Inst. Pasteur 84: 73, 1953.

4.1.8 AGGLUTINATION

4.1.8.1 SUMMARY

A method based on agglutination of antibody-coated particles in the presence of specific antigen has been evaluated. A number of methods for measuring agglutination have been considered, but experimental efforts have emphasized the use of a modified Coulter device for this purpose. The ability of this device to measure the concentration of particles of different sizes is based on changes in electrical conductivity caused by passage of the particles through a small pore through which an electrical current is generated.

It was estimated that as few as 10^4 virus particles could be detected by the use of the Coulter device and as little as 0.01 μ g of egg protein was observed to cause a visible agglutination of sensitized latex particles within 5 minutes. Experimental evidence also indicates that background interference resulting from atmospheric sources can be held to a minimum. These factors, along with advantages in specificity, multi-agent capability, logistic aspects, and virus detection would appear to rank the method as one of the more promising for BW detection.

4.1.8.2 INTRODUCTION

Detection methods based on agglutination of antibody-coated particles (polystyrene latex or other inert substances) in the presence of specific antigen are considered extremely promising. Agglutination can be measured by a variety of approaches which include the use either of a modified Coulter device or of a radiation counting device. In the Coulter device, measurement is based on differences in electrical conductivity between volumes of aqueous electrolytic solutions containing (1) agglutinated particles, and (2) unagglutinated particles. The alternate approach is based on measurement of radioactivity caused by retention of isotope-labelled, agglutinated particles on a filter with a pore size which allows the non-agglutinated particles to pass.

Detection by particle agglutination has the advantages of the specificity and multiagent capability of an immunological method and is sensitive, rapid, and readily adaptable to automation.

Experimental data have been obtained on the use of the Coulter principle and indications are that as few as 10^4 viruses per ml can be theoretically detected within a 5-minute period (as can bacteria). It is further indicated from these and other studies that background interference from atmospheric sources can be held to a minimum, that reagent requirements may be relatively small, and that unreacted reagent does not interfere appreciably with measurement of agglutinated particles and will probably not have to be removed prior to measurement.

Agglutination of antibody-coated particles has a number of advantages over other agglutination techniques such as those employing erythrocytes. The inert particles (polystyrene, bentonite, etc.) may be stored for long periods of time with little deterioration, thus eliminating the requirement of continued maintenance of animals such as would be required for supplying erythrocytes. Variation in the composition of these particles would be considerably less than that for biological materials. These particles can be easily coated with antibody and these sensitized particles can be stored for at least several months without loss of reactivity. Particles of uniform size, such as latex beads (of polystyrene or other polymers), are especially advantageous because the monomers, dimers, trimers and higher aggregates are so sharply distinguishable. Sensitized particles may be used for specific detection of viruses, as well as for all types of antigenic material including those from bacteria, molds, virus carrier medium, and nucleic acids.

4.1.8.3 STATUS

The principle of detecting antigen by agglutination of sensitized particles has been demonstrated by various investigators^(1,2). Various types of particles such as bentonite, polystyrene latex, and erythrocytes have been coated with antibody and used for detecting specific organisms. Indications are that methods employing particle agglutination are extremely sensitive when compared to other immunological methods, perhaps even more sensitive than the complement fixation test⁽³⁾.

Experimental work has been mainly concerned with determining the concentration of virus or egg carrier necessary for causing agglutination of antibody-coated polystyrene latex particles and the applicability of the Coulter principle for measuring particle agglutination. Experiments were also conducted to determine if sensitized ZnCdS particles would agglutinate in the presence of specific antigen. It was hoped that the agglutinated particles could be measured by determining an increase in concentration of fluorescence.

In initial experiments, 0.2 μ and 0.8 μ polystyrene latex particles and ZnCdS particles were sensitized by incubating appropriate concentrations of the particles with an appropriate concentration of anti-phage T4D rabbit globulin for 10 minutes at 56°C. The particles were washed 3 times in a glycine saline buffer (pH 8.2) and stored at 5°C until used (see Fifth Comprehensive Report⁽⁴⁾ for details). Final preparations were obtained in which 1 mg of 0.2 μ and 0.8 μ latex particles contained 75 to 10 μ g of antiphage globulin, respectively, and 1 mg of ZnCdS particles contained 9 μ g of antiphage globulin. Reaction of the sensitized latex particles with phage (10^6 to 10^8 /ml) resulted in visible agglutination within 5 to 10 minutes. Agglutination was not observed with sensitized ZnCdS particles.

For experiments in which the Coulter device was to be used, it was determined that best results would be obtained with latex particles of > than 1 μ in diameter. Latex particles of 1.3 μ in diameter were thus sensitized with antiphage globulin (K = 1300) and a high titered anti egg globulin (prepared against homogenized 11-day-old embryonated eggs) by the same procedure as used previously (see above). The sensitized particles were reacted with varying concentrations of phage T4D and of the soluble portion of homogenized 11-day-old chick embryos and the reactant solutions were observed microscopically for agglutination. Beads sensitized in an identical manner with normal rabbit globulin were used as controls. The results are presented in Tables 4-17 and 4-18 .

The data (Tables 4-17 and 4-18) indicate that sensitized particles were agglutinated within 3 to 12 minutes in the presence of as few as 3×10^6 T4D phage/ml (or $\sim 10^5$ total particles) and as little as 0.01 μ g of soluble egg protein ml (or 0.001 μ g of total protein). It was interesting to note that

Table 4-17

BEAD AGGLUTINATION WITH PHAGE T4D

Bead Conc. (nos./ml)	Phage Conc.* per ml	Phage Nos.**	Reaction Time for Agglutination (min)***
10^9	3×10^9	1.5×10^8	1
10^9	3×10^8	1.5×10^7	3
10^9	3×10^7	1.5×10^6	5
10^9	3×10^6	1.5×10^5	12
10^8	3×10^9	1.5×10^8	3
10^8	3×10^8	1.5×10^7	5
10^8	3×10^7	1.5×10^6	10

*Based on plaque counts.

**Based on 0.05 ml of phage mixed with 0.05 ml of beads.

***Time at which agglutination was first observed microscopically.

Table 4-18

BEAD AGGLUTINATION WITH SOLUBLE EGG HOMOGENATE

Egg Protein* (Conc.) (μ g/ml)	Total Egg** Protein (μ g)	Reaction Time for Agglutination (min)
12,000	1,200	Note observed after 15 minutes
240	24	11
120	12	3.5
60	6	3
30	3	3
0.1	0.01	3
0.01	0.001	7.5

*Final concentration of egg protein contained in the reactant solution as determined by the biuret method.

**Based on the fact that 0.1 ml of varying concentrations of homogenized egg was reacted with 0.1 ml of 1.3μ sensitized latex particles (10^8 /ml).

at high concentrations of egg protein, an inhibition of agglutination occurred, presumably due to saturation of the antibody sites.

Detection of bead agglutination was attempted with the Coulter device, which had been modified by Professor A. G. Marr of the University of California at Davis to permit rapid and accurate determination of size distribution of particles in suspension*.

The experimental procedure for determining size distribution with the present apparatus is as follows. A suspension of the reacted or unreacted sensitized particles in a weak electrolytic solution is placed in the outer chamber of the transducer, and the particles are drawn through a 30 μ orifice leading into the inner chamber by application of vacuum. A measured potential difference is maintained across the orifice, by means of electrodes in the two chambers. As the particles pass through the orifice, the resistance temporarily increases because of displacement of the electrolyte. The resulting decrease in current generates a voltage pulse. The pulse is amplified and differentiated. The magnitude of the derivative of the original pulse is directly proportional to the volume of the particle passing through the orifice. With the multichannel analyzer, the accumulation of size distribution can be observed continuously on an oscilloscope screen. In this device, a bead suspension can be passed through the orifice at a rate of 0.1 ml/minute and as few as 10² new dimers are detectable in 1 minute.

In initial experiments, it was determined that best results were achieved when coincidence (two or more particles in the aperture simultaneously) was minimized by keeping the concentration of particles to be measured below 10⁵/ml. Even though relatively small numbers of beads were used in the reaction (5×10^6 or less), these must be highly concentrated (10⁸/ml) to satisfy the kinetic requirement of a rapid reaction rate. It is thus necessary to react a concentrated suspension and then to dilute before measurement. However, dilution results in dissociation of the agglutinated particles. This dissociation

* For further details on this device see Contract DA MO 49-193-65-G160.

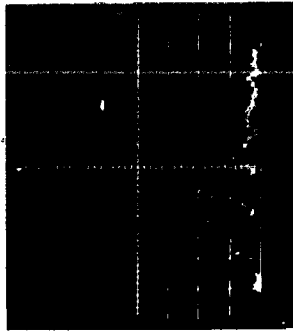
was partially inhibited by immersion of the mixture in boiling water for 30 seconds prior to measurement of size distribution.

Figure 4-42 shows photographs of oscilloscope tracings from agglutinated and unagglutinated sensitized 1.3 μ polystyrene latex particles which had been "heat-fixed" prior to detection. The location along the horizontal axis represents the size of the particle. From left to right the first peak represents the dimer, the second peak the trimer, and so on. The heights of the peaks are proportional to the accumulated number of particles of given size. Distinct differences in the trimer and other polymer peaks can be seen between the reacted beads and unreacted controls.

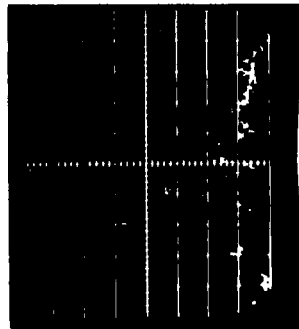
The present experiments suggest that the modified Coulter apparatus can be used for detection of agglutinated particles. Prof. A. G. Marr felt that the apparatus was about 100 times more sensitive in detection of agglutinated particles than was microscopic observation. It is therefore estimated that as few as 10^4 virus particles or other infectious agents per ml can be detected by this method.

4.1.8.4 CONCLUSIONS

It would appear that the agglutination method used in conjunction with the modified Coulter device has the potential of being rapid, sensitive, and specific and can readily and easily detect virus. Initial experiments on particle counts with air samples pretreated by liquid partition (see Section 4.2.3.1) furthermore suggests that background interference from atmospheric sources may not be a problem. The one major problem with the method is preventing the agglutinated particles from dissociating. Experimental evidence thus far, however, indicates that this problem can be solved. It would appear that the advantages of this method should merit further efforts on its development and application to BW detection.



Control



Agglutinated Beads

Figure 4-42. Oscilloscope Traces from Agglutination Studies

4.1.8.5

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- (4) Fifth Comprehensive Report, SGC 382R-7, Research Program on BW Detection, October 1964 - March 1965.

4.1.9 PHYSICAL METHODS

4.1.9.1 ELECTRON PARAMAGNETIC RESONANCE

4.1.9.1.1 SUMMARY

One possibility considered for detecting bacterial enzymes was the measurement of free-radical intermediates from enzyme-substrate reactions with electron paramagnetic resonance. Trials in the Varian laboratories failed to detect such intermediates by the EPR technique, and this approach was abandoned.

4.1.9.1.2 INTRODUCTION

Objectives and Approach - A limited research effort was conducted at Varian Associates, with the aim of testing the applicability of magnetic-resonance techniques to the detection of BW agents.

Preliminary analysis of present-day capabilities of EPR and NMR suggested the most promising approach to be measurement by EPR of free-radical intermediates in reactions catalyzed by enzymes in bacteria. Other approaches also considered were discarded as insufficiently sensitive.

Sensitivity and Limiting Concentrations - A roughly quantitative analysis was performed to determine the conditions needed to produce a signal. The following defining parameters were based on work on other programs in the Varian Laboratories: (a) As few as 10^{10} free radicals are detectable in liquid samples in research experiments, by use of special techniques, (b) Enzyme concentrations as low as 10^{-8} M have been detected, via free-radical intermediates, (c) As an indication of upper limits of free-radical concentrations attained in enzyme-substrate reactions, steady-state concentrations of 4×10^{-4} M have been measured.

The assumption of unduly large concentrations of free radicals within whole bacteria can be avoided by considering the free radicals distributed evenly throughout the entire reaction mixture, instead of concentrated only in the bacteria. Therefore, a bacterial suspension should be treated to release the contained enzymes into the aqueous medium by extraction, grinding, or other destructive methods.

The case of a hypothetical solution of enzyme and substrate was evaluated, by comparison with these criteria. A sample of 10^4 bacteria is assumed to be available, on the basis of collector capacity.

The number of free radicals is estimated as follows:

Assumed enzyme content = 0.01% ⁽¹⁾ $\approx 10^{-12}$ g. Volume, from Varian cell = 1.35×10^{-4} l. Molecular weight of enzyme = 10^5 . Whence molar enzyme concentration = $\frac{10^{-12}/10^5}{1.35 \times 10^{-4}} = 7 \times 10^{-14}$ moles/l. Free radical concentrations of 20 times the enzyme concentration were noted in some Varian experiments⁽²⁾. Whence numbers in mixing volume is $20 \times 7 \times 10^{-14} \times 1.35 \times 10^{-4} \times 6 \times 10^{23} = 10^8$ free radicals.

Although these estimated concentrations and numbers are at least two orders of magnitude lower than the present thresholds of detection, there appeared to be some hope of increasing them. Thus, the decision was made to perform a limited series of experiments in the Varian laboratories.

4.1.9.1.3 STATUS

First, the enzymatic oxidation of glutamic acid was examined under conditions most favorable for production of free-radical intermediates, but with negative results. The reaction proceeds as follows:



To perform this reaction, the following streams were prepared in a 0.04 M glycine/NaOH buffer at pH 8.5:

- 40 ml of enzyme* solution (containing 20 mg) + 10 ml of 0.1 M DPN solution
- 50 ml of 0.1 M glutamic-acid solution

Equal quantities of these streams were mixed, and scanned by EPR for free radicals under both flow and static conditions, using the apparatus and techniques described in the literature. No signal was observed. The reaction was next reversed by adding NH_4^+ ion to the spent reaction mixture with adjusted pH. Again, no signal was observed. It is concluded that no persistent free-radical intermediate of measurable concentration is formed in this reaction.

*L-glutamic-acid-dehydrogenase (bovine liver), Calbiochem A grade, specific activity approximately 3.8 EU/mg protein. (1 EU catalyzes the turnover of 1 micromole of substrate per minute).

Other experiments showed that free radicals were not detected when hydrogen peroxide is decomposed by catalase in the presence of a "third-agent" free-radical source (or without the third agent, as ascertained by previous experiments at Varian). Chlorpromazine and luminol (5-amino, 2, 3-dihydro-1, 4-phthalazinedione), were used as third agents. The experiment described below, performed with luminol, is typical.

The stream to be reacted consisted of the following solutions:

- A saturated solution of luminol in NaOH solution, mixed with an approximately-equal volume of catalase solution. Apparent catalase content was 1 to 5×10^{-5} M (although assay showed the activity to be about 1 percent of the theoretical value), pH was approximately 11.
- A 0.1 M H_2O_2 solution.

On pumping the first stream only, a slight signal due to a free radical was observed. However, on mixing equal quantities of the two streams, the original signal slowly disappeared. No new signal appeared, although phosphorescence was pronounced. The cause of the original signal (which is not due to luminol alone, but to some interaction with catalase) is not understood, and should be studied further.

For completeness, the mixed streams were also scanned for a detectable triplet molecule. The region of the Lande g-value of 4 was examined. No signal was observed.

4.1.9.1.4 CONCLUSIONS

In these preliminary studies the use of EPR to monitor enzyme-catalyzed reactions did not provide the sensitivity required for BW detection. Therefore, development could not be recommended, and no further work was performed on this topic during the program.

4.1.9.1.5 REFERENCES

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- (2) Yamazaki, I., Mason, H. S. and Piette, L., J. Biol. Chem. 235: 2444, 1960.

4.1.9.2 BIOELECTROCHEMICAL METHODS

4.1.9.2.1 SUMMARY

An amperometric detection method was developed by Magna, Incorporated. With improved techniques, it gave a characteristic response to B. globigii extracts of 10^{-16} amp/min per cell/ml. At the lowest measurable electrical signal, this corresponds to detection of 5×10^4 bacteria in 5 minutes. Intact cells gave even stronger responses than extracts. Neither general background particulates nor B. globigii spores masked the signals from bacterial extracts. This effort was discontinued to concentrate on other enzymatic methods which appeared easier to develop.

4.1.9.2.2 INTRODUCTION

A detection scheme based on simple electrical monitoring of a liquid sample would have many advantages. Design of a continuous, instrumented system should not be difficult. With the purpose of exploiting these advantages, Magna Corporation undertook to evaluate an amperometric method for detecting BW agents by means of their enzyme activity.

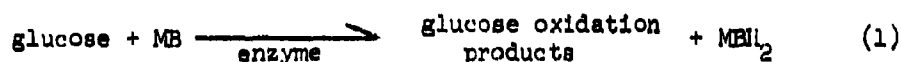
The evaluation involved (a) a determination that redox enzyme systems were present and active in pathogenic microorganisms (as shown by the literature), (b) selection of an enzyme-catalyzed biochemical reaction most amenable to amperometric measurement, (c) development of suitable equipment and procedures for integrating the biochemical and electrochemical aspects of the problem, (d) maximization of the rate of the biochemical and electrochemical reaction steps by selection of optimum operating conditions, (e) determination of possible interference from "normal" and non-biological background material removed from air; (f) reduction of the lower detection limit, in terms of the minimum number of cells per ml that can be detected in a few minutes time.

4.1.9.2.3 STATUS

A literature search was directed toward identifying redox enzyme activity in representative pathogenic microorganisms including the bacteria P. tularensis, P. pestis, B. anthracis, Brucella suis, and Pseudomonas (Malleomyces)

pseudomallei, the yeast Coccidioides immitus, the Rickettsia Coxiella burnetii, and Venezuelan equine encephalomyelitis and variola virus. Active redox enzymes were reported present in all of these organisms except the viruses. In summation, the redox enzymes considered important to this study were the tricarboxylic acid cycle enzymes generally, and specifically, succinoxidase, glutamic dehydrogenase, malic dehydrogenase as well as cytochrome oxidase, citrulline ureidase, and catalase. Urease is also of interest, but, being a hydrolytic enzyme, must be considered separately.

The Magna experiment was based on measuring the change of diffusion current due to enzyme activity in an electrochemical cell. For example, a reaction mixture may consist of glucose and methylene blue (MB) in a buffer solution. When the requisite voltage is applied across the cell, there is no biochemical reaction and a steady-state diffusion current results. If a dehydrogenase enzyme is now introduced, the glucose is oxidized and the methylene blue is reduced in the biochemical process which takes place in the bulk of the solution.



The MBH_2 is oxidized at a rotating anode, resulting in a simple response which is readily monitored instrumentally. The change in output current with time is proportional to the reaction rate of (1) which is in turn proportional to enzyme activity.

In preliminary tests, a large number of substrates were reacted with bacterial extracts (prepared in a French Press) in a large-scale amperometric cell. The substrates included glucose, pyruvate, succinate, acetate, citrate, α -keto glutarate, malate, and glucose plus DPN. Finally, under improved electrode and cell configuration, a "figure of merit" for B. globigii extracts in glucose media was determined to be:

$$10^{-16} \frac{\text{amp/min}}{\text{cell/ml}}$$

This permitted calculation of detectable numbers of cells per ml from the limiting response of the instruments. With the above figure of merit, and using existing laboratory instrumentation for readout, the lower extrapolated detection limit for B. globigii extracts was estimated to correspond to 5×10^4 cells per ml for a 5-minute observation time. Under improved conditions the corresponding limiting detectable response of the instrument was now 2.5×10^{-11} amp. (The lowest concentration of extracts that was subjected to actual test corresponded to 4×10^5 cells per ml.) With the electrolytic cell having a capacity of 1 ml used to obtain most of the data, at the lower detection limit of concentration the total number of cells detected would be 5×10^4 in 5 minutes. Limited testing of whole cells of B. globigii showed greater initial activity at a given cell count than for cell-free extracts.

Several tests were made on samples of background material removed from the air. Particulates removed from El Monte air were used, at levels of approximately 10^3 times the amount of bacteria. In no case was any significant interference observed. No signal was obtained in the absence of enzyme, and the desired reaction proceeded without inhibition with both background material and enzyme present. Several tests showed no output from this detector for spores of B. globigii, either before or after subjecting a spore suspension to the method used to prepare disrupted cells and extracts.

4.1.9.2.4 CONCLUSIONS

Research on bioelectrochemistry was discontinued in October 1963. The possibilities and limitations are considered to have been clearly demonstrated. In general, other types of enzyme reaction giving other types of readout were considered more suitable for eventual development into a field instrument.

4.1.10 RAPID DETECTION OF GROUP A ARBOVIRUSES

4.1.10.1 SUMMARY

Basic studies were undertaken at the University of Utah on the processing and properties of Group A arboviruses.

In basic studies conditions were established for optimal propagation of WEE, EEE, VEE, Sindbis and Semliki Forest viruses. Suspensions of viruses were obtained in most instances which titered greater than 1×10^9 PFU/ml. In replication in chick embryo cells, all viruses reached maximum titers in 10 to 12 hours.

The amount and type of protein added to the growth media affected both the growth rate and stability of these viruses; 5 percent calf serum appeared to increase titers and decrease inactivation.

Liquid partition in a two-phase system, density gradient centrifugation, column chromatography, and adsorption to aluminum phosphate were evaluated for concentration and purification. In liquid partition, bottom phase suspensions in some instances contained infectivity titers greater than 1×10^{11} PFU/ml. All of the viruses exhibited apparent densities of 1.195 in the density gradient.

Antisera were prepared against the purified agents, examined by hemagglutination inhibition techniques, and almost all were highly reactive with a high degree of specificity. The most cross-reactive was the Sindbis virus.

4.1.10.2 INTRODUCTION

Basic studies were undertaken at the University of Utah under the direction of Dr. D. W. Hill, on the growth, concentration, purification, and properties of several types of Group A arboviruses.

It was hoped that these studies would provide information on the application of immunological techniques for the purposes of detection. Specific information on this group of viruses is especially valuable in view of their great potential as BW agents because of their extreme virulence and neurotropic

properties and the unique problems involved in their detection. The latter are due mainly to small size (in many instances 50 mμ or less) and relative non-homogeneity in antigenic properties.

Since knowledge of the basic properties of the Group A arboviruses is a requisite for their detection and since the problems associated with detection of this group are common to all pathogenic viruses, full characterization of the arboviruses was felt desirable and was considered to apply to work at SGC for further development of instrumentation for virus detection.

4.1.10.3 STATUS

The present studies can be divided into those concerned with virus replication, concentration and purification, and elucidation of antigenic properties.

The viruses used in these investigations included Western Equine Encephalitis virus (WEE), Eastern Equine Encephalitis virus (EEE) Venezuelan Equine Encephalitis virus (VEE), Sinbis virus, Semliki Forest virus, and Chickungunya virus. In the case of WEE, EEE, and VEE more than one isolate of each virus was available and was under investigation.

4.1.10.3.1 VIRUS REPLICATION STUDIES

Following the receipts of all except the WEE viruses, all were passed by intracerebral inoculation into five to eight day old mice. Following the development of central nervous system symptoms, the animals were sacrificed, their brains removed aseptically, and 10-percent homogenates were prepared. The viruses in these suspensions were quantitated by a plaque assay technique using primary chick embryo cells*. These virus suspensions were used for subsequent passage in mice and for infection of primary chick embryo cell cultures which were prepared using ten day chick embryos. Melnick's medium containing 5 percent inactivated calf serum and 100 units of penicillin and 100 units of streptomycin per ml was used to support growth of the tissue cells. Unless

*The methods are described in detail in the Fifth Comprehensive and 20th Status Report.

otherwise stated, this medium was used in all subsequent studies. The infectivity titers of viruses grown in vitro are presented below.

INFECTIVITY OF VIRUS PROPAGATED IN PRIMARY CHICK
EMBRYO CELLS

Virus	Plaque Size	Titer (PFU/ml)*
EEE	Small	1.4×10^9
WEE (LP-7)	Large	1.4×10^9
WEE (SP-6)	Large	1.3×10^9
VEE	Medium and Small	2.2×10^8
Sindbis	Small	4.5×10^9
Semliki Forest	Minute	7.0×10^9
Chickungunya	Minute	5.5×10^2

As indicated, the plaque morphologies of each virus were noted and are recorded. The strain of VEE was found to consist of two populations of variants producing different size plaques. All viruses replicated to high titers in the chick cells except Chickungunya virus. The small amounts of infective virus detected could be attributed to carry-over from the original mouse brain homogenate. These suspensions were divided into aliquots and stored frozen at -20°C .

To gain insight into the temporal relationships of the replication of these viruses in chick embryo cells, single-step growth studies were initiated. Primary chick embryo cells were infected with chick-cell-propagated viruses at the following input multiplicities: both WEE viruses, 10; EEE virus, 10; VEE, 0.5; Semliki Forest virus, 1; and Sindbis virus, 10. These differences in multiplicities with the VEE and Semliki Forest viruses were necessary due to their relatively low titers. After an hour of adsorption at 37°C , growth medium

* Plaque forming units per ml.

was added and at timed intervals samples were removed and assayed for infective virus using the conditions for standard plaque assay of WEE virus. A typical growth curve as determined for a WEE virus is presented in Figure 4-43.

The results indicated that all viruses reached maximum titers at approximately the same times, but VEE, EEE, and Semliki Forest viruses failed to reach high titers comparable to those achieved by the WEE virus mutants and Sindbis virus.

On the basis of the hypothesis that the depression of yields of VEE, EEE, and Semliki Forest viruses may have been attributable to interferon contained in the infecting inoculum, extensive experiments with these viruses were initiated. The experimental design also included the propagation of all viruses in large volumes of high titers.

Chick embryo cells were propagated in large Pyrex baking pans (220 x 340 x 40 mm) using "Saran Wrap" to cover them. At 48 hours, when the monolayer cultures were confluent, the large pan cultures contained approximately 1.38×10^8 cells per pan as determined by hemocytometer counts following removal, using 0.25 percent trypsin solution. The cells were infected by the addition of diluted virus suspensions containing approximately 8×10^6 PFU per pan and the fluids harvested 22 to 24 hours later. These suspensions were titrated for infective virus and the data are summarized below.

INFECTIVITY TITRATIONS OF VIRUSES PROPAGATED IN CHICK
EMBRYO CELLS GROWN IN PYREX PANS

Virus	Titer (PFU/ml)
WEE (LP-7)	1.4×10^9
WEE (SP-6)	1.9×10^9
EEE	8.2×10^8
Semliki Forest	1.6×10^9
VEE	2.4×10^9
Sindbis	3.1×10^9

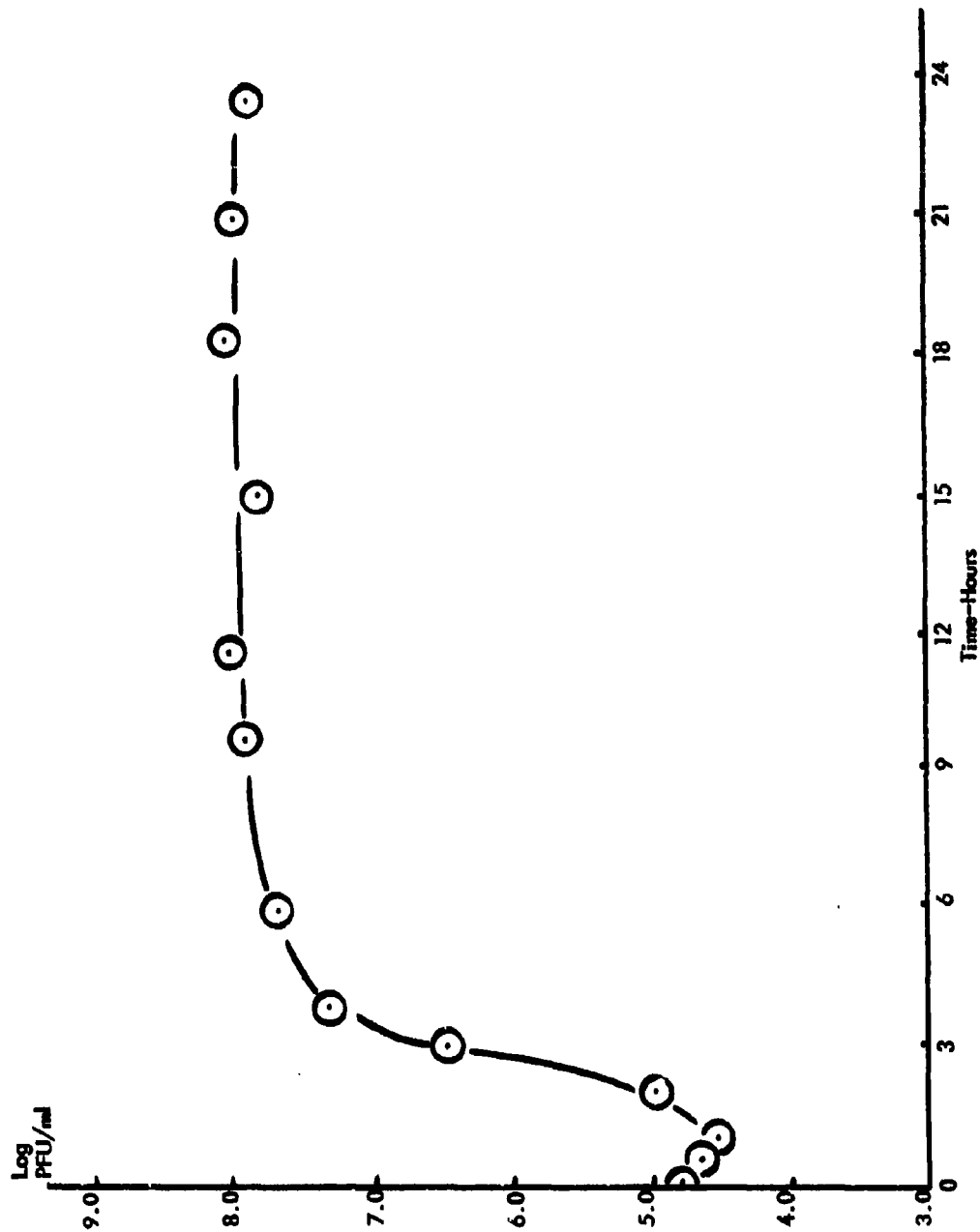


Figure 4-43. Growth Curve for Western Equine Encephalitis Virus

Other experiments in which input multiplicities (ratios of PFU to cells) of 35 were used, resulted in significantly reduced yields of infective virus.

A similar experiment to the above was performed using tissue culture medium (Melnick's) containing 0.1 percent gelatin rather than 1 percent calf serum for virus propagation. The infectivity titers of these suspensions are tabulated below.

INFECTIVITY OF VIRUSES PROPAGATED IN CHICK EMBRYO CELLS
WITH MELNICK'S MEDIUM CONTAINING 0.1 PERCENT GELATIN

Virus	Titer (PFU/ml)
WEE (LP-7)	9.1×10^8
WEE (SP-6)	1.3×10^9
EEE	7.0×10^8
VEE	2.2×10^9
Sindbis	4.1×10^8

Semliki Forest virus was not included in this study. It should be noted that these suspensions contained high titered hemagglutinating activities using male goose erythrocyte suspensions at pH 6.0.

Studies were undertaken to determine the effect of protein on replication and stability of the various group A arboviruses. In those studies determining the effect of protein on replication, aliquots of each infected culture were taken at time intervals during growth and assayed for infectivity. In those experiments determining the stability of virus in various media, following growth, volumes of each virus in each medium were placed in tubes at 37°C and, at time intervals, samples were removed and again assayed for infectivity. A typical example of the results obtained are shown for an EEE virus in Figures 4-44 and 4-45 .

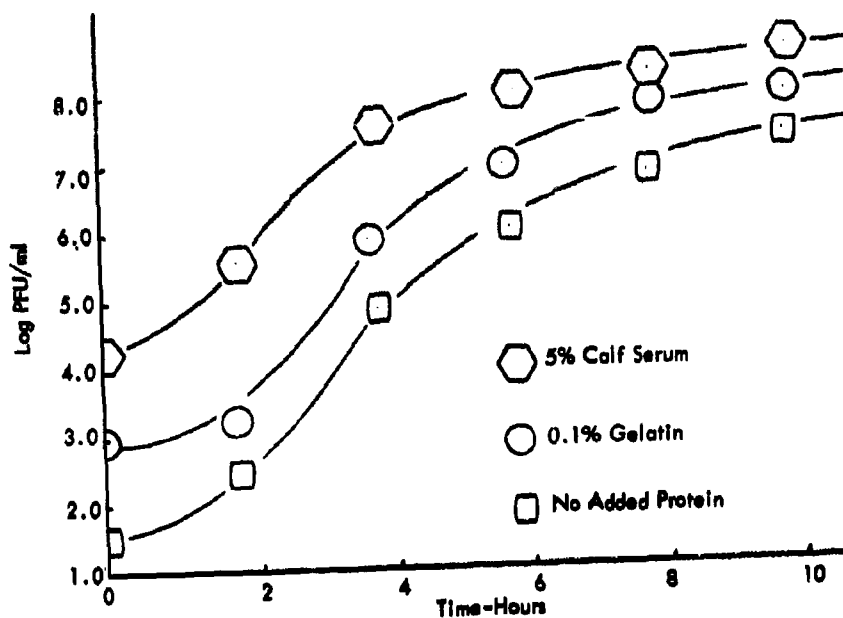


Figure 4-44. Replication of EEE Virus with Different Media

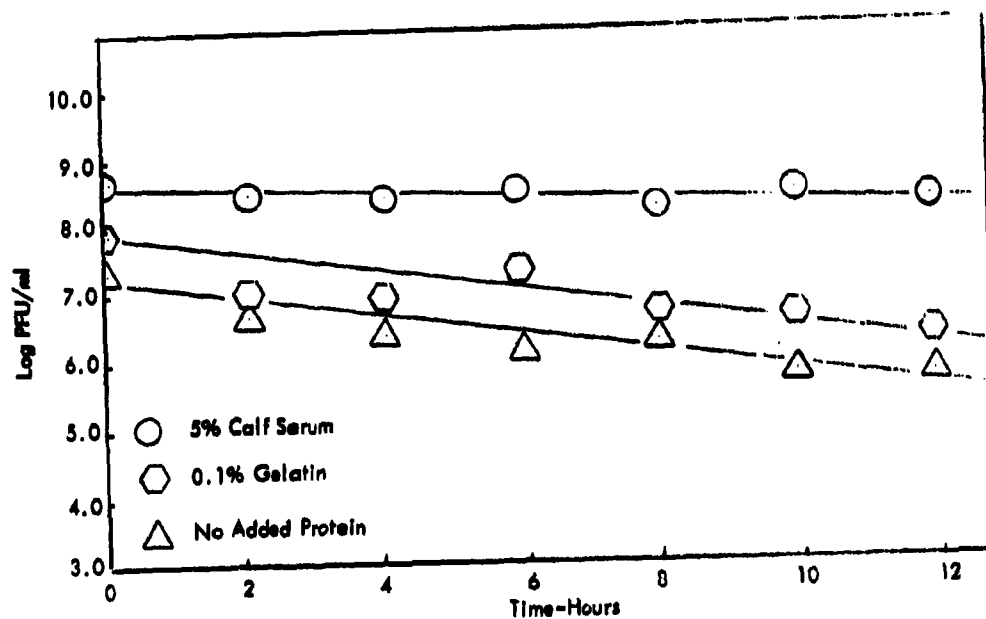


Figure 4-45. Inactivation of EEE Virus at 37°C in Different Media

These and other data indicated that 5 percent calf serum resulted in both increased virus titers and stability at 37°C. Examination of the kinetic details of the replication of the different viruses clearly indicated similarities.

The time of maximum infective virus in the fluids of infected cell cultures was between 10 and 12 hours. Using other media containing gelatin rather than calf serum or a medium containing no added protein, the results indicated similar patterns. The lower apparent yields of virus from cells in the medium with no added protein or with 0.1 percent gelatin could be explicable on the basis of greater rates of inactivation in the media without calf serum or on the basis of a requirement for some constituent of calf serum for maximum virus synthesis by primary chick embryo cells. In any event, it should be noted that high titers of infective virus were obtained from infected chick embryo cells maintained in media with no added protein or with 0.1 percent gelatin.

The results of numerous experiments designed to establish the optimal propagation of all of the viruses to high concentrations in large volumes are summarized as follows:

INFECTIVITY OF SUSPENSIONS OF ARBOVIRUSES
PROPAGATED IN PRIMARY CHICK EMBRYO CELLS

Virus	Infectivity (PFU/ml)
WEE-LP-7	5.2×10^9
WEE-SP-6	6.9×10^9
EVEE	8.3×10^9
VEE-SP	8.3×10^9
VEE-MP	6.4×10^7
Sindbis	9.8×10^9
Semliki Forest	1.5×10^8

In these experiments, primary chick embryo cells were grown for 48 hours in large Pyrex baking pans covered with a plastic wrap. The cell monolayers were washed with cold phosphate buffered saline solution before addition of the 100 ml of growth medium per pan. Based on cell counts made on similar cultures, diluted virus suspensions were added to give an input multiplicity of 10. The infected cells were incubated at 37°C for twelve hours; the virus suspensions were harvested and assayed for infectivity.

A similar experiment was done using tissue culture medium (Melnick's) containing 1 percent and 5 percent inactivated calf serum. Cells in milk dilution bottles were washed, infected, the virus suspensions harvested after 12 hours at 37°C and assayed for infectivity. The table below summarizes these data.

EFFECT OF CALF SERUM CONCENTRATION ON
YIELD OF VIRUSES FROM CHICK EM-
BRYO CELLS

Virus	Infectivity (PFU/ml)	
	1% Calf Serum	5% Calf Serum
WEE-LP-7	1.0×10^9	3.3×10^9
WEE-SP-6	1.3×10^9	4.4×10^9
EEE	4.2×10^8	3.0×10^9
Sindbis	2.6×10^9	7.7×10^9
VEE-SP	1.0×10^8	2.4×10^9
VEE-MP	1.8×10^7	4.8×10^7
Semliki Forest	5.0×10^6	6.2×10^8

The results indicate that the use of a 5 percent concentration of calf serum was considerably better than the lower concentration for supporting viral growth.

4.1.10.3.2 CONCENTRATION AND PURIFICATION

Liquid partition in a two-phase system, density gradient centrifugation, column chromatography, and adsorption to aluminum phosphate were evaluated for concentration and purification of these viruses.

Distribution of viruses in a two-phase system of sodium dextran sulfate and polyethylene glycol solution was studied using an adaption of the methods of Albertsson⁽¹⁾. Most of the viruses were found to concentrate in the bottom phase of the system (sodium dextran sulfate). Viruses were concentrated approximately 50 to 95 fold by this method. Bottom phase suspensions in some instances contained infectivity titers greater than 1×10^{11} PFU/ml.

Cesium chloride density gradient properties of the various viruses were compared. In these studies viruses were carefully layered on solutions of cesium chloride in phosphate buffer 7.0 and centrifuged, and fractions of the gradient were collected and assayed for density and infectivity. The data clearly indicated similar properties of apparent buoyant density of these viruses in cesium chloride. WEE, EEE, VEE, Sindbis, and Semliki Forest viruses all appear to exhibit densities of 1.195. Their apparent low density is compatible with data reported by others, e.g., Pfefferkorn and Hunter⁽²⁾, concerning the lipid content of these viruses.

An attempt was made to combine the two-phase polymer system with the aluminum phosphate gel concentration and purification methods of Pfefferkorn and Hunter⁽²⁾ and Miller and Schlesinger⁽³⁾. In these experiments, WEE virus, concentrated in the bottom phase (NA dextran sulfate) of the two-phase liquid partition system indicated above, was added to fresh aluminum phosphate gel. In one instance the virus was precipitated from the dextran sulfate with KCl prior to use. After a suitable incubation, the gel was centrifuged and washed 3 times with 0.01M phosphate buffer, pH 7.2. Virus was eluted by suspending the gel in 0.25M phosphate buffer (pH 7.2). Three elutions were done. The results are presented in Tables 4-19 and 4-20.

The above data shows that the virus adsorbed to the aluminum phosphate gel, as indicated by the amount of virus present in the supernatant after adsorption. However, the virus was not readily eluted under the experimental

Table 4-19

ADSORPTION AND ELUTION OF WEE VIRUS IN KCl-PRECIIPITATED
BOTTOM PHASE FROM ALUMINUM PHOSPHATE GEL

<u>Preparation</u>	<u>Volume</u>	<u>Titer (PFU/ml)</u>
KCl Precipitated Bottom Phase Virus	5 ml	1.51×10^9
Supernatant After Adsorption	6.3 ml	1.86×10^4
Supernatant After First Wash	6.1 ml	9.3×10^1
Supernatant After Second Wash	5.9 ml	1.06×10^5
Supernatant After Third Wash	5.4 ml	2.1×10^5
First Elution	5.3 ml	1.63×10^5
Second Elution	5.4 ml	3.28×10^6
Third Elution	5.1 ml	2.3×10^7

Table 4-20

ADSORPTION AND ELUTION OF WEE VIRUS IN BOTTOM PHASE
FROM ALUMINUM PHOSPHATE

<u>Preparation</u>	<u>Volume</u>	<u>Titer (PFU/ml)</u>
Bottom Phase	5 ml	6.3×10^9
Supernatant After Adsorption	5.9 ml	6.9×10^7
Supernatant After First Wash	5.6 ml	1.5×10^7
Supernatant After Second Wash	5.7 ml	1.75×10^7
Supernatant After Third Wash	5.8 ml	1.7×10^6
First Elution	5.4 ml	5.0×10^6
Second Elution	5.3 ml	2.3×10^7
Third Elution	5.3 ml	3.7×10^7

conditions described. One explanation was that virus was inactivated during the long period of elution. Further experiments indicated, however, that less virus was recovered from gel by elution for 90 minutes than by the previous methods using longer elution times.

The technique of column chromatography for the purification of virus preparations was introduced by Taverne, Marshall, and Fulton⁽⁴⁾. This method has an important advantage in that all operations can be carried out at constant pH and elution is accomplished by alteration in phosphate buffer concentration. Roizman and Roane⁽⁵⁾ demonstrated chromatographically on calcium phosphate that two strains of Herpes simplex virus gave elution patterns distinct for each variant.

In these studies WEE virus in KCI-precipitated bottom phase was adsorbed onto a column of calcium phosphate and eluted with a gradient concentration of phosphate buffer (pH 7.0). The eluate for the column was passed through a conductivity flow-through cell and the signal from the conductivity meter (Radiometer CDM₂ conductivity meter, Radiometer, Copenhagen, Sweden) was fed to a dual-channel recorder (Speedomax, Model G, Leeds and Northrup Co., Philadelphia, Pa.). The out-flow from the conductivity cell was collected volumetrically and samples were assayed for infectivity. The results are presented in Figure 4-46.

It appeared that most of the virus was eluted in a volume that has approximately three to four times that of the original sample. However, as a quantitative study was not conducted, the recovery rate was not determined. The first high peak in the conductivity curve was attributed to the high concentration of KCI present in the sample.

4.1.10.3.3 ANTIGENIC PROPERTIES

Rabbits, guinea pigs, and goats were used for preparation of antisera. Suspensions of WEE, EEE, VEE, and Sindbis viruses propagated in mouse brain or in chick embryo cells were injected subcutaneously and the animals bled by cardiac puncture. All virus suspensions used for immunizing rabbits were inactivated by heat or formalin. Goats were immunized with infective WEE virus

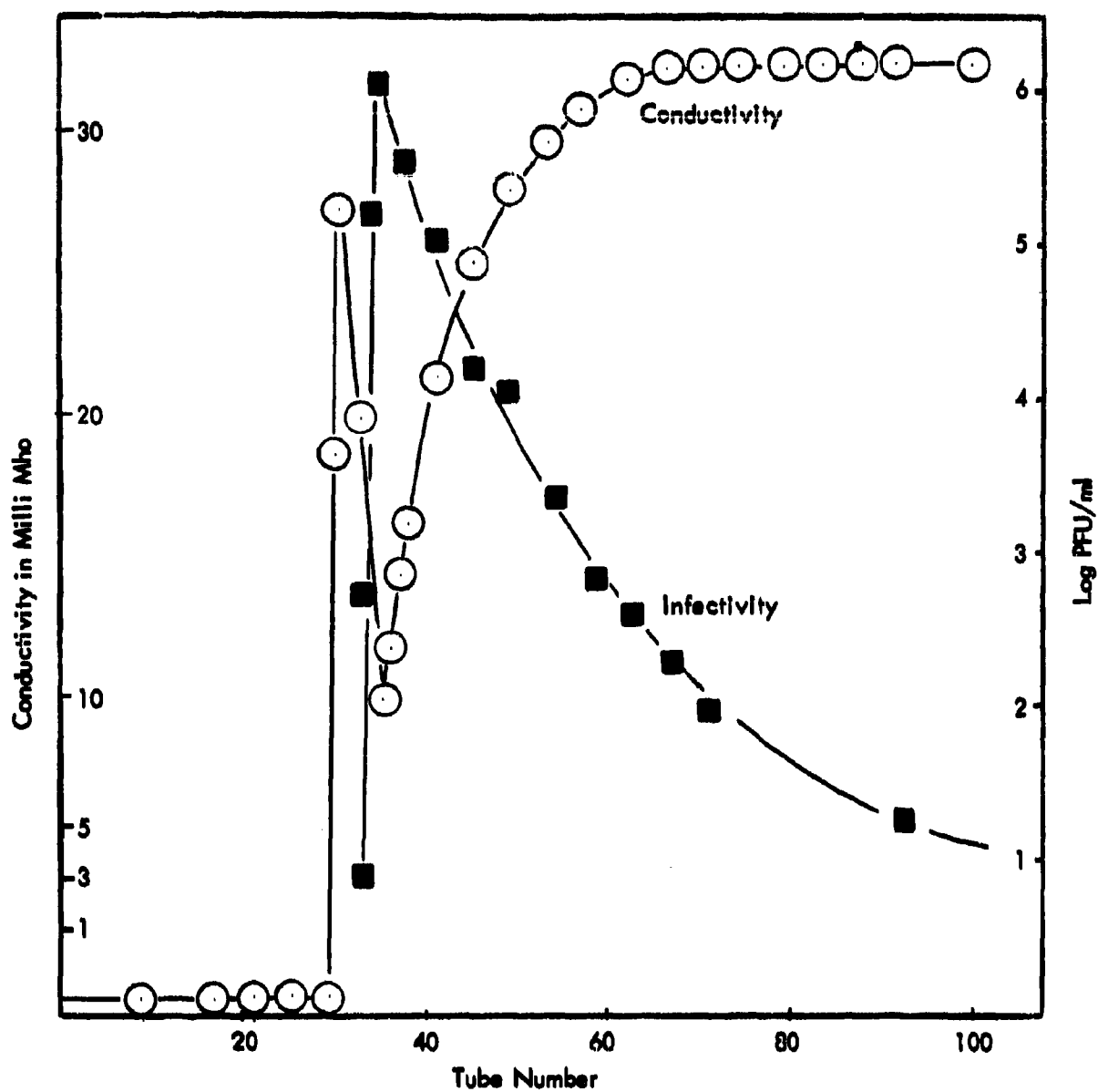


Figure 4-46. Elution of WEE Virus from a Calcium Phosphate Column

suspensions. Antiserum was also obtained from the horses which had been naturally infected with WEE virus.

The virus antisera were examined for antibodies using the technique of hemagglutination inhibition. The basic methods used were those described by Clarke and Casals⁽⁶⁾ using gander erythrocytes. The hemagglutinating antigens used were prepared from acetone-ether extracted suspensions of virus propagated in chick embryo cells in Melnick's medium containing 5 percent inactivated calf serum. All antisera were treated with acid-washed kaolin to remove non-specific inhibitors of virus hemagglutination.

With immunized rabbits, significant hemagglutination inhibition titers were evident as early as two weeks following a single dose of infective virus. Especially with animals which received EEE and VEE virus suspensions of infectivity titers of greater than 1×10^9 PFU/ml, central nervous symptoms were noted, but the animals survived.

The accumulated data on the hemagglutination inhibition titers of these sera are presented in Table 4-21. These data indicate that highly reactive antisera to all but the Sindbis virus were obtained, and that the antibody titers of individual animals varied considerably. The examination of these sera using six different virus antigen preparations support the conclusion that the cross-reactions of the viruses studied were at a relatively low level. The most-reactive virus studied was the Sindbis virus.

4.1.10.4 CONCLUSIONS

Data obtained from these studies have contributed much basic information concerning the growth, concentration and purification, and antigenic and other properties of several Group A arboviruses.

This type of information is considered requisite for development of methods for the detection of this group of viruses. It is felt, however, that further basic information will be required before adequate detection by immunological techniques can be developed.

Table 4-21

HEMAGGLUTINATION INHIBITION TITERS OF ANTISERA USING ACETONE-
ETHER TREATED ARBOVIRUS ANTIGENS

ANTISERUM	HEMAGGLUTINATION INHIBITION TITERS ¹						
	ANTIGEN						
	WEE LP-7	WEE SP-6	SINDBIS	EEE	VEE MP	VEE SP	
Rabbit Anti WEE-LP-7 (CC-8) ²	+ 640	+ 640	80	-	-	-	
Rabbit Anti WEE-LP-7 (CC-8)	640	640	+ 80	-	-	-	
Rabbit Anti WEE-LP-7 (MB-9) ³	1280	+1280	160	10	+ 20	10	
Rabbit Anti WEE-LP-7 (MB-9)	+ 640	320	40	-	-	-	
Rabbit Anti WEE-SP-6 (CCG-2) ⁴	+ 320	+ 320	-	-	-	-	
Rabbit Anti WEE-SP-6 (CCG-2)	160	160	20	-	-	-	
Rabbit Anti WEE-LP-7 (CC-Hyperimmune)	>5120	+5120	+1280	-	+ 20	-	
Rabbit Anti WEE-LP-7 (CC-Hyperimmune)	320	+ 320	+ 160	-	-	+ 10	
Rabbit Anti WEE-LP-7 (MB-?) ⁵	320	160	+ 160	-	-	-	
Rabbit Anti EEE (MB-9)	-	40	10	1280	-	80	
Rabbit Anti EEE (MB-9)	-	-	-	80	-	-	
Rabbit Anti EEE (MB-9)	+ 20	10	+ 20	160	10	-	
Rabbit Anti EEE (MB-?)	-	-	+ 10	40	-	-	
Rabbit Anti EEE (MB-?)	-	-	+ 10	40	-	-	
Rabbit Anti EEE (MB-?)	+ 40	+ 40	+ 10	40	-	-	

Table 4-21 (Continued)

HEMAGGLUTINATION INHIBITION TITERS OF ANTISERA USING ACETONE-
ETHER TREATED ARBOVIRUS ANTIGENS

ANTISERUM	HEMAGGLUTINATION INHIBITION TITERS ¹					
	ANTIGEN					
	WEE LP-7	WEE SP-6	SINDBIS	EEE	VEE AMP	VEE SP
Rabbit Anti VEE-SP (CCG-2)	+ 10	+ 10	-	-	2560	1280
Rabbit Anti VEE-SP (CCG-2)	20	20	+ 40	10	1280	1280
Rabbit Anti VEE-AMP (CCG-2)	20	20	+ 20	-	1280	640
Rabbit Anti Sindbis (CC-8)	+ 40	+ 40	160	-	-	-
Rabbit Anti Sindbis (CC-8)	+ 40	+ 40	+ 40	-	-	-
Rabbit Anti Sindbis (MB-9)	20	+ 20	20	-	-	-
Rabbit Anti Sindbis (MB-9)	20	+ 20	+ 20	-	-	-
Rabbit Anti Sindbis (CCG-2)	-	-	+ 10	-	-	-
Goat Anti WEE-LP-7 (CC-Hyperimmune)	5120	2560	640	-	-	-
Goat Anti WEE-LP-7 (CC-Hyperimmune)	5120	2560	1280	-	-	-
Horse, Naturally infected	160	80	40	-	-	-
Horse, Naturally infected	+ 640	+ 320	640	+ 10	+ 10	-

Table 4-21 (Continued)

HEMAGGLUTINATION INHIBITION TITERS OF ANTISERA USING ACETONE-
ETHER TREATED ARBOVIRUS ANTIGENS

- ¹ Reciprocal of endpoint titer. + = partial inhibition. - = neg. at 1:10.
- ² CC-8 = Animal immunized with virus propagated in chick embryo cells and bled eight weeks later.
- ³ MB-9 = Rabbit immunized by injection of infective mouse brain homogenate and bled nine weeks later.
- ⁴ CCG-2 = Rabbit immunized by injection of virus propagated in chick embryo cells in Melnick's medium with 0.1% gelatin.
- ⁵ MB-? = Animal immunized by injection of infective mouse brain homogenate and bled at some unknown time later.

It is hoped that the information obtained here can be applied for development of detection methods of pathogenic viruses other than those included in the arbovirus group.

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4.1.11 STUDIES ON IMMUNOLOGICAL DETECTION OF VIRUS AND VIRUS CARRIER MEDIUM

4.1.11.1 SUMMARY

A study conducted at UCLA sought to develop methods for obtaining purified antigen and antibody preparations. These could increase specificity and sensitivity in the immunological detection of virus and virus carrier medium.

Fowlpox virus purified by absorption to and elution from cation and ion exchange resins was relatively free of contaminating materials and was both serologically and biologically active. Methods utilizing DEAE-cellulose resulted in the purest virus preparations. The preparative methods preceding column chromatography of the crude virus had little effect on recovery following chromatography. The passive hemagglutination test was of little or no value in the detection and characterization of virus antigen fractions. The complement fixation, latex agglutination, and latex agglutination-fluorescence tests were very useful and specifically reactive.

Similar procedures were used to fractionate egg antigens. The various fractions were characterized and compared by two-dimensional double diffusion in gel. Antigens common to all of the above-mentioned materials were found prior to discontinuation of this effort.

4.1.11.2 INTRODUCTION

The purpose of the present study was to develop methods for the improvement of specificity and thus of sensitivity in the immunological detection of viruses and virus carrier medium. The detection of virus particles as implied through detection of their carrier medium would be advantageous in that far fewer types of materials would have to be detected. Some information was also obtained on the application of specific serological tests to virus detection. These studies were conducted at the Department of Bacteriology, UCLA, under the direction of Dr. M. J. Pickett.

It was felt that specificity could be improved by development and application of methods for purifying antigenic materials. Fowlpox virus and various types of chicken egg materials were used in these studies.

In investigations of egg carrier medium, it was hoped that one or a few antigens could be isolated which were common to various components of embryonated eggs of various ages and grown under various conditions.

Isolation of a single common antigen would provide a simple and easy method for obtaining a specific high titered reagent for immunological detection of egg carrier medium and could lead to the development of a specific immunological system which could detect virus carrier medium under all conditions required for growth and dispersion of almost all types of egg-grown virus aerosols.

It was hoped that methodology developed here would have application to the detection of aerosolized pathogenic viral agents.

4.1.11.3 STATUS

4.1.11.3.1 ISOLATION, PURIFICATION, AND STUDY OF FOWLPOX VIRUS

In these studies, fowlpox virus was grown by inoculation of the chorioallantoic membrane (CAM) of 11-day old chick embryos, and incubation of the infected eggs for 3 days at 37°C. The infected CAM membranes were removed and in most instances virus was isolated from them by the selective fluorocarbon deproteinization method described by Gessler, et al⁽¹⁾ and/or extracted and pelleted according to the method of Jollik⁽²⁾. Virus pellets were variously prepared utilizing sonication and pelleting in 36 percent sucrose, followed by centrifugation in sucrose gradients (25 to 40 percent sucrose in Tris buffer). Virus pellets prepared by these methods were stored at -20°C.

The infectivity titers of the virus preparations were determined by (1) inoculation of chick embryos and observing for either death of the embryo and/or typical pocks on the CAM and/or pathological changes of the CAM, (2) inoculation of 3-day-old cockerels and observing for the presence of typical pocks and/or lesions of the skin and/or mucosal membranes, and (3) a tissue culture plaque technique as recommended by Porterfield and Allison⁽³⁾.

Both ion exchange and gel filtration column chromatography were used for purifying the fowlpox virus. In the former instance, both a diethylaminoethylether (DEAE-cellulose) anion exchange resin and carboxymethyl cellulose

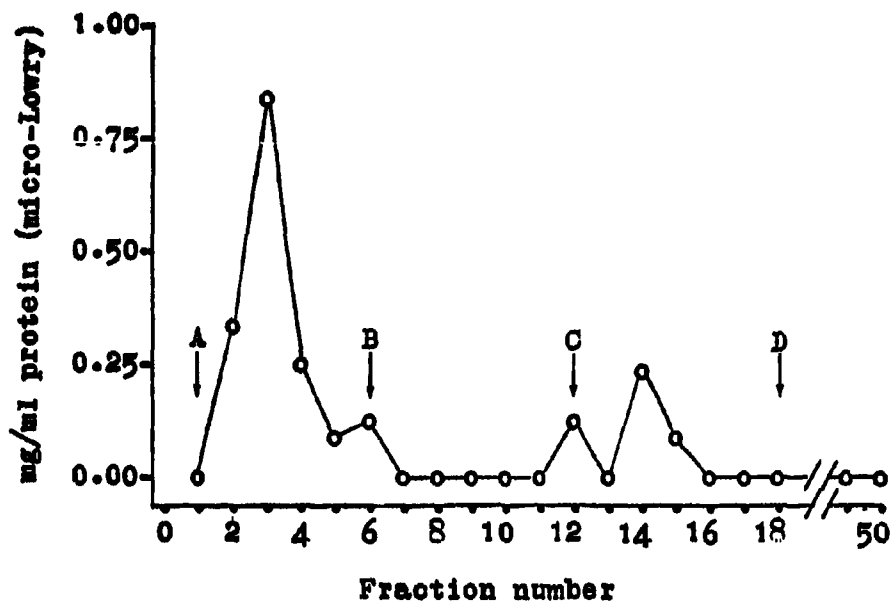
(CM-cellulose) cation exchange resin were used and, in the latter instance, Sephadex (G75, G100 and G200) and pearl-condensed agar⁽⁴⁾ were employed. The virus suspensions were added to the column and eluted with appropriate buffer solutions. Single or gradient concentrations of phosphate buffers were employed for virus elution.

The various fractions eluted were tested for protein by absorption at 200 m μ , Biuret, and/or the micro-Lowry⁽⁵⁾ methods, and were subjected to various serological tests. Several types of serological tests were employed, including those based on (1) two-dimensional double diffusion in gels⁽⁶⁾, (2) direct, passive, and tanned red blood cell hemagglutination, (3) complement fixation, (4) latex agglutination and latex agglutination-fluorescence⁽⁷⁾, and (5) virus neutralization (for details see Fourth Comprehensive Report⁽⁸⁾). The antibodies required for these tests were obtained by injecting rabbits subcutaneously with specific antigens suspended in Freund's complete adjuvant.

Figure 4-47 represents a typical distribution of protein in the various fractions collected after absorption of virus and eluting from a DEAE-cellulose column. In these experiments, extracted virus had been subjected to centrifugation in a sucrose gradient prior to absorption on the column. The various fractions were also tested for virus by a number of specific serological methods. Antisera to both CAMV (infected chlorioallantoic membrane) and CAM were also used in these tests to further evaluate the purity of the various fractions.

It can be seen that three peaks were obtained, the first of which contained infectious virus as indicated by the inoculation of embryonated eggs. Fractions from the first peak also reacted specifically in the complement fixation and latex agglutination tests. Tanned red blood cell hemagglutination and direct hemagglutination tests (not shown here) were of no value in determining the presence of viruses.

Figure 4-48 represents the results obtained with a "batch" fractionation of fowlpox virus. In this experiment, a virus-infected chlorioallantoic membrane (CAMV) was sonicated for 3 minutes to disperse the virus and then absorbed onto a DEAE-cellulose column and eluted with a single phosphate buffer (0.02 M,



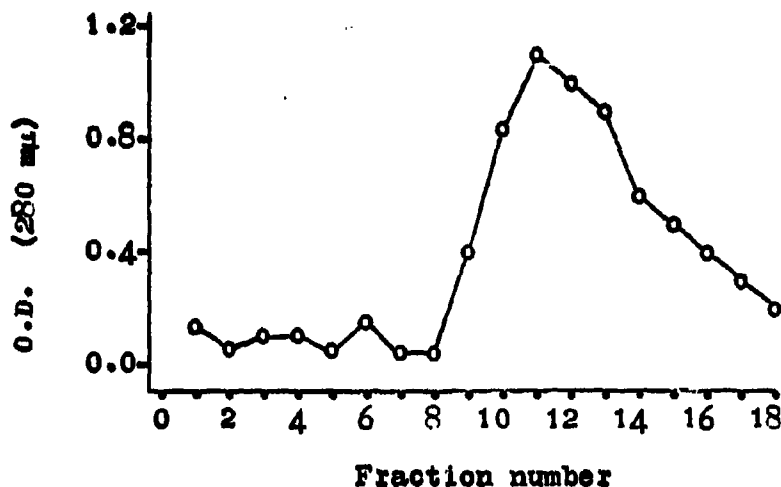
 Embryonated egg inoculation results as indicated by pocks
 and/or pathological changes of the chorioallantoic membrane
 and/or death of the chick embryo (only fractions 1-14 done)
 0 + + 0 0 0 0 0 0 0 0 0 0 0 0

 Qualitative complement fixation tests with known serums
 anti-CAMV (#3) 0 + + + + + + + + 0 0 0 0 0 0 0 0 0 0 // + to 50
 anti-CAM (#7) 0 + 0 + 0 0 0 0 0 0 0 0 0 0 0 0 0 0 // ± to 50

 Qualitative latex agglutination tests with known serums
 anti-CAMV (#2) 0 + + 0 0 0 0 0 0 0 0 0 0 0 0 0 0 // 0 0
 anti-CAM (#7) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 // 0 0

 Tanned red blood cell hemagglutination with known serums
 anti-CAMV (#3) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 // 0 0

Figure 4-47. Virus Purification by DEAE-Cellulose Column
 Chromatography (Elution Via a Gradient Concentration
 of Phosphate Buffer and Phosphate Buffer Plus
 NaCl, pH 7.1)



 Embryonated egg inoculation results as indicated by pocks
 and/or pathological changes of the chorioallantoic membrane
 and/or death of the chick embryo

0 0 0 0 0 0 0 0 + + + + + + + + + +

 Young chick infectivity indicated by pocks on their necks

0 0 0 0 0 0 0 0 + 0 0 0 0 0 + 0 0 0

 Qualitative complement fixation tests with known serums

anti-CAMV (#3) 0 0 0 0 0 0 0 0 + + 0 0 0 0 0 0 0 0

anti-CAM (#7) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

 Tanned red blood cell hemagglutination with known serums

anti-CAMV (#3) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

Figure 4-48. Virus Purification by DEAE-Cellulose Column
 Chromatography (Elution by the Batch Fractionation
 Method Using a Single Concentration of 0.02M
 Phosphate Buffer, pH 7.1)

pH 7.1). A single protein peak, as determined by absorption at 280 m μ , revealed the presence of virus by egg and chick inoculation and by complement fixation tests. The tanned red blood cell hemagglutination test again did not yield usable results.

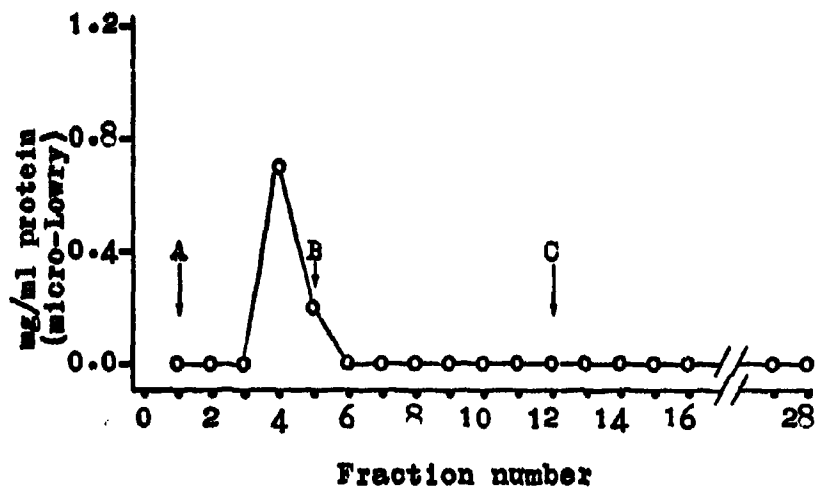
Figures 4-49 and 4-50 represent similar experiments when using CM-cellulose. Virus isolation was possible with the cation exchange resin but results from the complement fixation tests indicated that isolated fractions were not as free of contaminating CAM antigen as those isolated on DEAE-cellulose.

Additional experiments were run with the CM-cellulose resin in which 0.05 percent Tween 80 was added to the eluting buffer. The results indicated that the use of Tween 80 does permit the demonstration of several peaks and the recovery of significantly more virus.

Molecular filtration of crude fowlpox virus preparations on Sephadex G-100 or on pearl-condensed agar columns was not successful as a method of separating fowlpox virus from contaminating host materials. These procedures are probably best used as adjuncts to other purification methods (i.e., chromatography on DEAE-cellulose) for the removal from virus preparations of interfering host materials which are of small molecular weight (viz., less than 100,000 MW), and probably non-antigenic.

The above experiments show that fowlpox virus can be purified by adsorption onto and elution from both anionic and cationic exchange columns. Virus obtained by these methods was relatively free of contaminating host tissue antigens and was both serologically and biologically reactive. It has been further demonstrated that a buffer-saline gradient need not be used and that a single phosphate buffer (0.02 M, pH 7.1) will serve to elute all of the virus. Additionally, DEAE-cellulose-eluted virus was more free of contaminating antigens than was the virus recovered from CM-cellulose columns.

The preparative procedures used prior to the column chromatography of the crude virus had little effect on the amount of virus recovered following chromatography. The use of fluoro-carbon extraction, sucrose gradient centrifugation, and/or pelleting of the virus in sucrose did little more than remove the gross contaminants from the virus suspensions. Hence, chromatography can be performed equally well on crude unprocessed virus containing tissues. The only

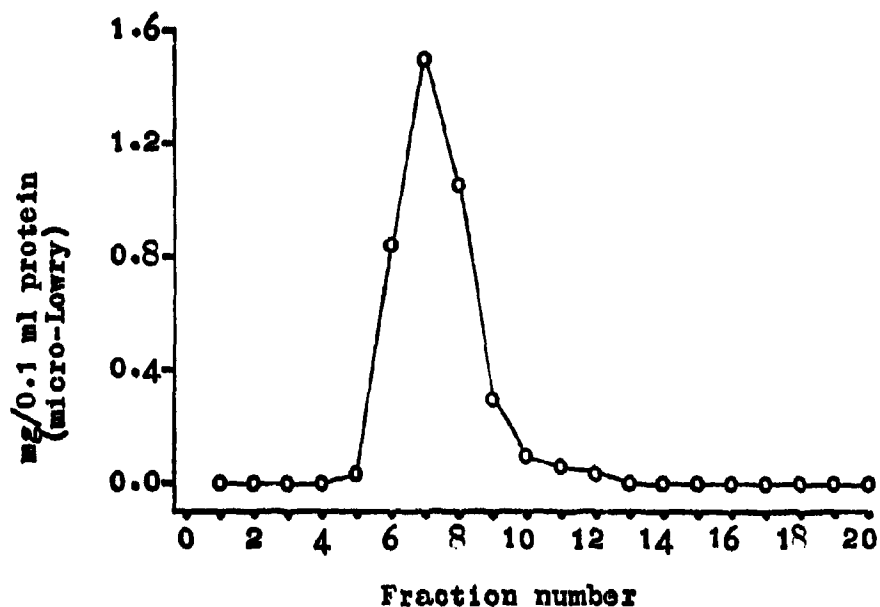


 Embryonated egg inoculation results as indicated by pocks
 and/or pathological changes of the chorioallantoic membrane
 and/or death of the chick embryo (only fractions 1-10 done)
 0 0 + + + 0 0 0 0 0

 Qualitative complement fixation tests with known serums
 anti-CAMV (#3) 0 0 0 + + + + 0 0 0 0 0 0 0 0 0 0 0 0
 anti-CAM (#7) 0 0 + + + + 0 0 0 0 0 0 0 0 0 0 0 0

 Qualitative latex agglutination tests with known serums
 anti-CAMV (#2) 0 0 0 0 + + + 0 0 0 0 0 0 0 0 0 0 0 0
 anti-CAM (#7) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

Figure 4-49. Virus Purification by CM-Cellulose Column
 Chromatography (Elution by a Gradient Concentration
 of Phosphate Buffer and Phosphate Buffer Plus Saline,
 pH 7.1)



Qualitative complement fixation tests with known serums																			
anti-CAMV (#5)	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0
anti-CAM (#7)	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0

Qualitative latex agglutination tests with known serums																			
anti-CAMV (#5)	0	0	0	0	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0
anti-"V" (#11)	0	0	0	0	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0
anti-CAM (#7)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 4-50 Virus Purification by CM-Cellulose Column Chromatography (Elution by the Batch Fractionation Method Using a Single Concentration of Phosphate Buffer, pH 7.1)

advantage in these preparatory procedures is that they extend the life of the columns by preventing their contamination with large quantities of colloidal elements.

Fowlpox virus is not a hemagglutinating virus when tested in a direct hemagglutination test with human O, Rh negative, chicken, or sheep red blood cells. The tanned and untanned red blood cell tests using human O, chicken, or sheep cells in a passive hemagglutination test were of little or no value in the detection and characterization of virus antigens. The complement fixation, latex agglutination, and latex agglutination-fluorescence tests were very useful and specifically reactive in these studies. The latex tests have the additional advantage of allowing the determination of quantitative results. Another quantitative method which has shown promise of enumerating fowlpox virus and studying neutralizing antibody is a tissue culture plaque technique utilizing chick fibroblasts. Additional work must be done with the tissue culture methods, however, before any definitive conclusions can be made concerning their value and usefulness.

4.1.11.3.2 ISOLATION, PURIFICATION, AND STUDY OF ANTIGENS FOUND IN EMBRYONATED AND NON-EMBRYONATED HENS' EGGS

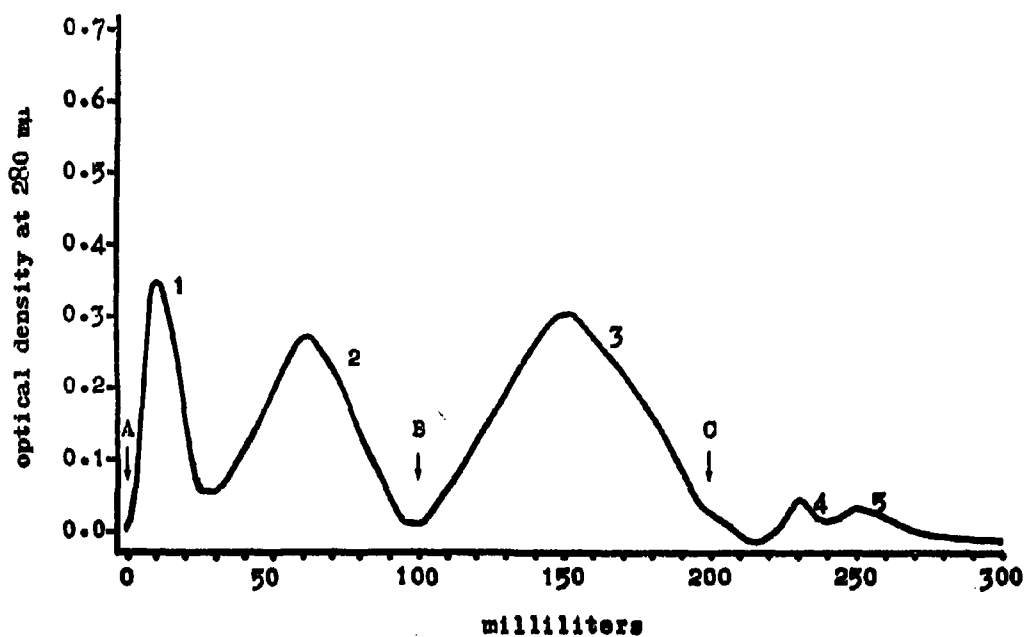
Antigens for egg white, normal whole egg, and 11-day-embryonated whole egg were prepared and fractionated by ion exchange chromatography using a DEAE-cellulose resin and procedures similar to those described above. Diluted egg white, homogenized whole egg, and 11-day-embryonated egg were absorbed on the column and eluted with a gradient concentration of phosphate buffer media. The various fractions were evaluated for protein concentration by the same procedures as indicated above. The various fractions were also characterized and compared by the two-dimensional double diffusion in gel. Antibodies used for these tests were obtained by subcutaneous injection of the various specific antigens into rabbits. The column fractions and other materials which were too dilute for use in serological and chemical procedures were concentrated by dialysis against polyethylene glycol (Carbowax-6000).

The results of the fractionations of egg proteins in egg white, normal whole egg, and 11-day-embryonated whole egg are shown in Figures 4-51, 4-52, and 4-53, respectively. It was shown serologically by means of gel diffusion that precipitating antigens were present in every peak. Reactions of identify and non-identify were used for the determination of each antigen. Time did not permit a complete comparison of all antigens isolated, so an antigen which was common to all egg materials and which was found in quantity in these materials was arbitrarily chosen and characterized. This is the antigen found in peaks No. 3, 8, and 17 (Figures 4-51 through 4-61). The batch fractionation of egg white permitted the isolation of this same antigen (Figures 4-60 and 4-61).

Serological identification of this and other egg antigens was not completed because of the time limitations. Similarly, animals immunized with this purified antigen had insufficient time to produce significant antibody titers and this portion of the project was deferred.

4.1.11.4 CONCLUSIONS

Studies at UCLA have provided much useful information on the methodology for obtaining purified virus and egg antigens. Some information was also obtained on the various types and distributions of egg antigen fractions in different egg preparations and on the application of various serological tests for detection of virus. This information may have application in the present or future programs for the detection of viruses. Serological identification of fractionated egg antigens was not completed and insufficient data was thus available to determine if one or a few of the various antigen fractions could be used to elicit antibodies for the detection of various kinds of egg carrier medium.



Fractionation of Egg Protein and Antigen Map

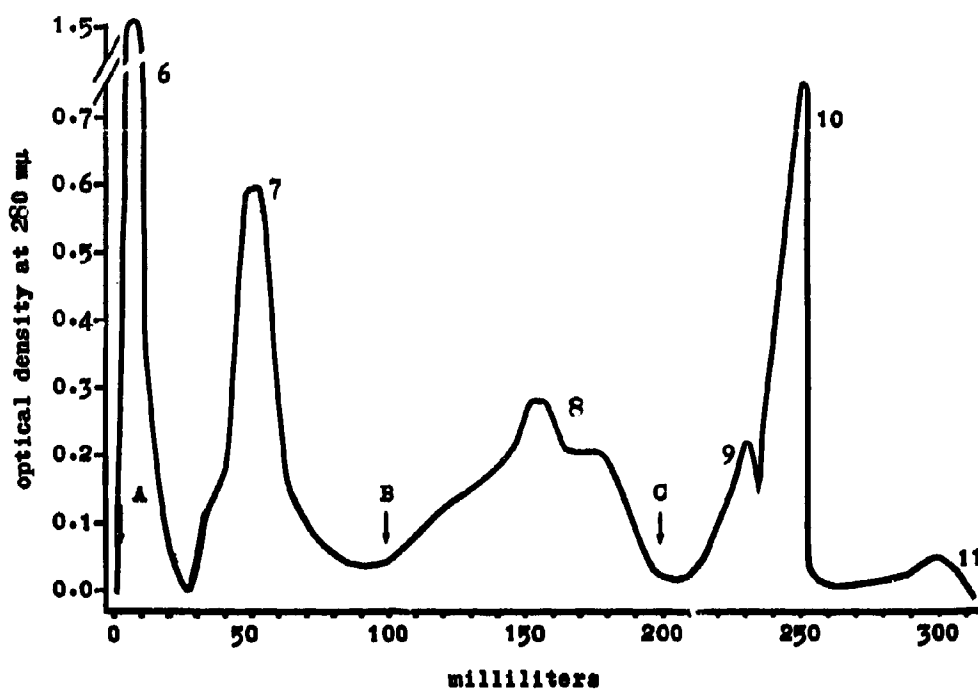
Gradient Elution Schedule

Initial buffer - 750 ml containing 0.02M glycine

- A. 0.02M KH_2PO_4 , 0.02M K_2HPO_4 , and 0.02M glycine in 1 liter
- B. 0.1M KH_2PO_4 , 0.1M NaCl, and 0.02M glycine in 1 liter
- C. 0.1M KH_2PO_4 , 0.1M NaCl, 0.03M HCl, and 0.02M glycine in 1 liter

Graph shows the fractionation of egg white (diluted 1:2 in 0.02M glycine). Antigens present in each peak are shown beneath the graph. The width of the line indicates the quantity of antigen present as determined by double diffusion in two dimensions in gels.

Figure 4-51. Fractionation of Egg Proteins and Antigen Map



Fractionation of Egg Proteins and Antigen Map

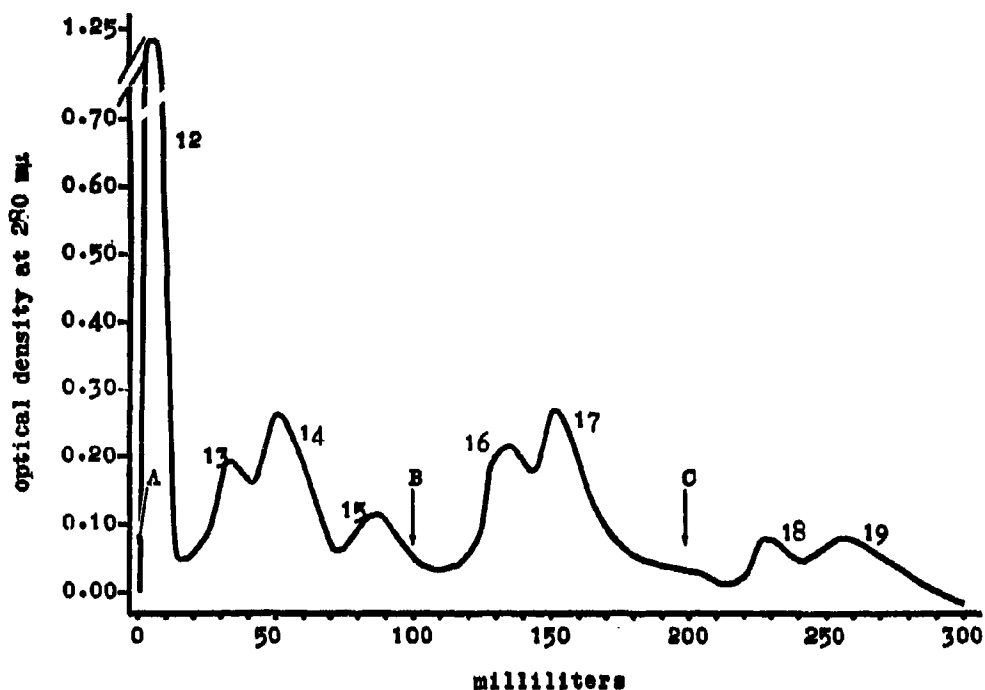
Gradient Elution Schedule

Initial buffer - 750 ml containing 0.02M glycine

- A. 0.02M KH_2PO_4 , 0.02M K_2HPO_4 , and 0.02M glycine in 1 liter
- B. 0.1M KH_2PO_4 , 0.1M NaCl, and 0.02M glycine in 1 liter
- C. 0.1M KH_2PO_4 , 0.1M NaCl, 0.03M HCl, and 0.02M glycine in 1 liter

Graph shows fractionation of normal whole egg (diluted 1:2 in 0.02M glycine). Antigens present in each peak are shown beneath each graph. The width of lines indicates the quantity of antigen present as determined by double diffusion in two dimensions in gels. Dotted line indicate partial identity between antigens.

Figure 4-52. Fractionation of Egg Proteins and Antigen Map



Fractionation of Egg Proteins and Antigen Map

Gradient Elution Schedule

Initial buffer - 750 ml containing 0.02M glycine

- A. 0.02M KH_2PO_4 , 0.02M K_2HPO_4 , and 0.02M glycine in 1 liter
- B. 0.1M KH_2PO_4 , 0.1M NaCl, and 0.02M glycine in 1 liter
- C. 0.1M KH_2PO_4 , 0.1M NaCl, 0.03M HCl, and 0.02M glycine in 1 liter

Graph shows the fractionation of 11-day embryonated whole egg (diluted 1:5 in 0.02M glycine). Antigens present in each peak are shown beneath each graph. The width of lines indicates the quantity of antigen present as determined by double diffusion in gels.

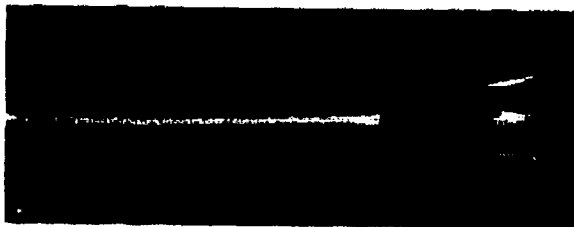
Figure 4-53. Fractionation of Egg Proteins and Antigen Map

IDENTIFICATION OF EGG ANTIGENS BY GEL DIFFUSION

The wells in the following figures will be lettered from left to right (viz. A-E or F) for easy identification. Peak number identities are given in Figures 4-51, 4-52, and 4-53.

Figure 4-54

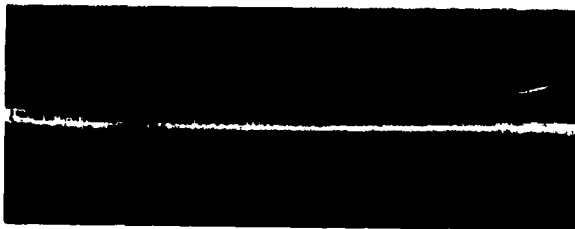
Egg proteins from egg
white fractionation.
Reference T5-B51



A - antigens from peak #1
B - antigens from peak #2
C - antigens from peak #3
D - antigens from peak #4
E - antigens from peak #5
F - antigens from peak #5
Gutter - anti-normal whole
egg

Figure 4-55

Egg proteins from egg
white fractionation.
Reference T6-B51.



SGC/405

A - antigens from peak #1
B - antigens from peak #2
C - antigens from peak #2
D - antigens from peak #3
E - antigens from peak #4
F - antigens from peak #5
Gutter - anti-normal whole
egg

Figure 4-56

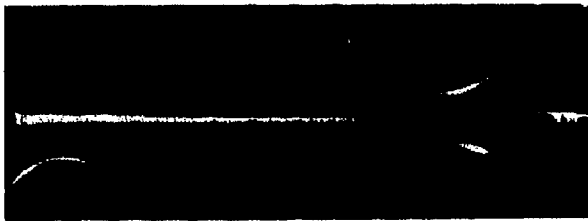
Egg proteins from normal whole egg fractionation.
Reference T7-B51.



A - antigens from peak #6
B - antigens from peak #7
C - antigens from peak #8
D - antigens from peak #9
E - antigens from peak #10
F - antigens from peak #11
Gutter - anti-normal whole egg

Figure 4-57

Egg proteins from normal whole egg fractionation.
Reference T8-B51



A - antigens from peak #6
B - antigens from peak #7
C - antigens from peak #9
D - antigens from peak #10
E - antigens from peak #8
F - antigens from peak #11
Gutter - anti-normal whole egg

Figure 4-58

Egg proteins from 11-day embryonated whole egg fractionation.
Reference T9-B51



A - antigens from peak #12
B - antigens from peak #13
C - antigens from peak #8
D - antigens from peak #14
E - antigens from peak #15
F - antigens from peak #16
Gutter - anti-normal whole egg

SGC/404

Figure 4-59

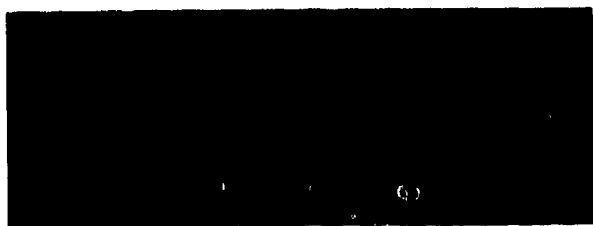
Egg proteins from 11-day
embryonated whole egg
fractionation.
Reference T10-B51



- A - antigens from peak #16
- B - antigens from peak #17
- C - antigens from peak # 8
- D - antigens from peak #18
- E - antigens from peak #19
- Gutter - anti-normal whole
egg

Figure 4-60

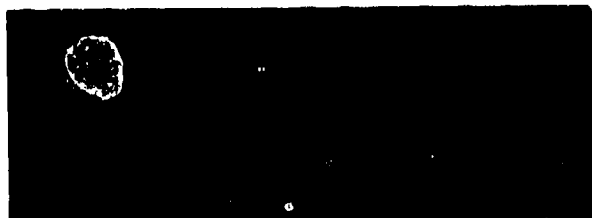
Common antigens in raw
samples.
Reference T11-B51.



- A - antigens from peak # 8
- B - raw eggwhite (diluted
1:100 in 0.85% NaCl)
- C - whole normal egg (diluted
1:100 in 0.85% NaCl)
- D - whole 11-day embryo-
nated egg (diluted 1:100
in 0.85% NaCl)
- E - batch fractionated egg
white
- Gutter - anti-normal whole
egg

Figure 4-61

Common antigens in raw
samples.
Reference T12-B51.



- A - eggwhite (diluted 1:100
in 0.85% NaCl)
- B - whole normal egg (dilu-
ted 1:100 in 0.85% NaCl)
- C - whole 11-day embryonated
egg (diluted 1:100 in
0.85% NaCl)
- D - antigens from peak # 8
- E - batch fractionated egg
white
- Gutter - anti-normal whole
egg

SGC/403

4.1.11.5

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- (6) Mansi, W., J. Comp. Path., 67: 297, 1957.
- (7) Manclark, C. R. and Pickett, M. J., "Pattern of the Immune Response in Vibriosis", In manuscript, 1964.
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The ultimate detection of one pathogen or less per liter of air will require that the air sample be sufficiently concentrated to permit statistical assurance of sample reliability and to allow a detection system of reasonable sensitivity to be used. Therefore the characteristics of a practical air sampling device must include the collection of a representative sample of air, the subsequent concentration of the particulate matter to a small volume (by about 10^5 -fold or more) in a form (preferably liquid) compatible with the sensor, and the separation of the pathogenic microorganism from extraneous background material and other debris which might interfere with the sensing technique. Furthermore, rapid and specific separation and concentration of microorganisms from a background containing as much as 10^6 times as many organic and inorganic particulates is desired. The separation should also be made in a manner which will meet the viability requirements of the sensor.

The objectives of the research in aerosol sampling can be further characterized. The volume of air processed for optimum detection conditions can vary from a few l/min for the FAST system to several thousand l/min for some of the new sensing systems under study. The form in which the aerosol is collected must be compatible with the sensor and the viability must be preserved to the degree necessary for sensing. The ratio of volume of air sampled to volume of concentrated effluent should be high and the separation of the pathogenic microorganism from background should be as specific as possible. Indeed, the objective is to provide a completely specific separation of organism from background; this specific separation in itself could serve as a sensor. The system should also be simple, of small size, require low power and be capable of unattended operation for at least 24 hours.

The progress attained on the current research program on aerosol sampling can be summarized in terms of the components of the subsystem above.

The substantial mass of background debris particulates larger than 5 to 10μ can be separated with a primary separator. The new separators developed by Metronics use open-cell polyurethane foam for this purpose. Good efficiency, low power requirements, simplicity, low cost, and good capacity are some of the attributes of this device.

The system of collection and concentration into an aqueous medium has been the selected approach for SGC research. The new porous-electrode electrostatic precipitator developed by Metronics has been giving outstanding results for this purpose.

The separation of the pathogenic organism from background debris is considered to be one of the most important problems in that it may enable a substantial increase in selectivity and sensitivity of sensing. The technique of liquid partition between non-miscible aqueous phases has shown a significant and consistent distribution of the background in one phase and microorganisms in the other phase. The method is currently of great interest for separation of background from the pathogenic microorganisms.

The research to be described in the sections to follow has been arranged according to the principle to be used for collection, concentration, or separation. The first group is entitled Electrical Field Effects and describes research on separation by means of modified electrophoresis techniques and collection/concentration with the new porous electrode electrostatic precipitator. The phenomenon of electrophoresis was believed to offer one of the best hopes for a specific separation of pathogenic microorganisms and, therefore, effort to develop rapid and specific techniques of electrophoresis was felt to be warranted. The new porous electrode electrostatic precipitator likewise offered an efficient, compact collector with low power requirements.

The second category involves the use of Inertial Effects, especially for separation. The inertial differences between the pathogenic microorganism to be detected and the non-biological particulates larger than about 5μ (comprising over half of the mass of atmospheric particulates) make inertial methods particularly attractive for use as primary separators.

The third category has been termed Combined Force Effects because the behavior is generally a combination of surface effects as well as other physical or chemical properties. The very promising liquid partition work is described, as well as interesting experiments in foam separation.

4.2.1 APPLIED ELECTRICAL FIELD METHODS

4.2.1.1 MAGNETICALLY STABILIZED ELECTROPHORESIS

4.2.1.1.1 SUMMARY

The technique of magnetically stabilized electrophoresis has been developed to the point where rapid and selective separations of organisms from background and background simulants has been made. This development has been possible because of significant improvements in technique and instrument development. Some of the improvements include an added hydrodynamic flow to decrease the residence time and allow collection control, the use of ion exchange membranes to seal the electrophoretic zone from the electrode pockets where electrolysis products are formed, and a closed system in which sample injection and collection are coupled. In a run with B. globigii cells and Arizona road dust, 95 percent of the organisms were retained in the correct collection tube, while 98 percent of the background was found in the reject collector stream. A similar enrichment at flow rates as high as 1.2 ml/min was shown for BG with actual atmospheric background particulates.

4.2.1.1.2 INTRODUCTION

It has been known that bacteria, viruses, and other cellular structures can be made to migrate selectively in an electrophoretic field⁽¹⁾. However, the migrations under reasonably low electric fields were slow and it remained to be shown that rapid migrations could be achieved. One modification of the electrophoresis technique which offered promise as a rapid and specific separation device was derived from the work of Dr. Alexander Kolin at UCLA and is termed magnetically stabilized electrophoresis. This technique makes use of the electromagnetic rotation of an annular fluid column to stabilize the solution to thermal gradients⁽²⁾. Thus, an increased electric field can be used to increase the rate of particle motion without loss of definition. In a demonstration of this effect, three dye fractions were clearly separated into their separate colors in a period of about one minute⁽³⁾. Although the molecular-size, dissolved dye particles were shown to migrate rapidly, it

remained to be established on the Space-General program that microorganisms of larger dimensions can be selectively and rapidly separated from background material. The observation that the molecular-size dye particles migrated rapidly, whereas in some other accelerated electrophoresis techniques mobility required a significant particle size, was the basis for the effort on this technique.

4.2.1.1.3 STATUS

Initial studies with the technique of magnetically stabilized electrophoresis were made with a unit similar to that described earlier by Kolin⁽³⁾. In this unit, particles migrate through a horizontal annular column of buffer solution, which is rotated by an electromagnetic field. Two cylindrical bar magnets inside the annulus (with similar poles opposed) set up this radial magnetic field, while an electric field parallel to the axis is established by electrodes at the two ends. The electrical field both contributes to the electromagnetic rotation of this column, and drives the particles toward the positive electrodes, by virtue of electrophoretic mobility. This device served to acquire operating experience and to define a number of important experimental parameters.

Initial tests with dye mixtures showed the effect of several variables on the electrophoretic migration. In line with the experience of Professor Kolin at UCLA, the definition of the separated dyes was shown to be sharper when the cell was kept cold. Bath temperatures of about 4 to 6°C appeared best. The effect of pH was shown to be significant and related to the isoelectric point (or transition point of dyes and indicators) in the expected manner. The rate of migration decreased sharply as the transition pH was approached.

The effect of voltage and current was also briefly examined. The separation of the dye colors was greater as the voltage and current increased, up to the point where the magnetic rotation could no longer stabilize the column against thermal gradients and resultant turbulence. In one case, 500 volts at 40 ma was impressed across the cell (more than 60 volt/cm) with the result that

the dye streams were each separated by 1.5 mm in about a quarter turn. Approximately 5 seconds were required for this separation. A pH 2 buffer was used in this case.

The rate for rotation of the liquid column could also be accelerated by the use of higher voltage and current. Increasing the voltage from 350 to 500 volts and the current from 20 to 40 ma decreased the time per revolution from 30 to 35 seconds to 20 seconds. It has been noted that a minimum current apparently exists for liquid rotation. When distilled water was placed in the cell, a current of 3 ma at 500 volts was obtained and liquid rotation was not achieved; the dye mixture merely streamed electroosmotically towards the electrodes. When a few NaCl crystals were added to bring the current up to 17 ma, liquid rotation was restored.

The sensitivity of the technique was shown in runs in which the chemically related dyes fluorescein and eosin (tetrabromofluorescein) were clearly separated after one turn (20 seconds) of the liquid column (500 v, 30 ma, pH 2, temperature 4 to 6°C). The first separation of bacteria was also performed under similar conditions.

Following initial standardization of these variables, aqueous suspensions of the colored bacteria S. marcescens and Chromobacterium violaceum were separated successfully. The two species formed two streams about 1 mm apart after three 20-second turns of the liquid column. In these runs, values of pH of 4.65 and 7.0 were used with a voltage of 500 volts and currents of 30 and 40 ma, respectively. The temperature ranged from 4 to 7°C. The pink S. marcescens migrated more rapidly than the violet bacterium. Most recently, streams carrying the two species were separated by 1 mm in approximately five seconds. The rapid separations were made with transverse flow (explained below).

Following the first separation of bacteria, effort has been directed toward modifying the apparatus for quantitative evaluation of selected process variables. One of the more important of these is the transverse flow effect. Dr. Kolin has shown that continuously adding electrolyte to the positive electrode chamber (with equivalent draw-off from the negative electrode chamber) increases the pitch of rotation which is characteristic of an individual material.

This is due simply to superimposing a new component of flow. It has been shown that the pitch can be changed continuously until the streams separate completely, so that the leading component of one turn does not interfere with the trailing portion of the preceding turn. A device which adds buffer at a controlled rate (~30 drops/min) has been found satisfactory, when used with solution overflow from the negative chamber.

The collection of separated fractions was next demonstrated. The collection device which was prepared was modeled after the unit developed by Dr. Kolin of UCLA on studies carried out under contract to the Office of Naval Research⁽⁴⁾. This collector consists of a flat bundle of ten fine polyethylene tubes cemented together and cut at a 45° angle. The bundle of tubes is inserted into the annulus of the magnetically-stabilized electrophoresis cell at an oblique angle, opposing the advancing spiral streams. The separate fractions can then be removed at the appropriate points. In one set of experiments, the collector was found effective in separating dye and starch fractions. In another experiment, milk was separated into at least three separate but diffuse streams.

On the basis of the experience garnered on the initial unit, an improved magnetically stabilized electrophoresis unit was designed and constructed. Salient features of the improved instrument, shown in Figure 4-62, include the following:

The electrodes are isolated from the working fluid by ion exchange membranes, as shown at the back of the buffer pockets. In this manner, the electrode pockets are hydraulically separated from the "working volume" by the membranes. This concept is an extension of the use of dialysis membranes by Dr. Kolin, on studies carried out under contract to the Office of Naval Research.

Further, the working volume is sealed so that forced injection of the feed stream can be used. The forced injection allows continuous operation of the injection-collection unit.

A syringe drive for providing the constant-displacement fluid feed can be seen in the lower edge of the figure. A small pump provides buffer feed (out of view in figure). Palladium sheet electrodes are used to reduce the contamination (electrolysis products) experienced with stainless steel electrodes.

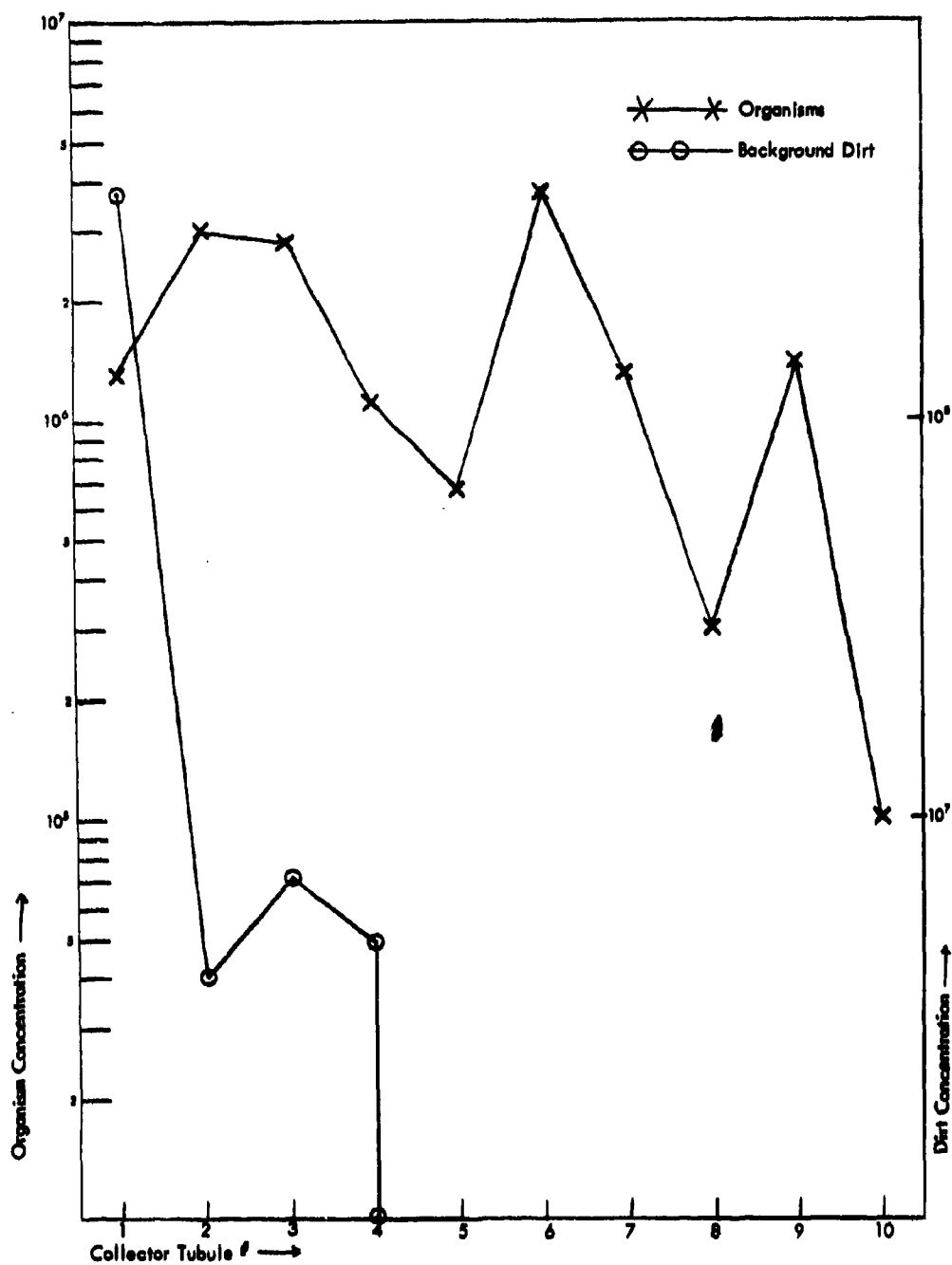


Figure 4-62. Improved Apparatus for Magnetically Stabilized Electropho

Other improvements in the new device are concerned with improved temperature control, balance of transverse flow by buffer addition, and improved injection.

An initial benefit from the improved apparatus for magnetically stabilized electrophoresis is a high degree of long-term stability. A mixture of three National Aniline dyes (Rose Bengal, Fast Green FCF, and Evans Blue) in 0.25 percent aqueous solution underwent continuous separation for over one hour, and the last 10-ml sample collected from any particular collector tube appeared visually to be the same color as the first sample from the same tube. (Each sample represented an approximately 15-minute collection interval.) The separation was performed in a pH 10 buffer solution at room temperature. No operating adjustments were made during this one-hour experiment.

The stabilization is attributed to several factors. These include the closed system of liquid flow, with forced injection and no surfaces vented to the air, and the separation of electrodes from the working volume by ion exchange membranes, so that bubble formation takes place harmlessly outside of the working volume. Another factor is introduction of the epoxy-coated (0.005-inch) aluminum tube housing the magnets. This not only improves the dimensional uniformity, but also helps suppress temperature variations by its cooling efficiency. The new apparatus was also observed to be reasonably insensitive to vibration and shock, as a consequence of the closed-system construction. A further increase in the sharpness of separation was achieved when the annulus opening was increased from 1.13 to 2.0 mm.

A number of successful separations of organisms, background simulants, (Arizona road dust), and dye tracers has been made with this device. As an example, the improved device gave a clear separation of B. globigii (spores) from Arizona road dust. The results and experimental parameters are shown in Figure 4-63.

Numerically, the segregation is much better than the logarithmic scale suggests. For example, if tubes 5, 6, 7 are considered retained and the rest discarded, the measures of effectiveness are

Recovery of organisms: 95 percent
Exclusion of contaminant: 98 percent

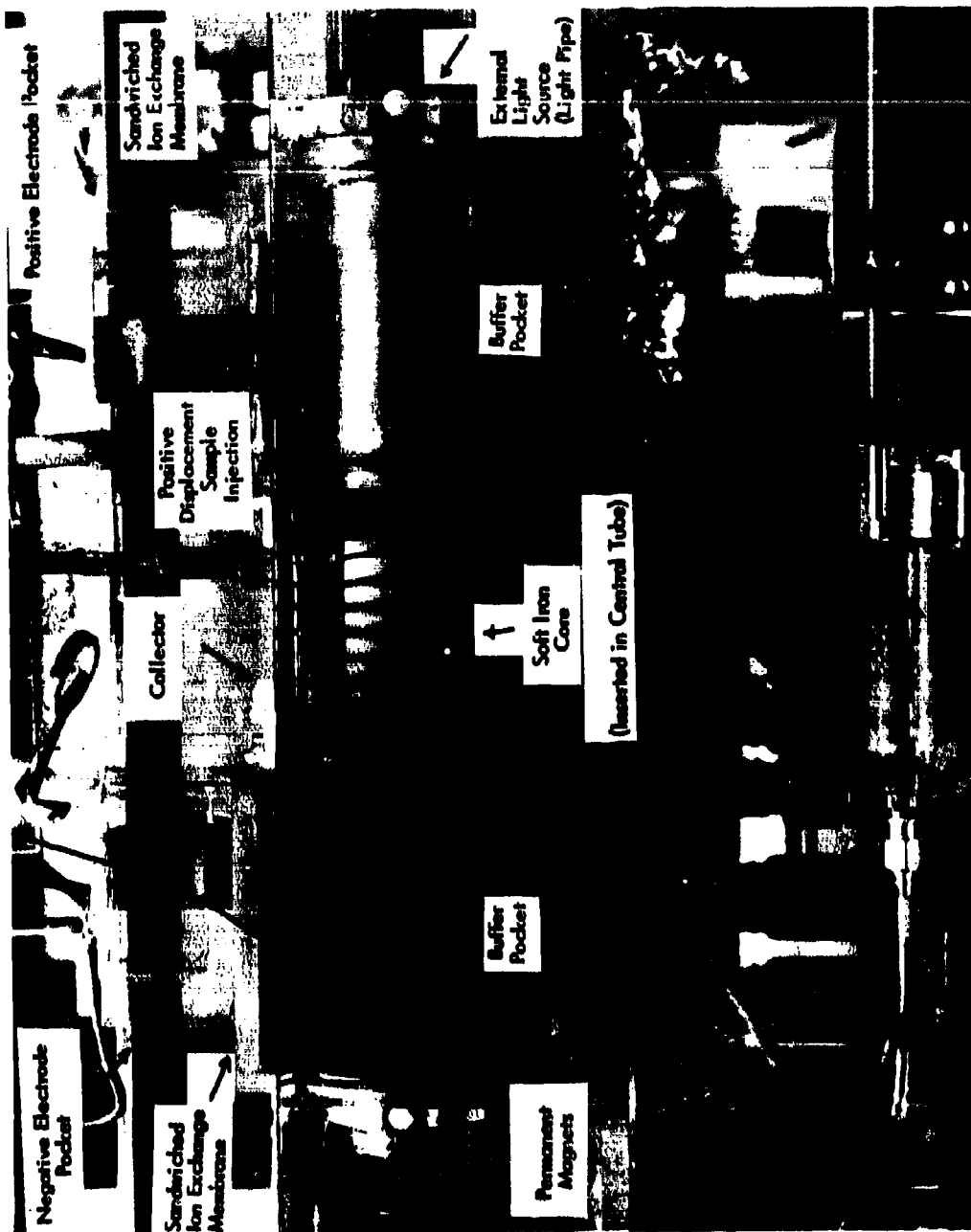


Figure 4-63. Separation of *B. globigii* from Arizona Road Dust with Magnetically Stabilized Electrophoresis

This experiment employed a low sample injection rate (0.007 ml/min), because the changes which permit the higher rates had not yet been made.

In an effort to increase the sample throughput rate to values matching the output of collectors to which the unit might be attached, the annular spacing was increased from 2.0 to 2.54 mm and larger injectors were used. A number of runs were made at 0.41 ml/min, a capacity sufficient to handle the output of a collector such as used on the FAST detection device, and additional runs were made at 1.2 ml/min and 5.0 ml/min sample injection rates. While satisfactory separations were made at 1.2 ml/min, the separations at 5.0 ml/min were not clearly defined. As an illustration of possible application to a collection-concentration subsystem, the separation of bacteria from actual background was demonstrated. In this experiment 5.8×10^7 BG vegetative cells were suspended in 20 ml of phosphate sucrose buffer through which 3000 liters of El Monte atmosphere had been passed. The mixture was injected into the device at a rate of 1.2 ml/min through a 1.4 mm injector. The device was not cooled. It is evident from the results (Figure 4-64) that the organisms migrated selectively, while the atmospheric particulates showed no such tendency. This in effect increased the organism-to-background ratio by a factor well in excess of 10 in tubes 5 through 10.

4.2.1.1.4 CONCLUSIONS

Work on the magnetically stabilized electrophoresis technique was concluded to allow concentration of effort on the liquid partition technique and the technique of thin film microelectrophoresis which appeared to be further along in development.

However, the technique has shown good separation capabilities. Current design has been somewhat complex but it is envisioned that a much simpler version can be engineered. Yet to be fully established, though, is the general usefulness of electrophoresis and whether there is a usable difference in the mobilities of organisms and background material.

The technique of magnetically stabilized electrophoresis has during this contract been shown to provide efficient and rapid separation. It appears to require a moderately low power, and is believed to be logistically practical. In view of these conclusions, the effort should be considered as a backup to other separation techniques, should their progress in development be less than desired.

- a) Sample Injection Rate .007 ml/min
- b) 1:30 Dilution of Hydrion pH 10 buffer
- c) Buffer Flow Rate 4.5 ml/min (Gravity)
- d) Current Through Cell 45 ma

- e) Diameter of Injector Tip 0.24 mm
- f) Circulating Ice-Water Bath
- g) Collecting Time 25 minutes
- h) Annular Clearance 2.0 mm
- i) Residence Time (Injection to Collection) 45 sec

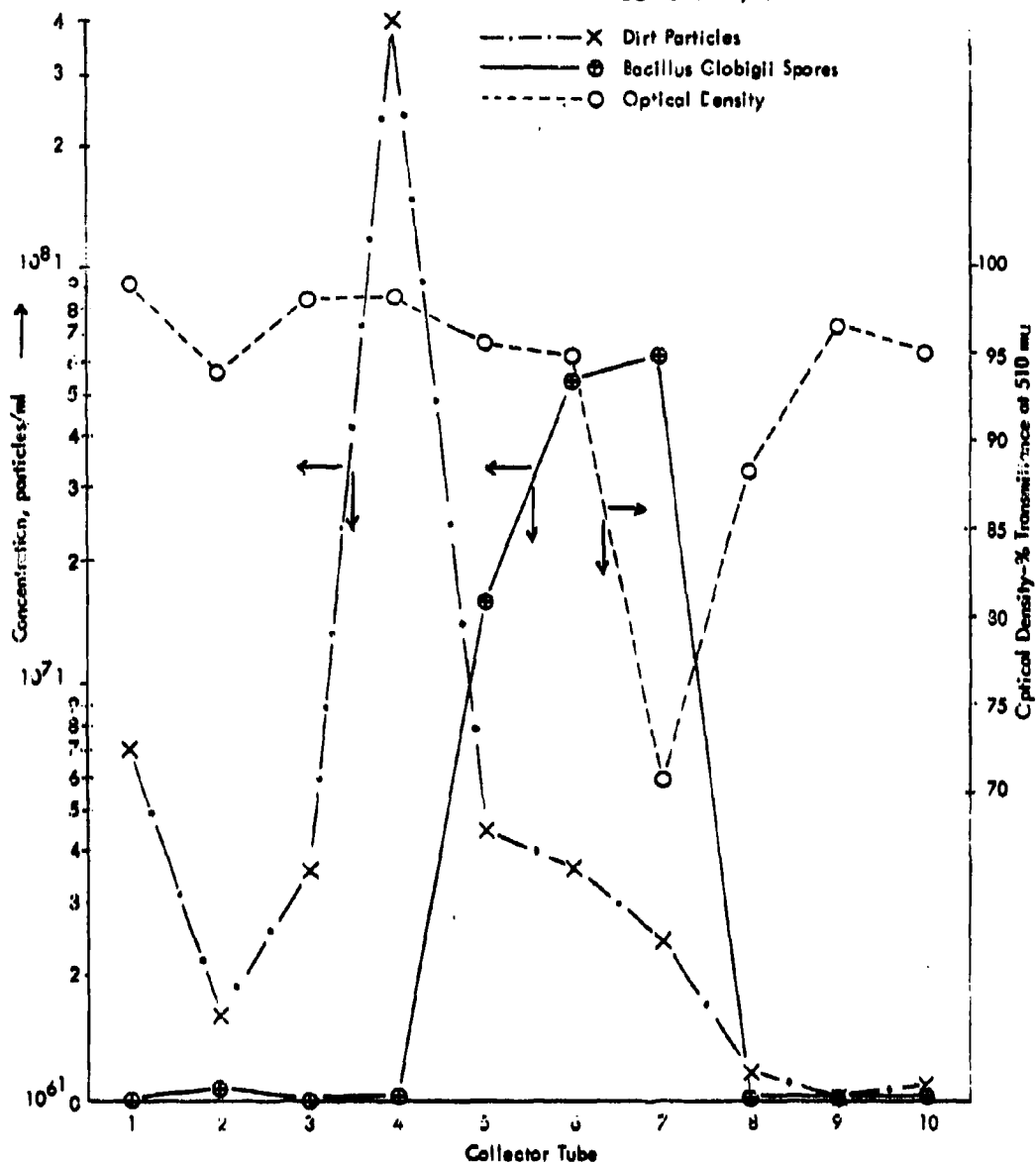


Figure 4-64. Electrophoretic Separation of B. globigii from El Monte Atmosphere

4.2.1.1.5 REFERENCES

- (1) "The Electrophoresis of Viruses, Bacteria and Cells, and the Microscope Method of Electrophoresis", Brinton, C.C. and Lauffer, M.A., in Electrophoresis, Theory, Method and Applications, M. Bier, ed. (Academic, New York, 1959) Ch. 10.
- (2) Kolin, A., J. Appl. Phys., 25: 1442-3, 1954.
- (3) Kolin, A., Proc. Natl. Acad. Sci. 46: 509-23, 1960.
- (4) Personal communication from Dr. A. Kolin.

4.2.1.2 THIN-FILM MICROELECTROPHORESIS

4.2.1.2.1 SUMMARY

Studies of continuous electrophoretic separation concluded with the recommendation for further exploratory development of the thin-film electrophoresis device studied in the Beckman laboratories.

A small vertical thin-film apparatus with transverse electric field was shown to perform accurately and reproducibly in measuring mobilities of reference particles, including bacteria. (Each type of particle zoned in a characteristic fine line.) As indicated by measured deflections, mobility was invariant with times of operation longer than one-half hour, with field gradient, and with time (or distance) of travel. This behavior appears to confirm the elimination of effects interfering with electrophoresis.

A scaled-up version cleanly fractionated 3- and 4-component mixtures of bacteria and inorganic particulates, injected at 0.05 ml/min. The patterns observed, with additional results on individual inorganics, suggest some hope for a general separation of bacteria from background. Separated bands were photographed. Improved methods of control were operated, including completely adjustable positioning for sample injection, positive elimination of contaminants from electrodes, a reliable system for lateral biasing of flow, and many other features.

4.2.1.2.2 INTRODUCTION

The general aim of this investigation conducted in the Beckman laboratories is to study the application of electrophoresis to BW detection by continuous separation of bacteria and other particulates in thin-film liquid media. A successful electrophoretic process can provide a fractionation stage in future collection-separation-concentration subsystems. It will also be useful for special separations in other sections of the program.

With free-flowing electrophoresis, particle fractions can be collected directly on a moving porous tape or isolated in separate liquid streams. The operation may be rapid for several reasons. Electrolytic

displacement is more rapid in a free liquid than in liquid on a porous curtain or in a gel. In a stream of particulates (as contrasted with a stream of small molecules), diffusion broadening is small, resulting in narrow bands which can be completely separated over short distances. Relatively high voltages can be employed with the technique because cooling of a thin flowing film can be much more efficient than cooling of fixed paper or gel curtains, and buffer concentration can be quite low with a particulate suspension, permitting lower currents at given potential gradients and, therefore, less heating.

This approach required the development of new techniques and apparatus. It follows the principle of Hannig⁽¹⁾ in which electrophoresis is made to occur in a free-flowing liquid curtain, established between two inclined glass plates. A preparative version of the Hannig apparatus is marketed commercially as the "Elphor FF", which, according to claims, can separate bacteria. However, the Hannig apparatus is too large and cumbersome (plates 50 x 50 cm) and the residence times are too long (about 20 to 30 min) for it to be directly adaptable to this program. Obviously, new adaptations of the flowing-curtain principle were needed.

4.2.1.2.3 STATUS

The development of apparatus and its use in performing separations proceeding concurrently, but are discussed separately for the sake of clarity.

4.2.1.2.3.1 APPARATUS DEVELOPMENT

Development of a satisfactory form of continuous thin-film apparatus proceeded through several stages of modification and evaluation. From the first, the conception of this device envisioned small apparatus size, fine control of operating conditions, and the possibility of a separation time measured in seconds.

In the first-generation experimental device the film flowed a distance of 3.5 cm between two inclined glass plates. The deflection of streams of blood cells and other particulates was demonstrated, and a mixture of dye molecules was separated. However, it was found early that vertical orientation

of the flowing film would nearly always be preferred. This essentially eliminates settling of particles on the back plate, and makes it possible to accommodate particles such as minerals of relatively high density. One version of the apparatus used in many early tests was constructed as shown in Figure 4-65.

The buffer solution forming the vertical film of approximately 0.1 mm thickness feeds into the top at a precisely controlled rate. Vertical electrodes on both sides are bathed in the solution. (The film is not sealed on the sides, but merely confined by capillary effect.) The sample stream is introduced near the top of the film, in a zone width of 0.13 mm or less. The sample medium is similar to that of the flowing film. It is also important to match its entering velocity with that of the film. On flowing down, it is deflected by the horizontal electrical field. Separated fractions follow characteristic paths, determined by their mobilities, and under proper conditions, these paths are distinct and sharply defined lines. Transit times are typically 10 to 30 seconds. One particle pick-up system used in early tests depends upon deposition of separated fractions on Millipore tape, which rests on the sintered glass top of a small vacuum chamber.

At an early point in development of the apparatus, a correct reading of zero mobility was demonstrated by tests with the known zero-mobility substance, o-nitroaniline (in the form of a fine suspension). The relatively sharp line and absence of lateral deflection by the electric field indicate the absence of electro-osmotic effects in these measurements.

To provide an alternative quantitation of separation, a simple photoelectric scanning and recording device was also put into operation. Recorded traces showed clearly the profile of light with distinct bands scattered from separated streams of E. coli and B. globigii.

However, the performance of accurate measurements has made necessary a further revision of techniques and methods of operation. In particular, two faults in earlier apparatus needed correction, namely (1) a lateral drift in the flowing stream, and (2) contamination of the film by electrode reaction products. With respect to (2), the intrusion of H^+ ion liberated at the anode was proved by withdrawing test samples through probes inserted into one glass

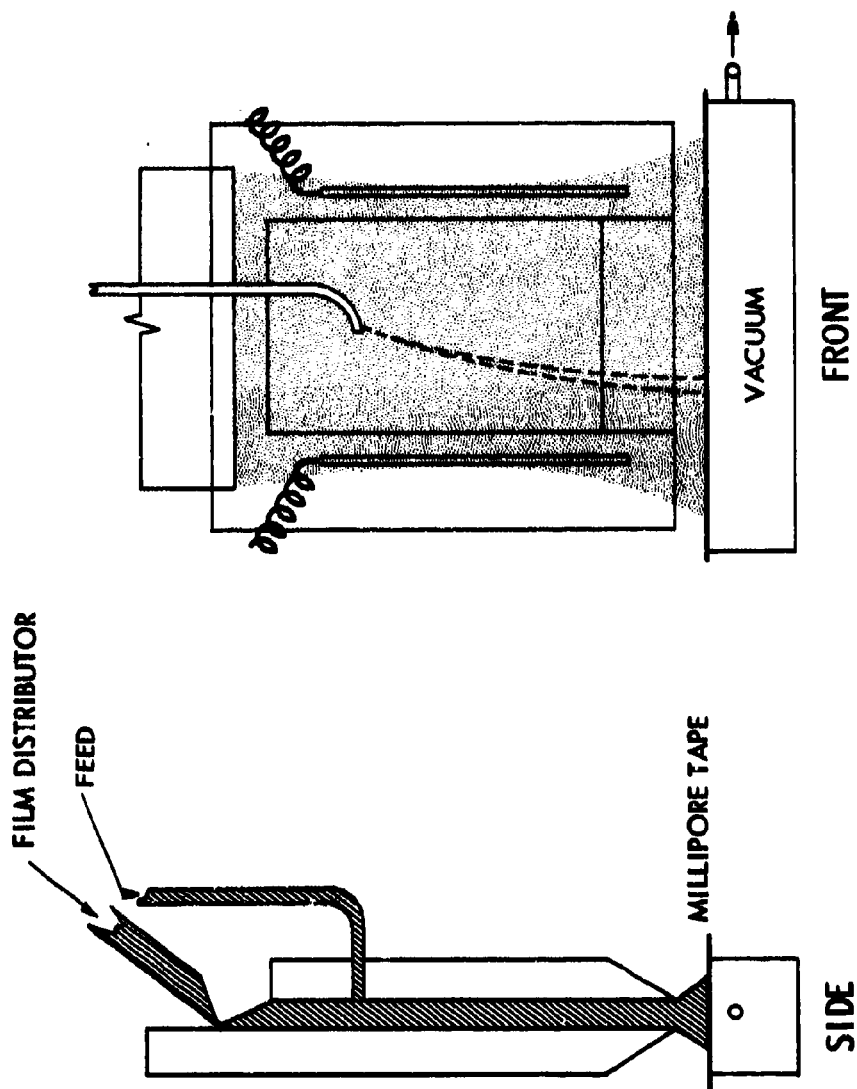
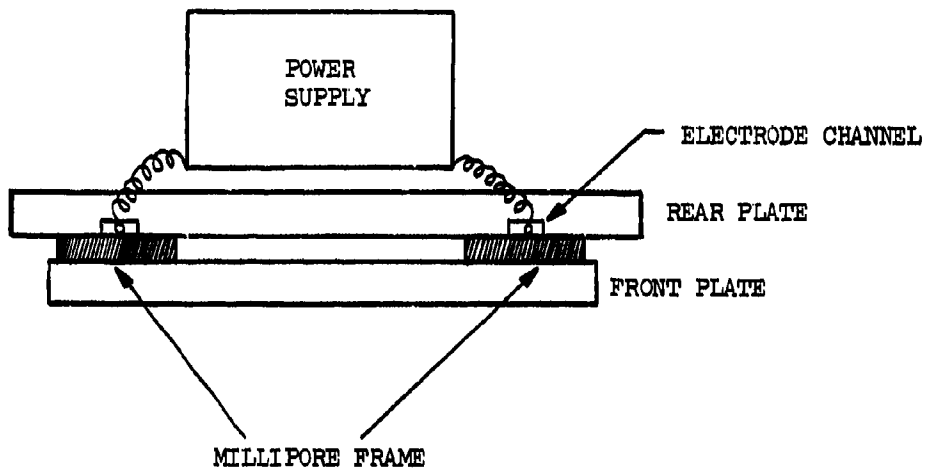


Figure 4-65. Early Continuous Thin-Film Apparatus

plate. It is a persistent contaminant. Unfortunately, the electrophoretic mobility of the particles being studied is a function of pH, and cannot be measured accurately without good control of the pH environment.

A later form of apparatus solves these problems and permits accurate quantitative measurements. Lateral drift in the flowing stream was stabilized by framing the separation zone with a Millipore shim and feeding and withdrawing the film at single points within the frame, respectively, at the top and bottom. The film thickness, determined by the gauge of the Millipore shim, is 0.15 mm. A preferred method of coupling the electrodes to the buffer film was also adopted. Vertical grooves were cut in the backing plate to provide electrode chambers containing flowing electrolyte on either side of the electrophoresis space and behind the Millipore frame surrounding it. The new arrangement is illustrated below:



TOP VIEW OF HORIZONTAL SECTION

In the revised apparatus, added stability and reliability were accompanied by a noticeable electro-osmotic flow. This occurs as a result of enclosure of the film by the Millipore frame, which causes a lateral circulation within the liquid. However, this did not blur the sharpness of the particle streams.

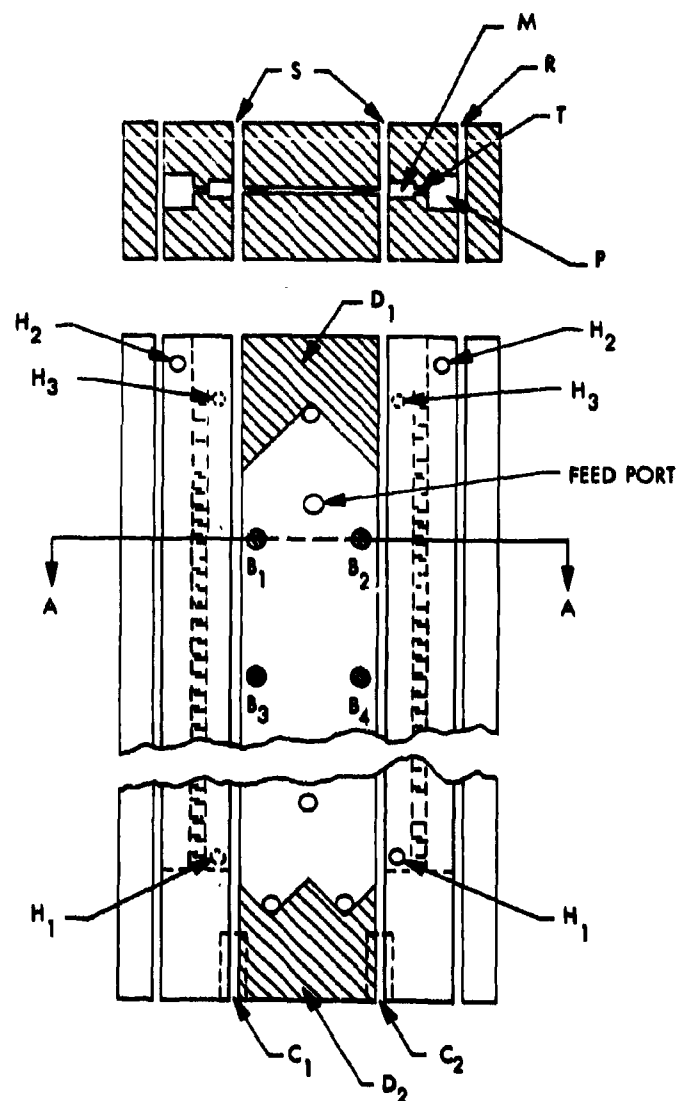
Several successor versions of the vertical thin-film device were constructed and operated during the last part of the program. Each new model was designed to exploit the experience gained with previous units. The general aim of this development was, besides stabilization of flow and elimination of contamination in the film, an increase of sample injection rates, nominally to several tenths milliliter per minute. In these models, contamination was minimized by inserting dialysis membranes to separate the flowing film from the vertical electrode compartments on either side. These electrode compartments were flushed continuously with separate supplies of buffer. In Models C and D, the bottom of the Millipore frame was cut in the shape of a W, to allow controlled take-off of the curtain through two outlets at the bottom, and consequent lateral regulation of flow. Construction of one of these (Model D) is illustrated in Figure 4-66. Figure 4-67 is a photograph of Model E, assembled and with auxiliary parts in readiness for use. Lucite replaced glass as the construction material for the parallel plates forming the film. To accommodate higher flow rates of buffer curtain and sample, film thicknesses were increased to 1 to 2 mm.

Specifically, the following units were constructed and used:

- a. Model C, for studies of buffer stability, resolution, bandwidth, feed capacity, and other factors.
- b. Model D, for multicomponent separations described in a following section.
- c. Model E, similar to D except for a longer path length (40 cm instead of 12 cm). This also incorporated a swivelling sample feed tube, which can be aligned so that any desired fraction is removed through a port at the bottom of the film.
- d. Model F, similar to E, with the addition of channeling of plates for carrying cooling liquid.

4.2.1.2.3.2 MEASUREMENT OF ELECTROPHORETIC MOBILITIES

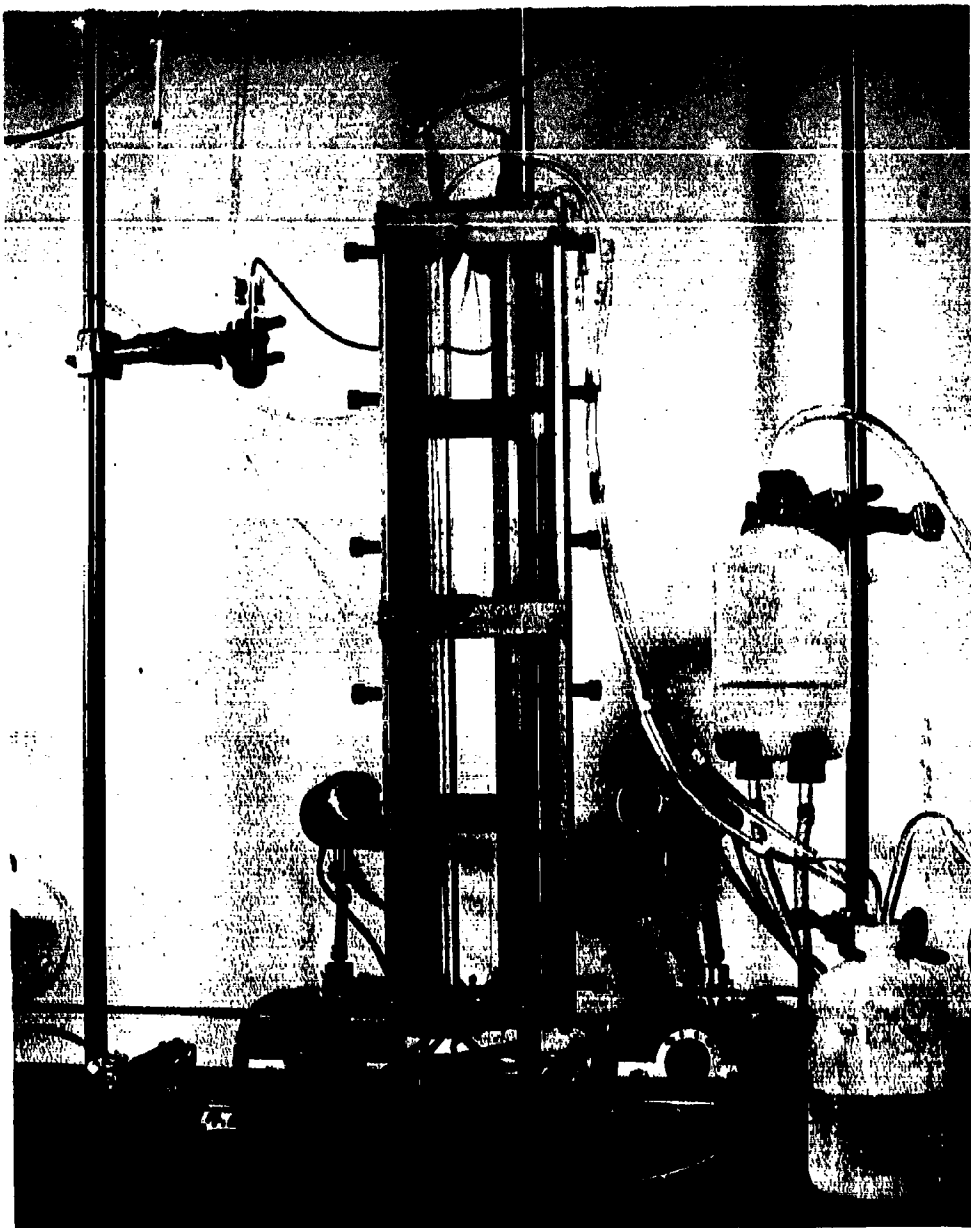
The mobility, μ , expressed in microns per second divided by volts per centimeter, is an intrinsic property of the particle and the suspending liquid. The mobility of the particle is thus the basic measure of its separability from other types of particles. Similarly, if an apparatus measures mobilities correctly, then instrumental effects interfering with electrophoresis will have been eliminated, and



NOTES

1. Dialysis membranes at S not shown.
2. Rubber gasket seal at R not shown.
3. Spacer shims at B₁, B₂, etc.
4. D₁, D₂ end seals and flow guides.
5. Rinse fore-chamber at M; electrode chamber at P; connecting jets at T.
6. Rinse electrolyte inlet at H₁, outlets at H₂, H₃.
7. Bottom seals completed at C₁, C₂ by cast sealing compound..

Figure 4-66. Thin Film Electrophoresis Apparatus
(Model D) with Counterflow Electrode Isolation



SGC/634

Figure 4-67. Thin-Film Electrophoresis Apparatus, Model E

separations based solely on electrophoresis can be performed. This criterion was applied, using the improved microelectrophoresis apparatus with Millipore frame and grooved electrode compartments.

No standard values for particle mobilities are available for comparison at the particular ionic strengths used in the Beckman experiments. Therefore, it was necessary to demonstrate the expected behavior indirectly, through the self-consistency of measurements. That is, it was necessary to show that indicated mobilities are invariant with voltage, duration of measurement, and prolonged operation over repeated measurements, and that mixtures of particles separate according to the individual mobilities of the components. From the numerous measurements which were performed, some examples are cited below.

To show the invariance of mobility with time of operation, a suspension of E. coli was fed continuously to the instrument for one hour, with the voltage turned alternately on and off at 5-minute intervals. Deflections were read at a fixed point near the bottom of the curtain with a horizontal microscope. The maximum departure from the average deflection did not exceed ± 3 percent. Figure 4-68 illustrates a similar test of stability made with ultramarine blue particles.

As a further indication of consistency, the terminal deflections of E. coli and ultramarine blue particles were shown to be proportional to potential gradient. Under conditions of uniform electrical field distribution and flow, this result would show the invariance of mobility with potential gradient. The flowing film was formed by a 0.003M phosphate medium, of 5.0 pH. The potential gradients used varied from 0 to 57 volts/cm, as measured by probes inserted through one glass plate. Deflections (at the bottom of the flow path) are plotted as a function of potential gradient in Figure 4-69.

A binary mixture of the two particles separated into two distinct streams corresponding to deflections of the individual components. The deflections of the two streams are also plotted on Figure 4-69.

The deflection of particles is also approximately constant in each interval of time (i.e., unit distance of travel). Therefore, mobility calculated from these measurements is also invariant with time, indicating that a true electrophoretic deflection is measured. An actual plot of particle deflection against vertical distance of travel for ultramarine blue was approximately linear, except for end effects.

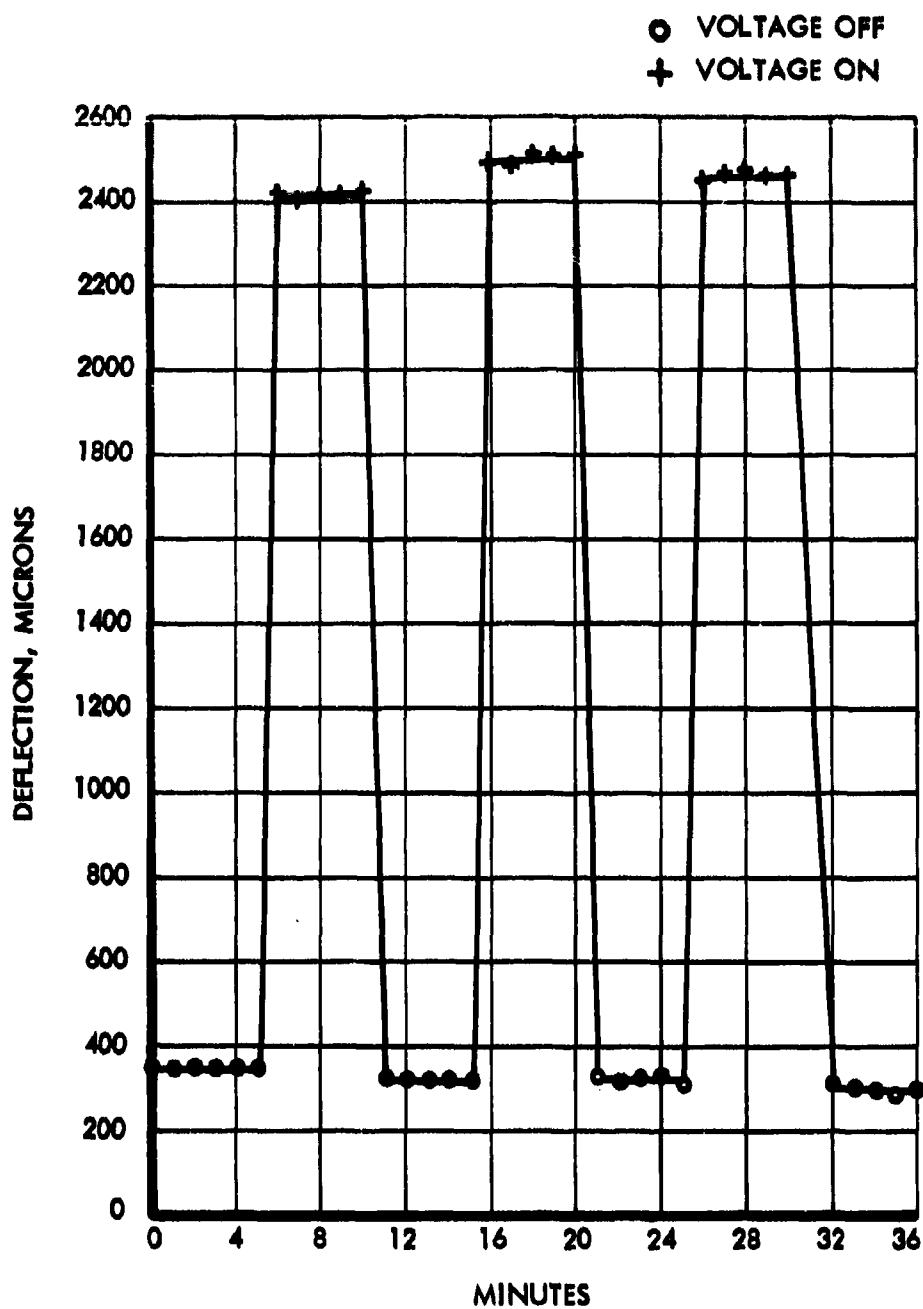


Figure 4-68. Improved Electrophoretic Film Stability

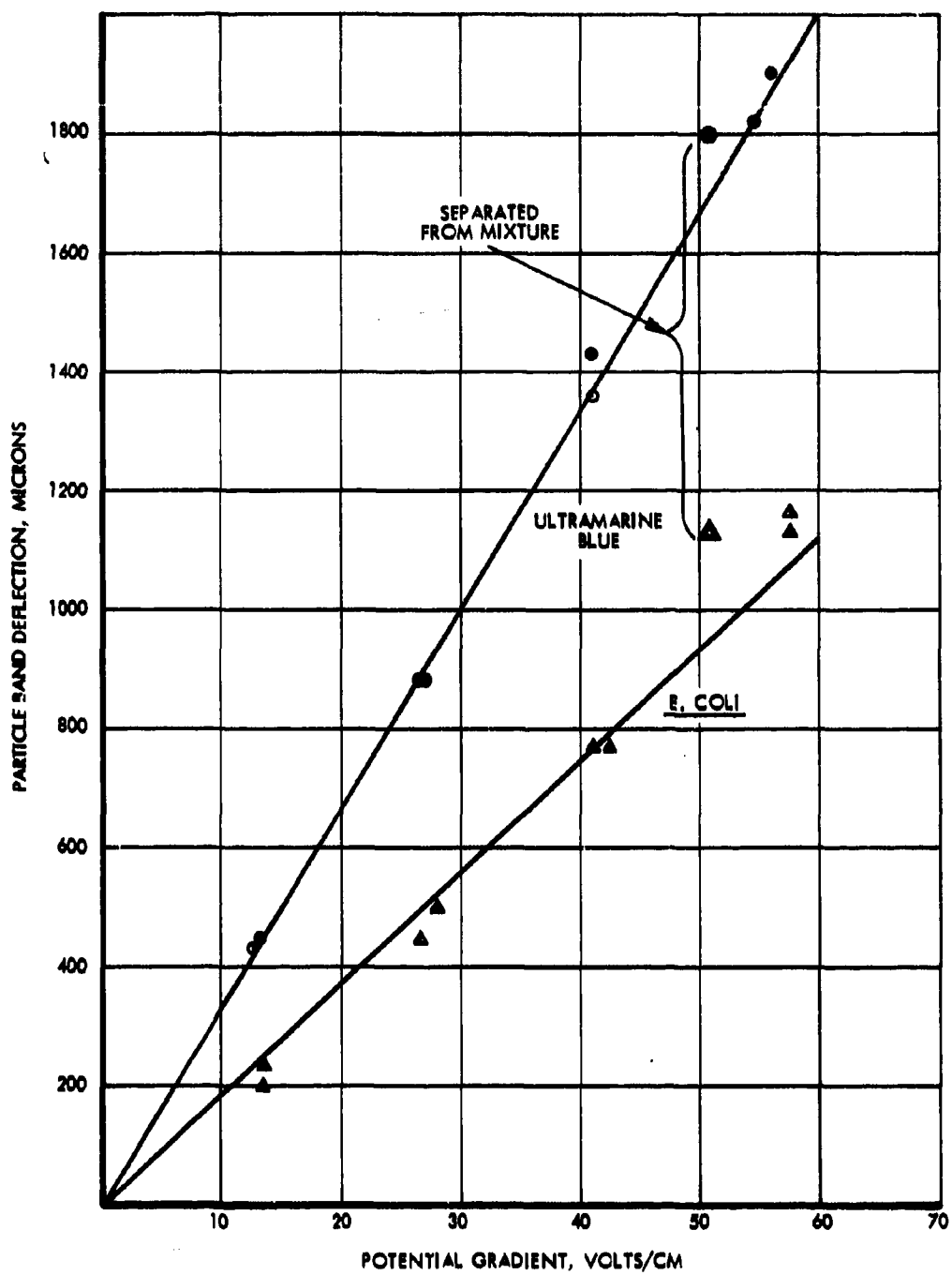


Figure 4-69. Linearity of Particle Deflection with Field Gradient

The absolute value of mobility may be calculated from such a plot with the aid of one more datum, i.e., the correction for the electro-osmotic velocity acting on a particle stream. (As previously noted, electro-osmotic currents are produced by the restrictive presence of Millipore strips framing the sides of the separation zone.) Electroosmotic velocity is determined by analyzing the deflection of a known zero-mobility standard substance, such as particles of o-nitro aniline. The method of applying this correction is described in a comprehensive report⁽²⁾; the mobility of the ultramarine blue particles was calculated to be

$$\mu = 6.5 \mu/\text{sec per volt/cm.}$$

A group of measurements of mobilities of background simulant particles was compiled. The measurements were made at different times in the improved apparatus with grooved electrode compartments and were not corrected for electro-osmosis because this correction is considered less accurate than the measurements. (For tentative correction, approximately -2.6 can be added.) The medium was 0.003M, pH 5.0 phosphate solution. The direction of migration is toward the anode in each case, so that the signs are uniformly negative.

	Uncorrected Particle Mobilities $\frac{\mu/\text{sec}}{\text{volt/cm}}$		
	<u>Series 1</u>	<u>Series 2</u>	<u>Series 3</u>
Putnam Clay	-2.68	-2.43	-
Arizona Road Dust	-3.53	-2.52	-
Puffball Spores	-2.53	-2.24	-
Carbon Black	-3.66	-4.26	-4.11 $\pm$ 0.27
Ultramarine Blue	-3.88	-	-3.60 $\pm$ 0.28
	<u>Field Gradient, volt/cm</u>		
	35.0, 31.2	37.8	39.4

Further work suggested that the differences observed within each material are associated more with the histories of the particulate suspensions than with instrumental irregularities. Similar day-to-day variations of mobility were noted in otherwise highly precise measurements made on spores in the Naval Research Laboratory⁽³⁾.

4.2.1.2.3.3 RESOLUTION OF PARTICLE MIXTURES

Numerous separations of many-component mixtures of bacteria and other particles were demonstrated during the development of thin-film electrophoresis. A recent series performed with an advanced model (Model D) is noteworthy both for effective separation, and for illustrating the processing of reasonable feed quantities in stable, long-time operation. These experiments, performed on different bacteria and inorganic pigments and their mixtures, are summarized in Figure 4-70. Figure 4-71 is an enlarged photograph illustrating the resolution achieved in separation. The sample feed rate was approximately 0.25 ml/min in all cases. The following conclusions may be drawn from the data:

- a. The principle of superposition appears to hold strictly. That is, component bands separated from a mixture occupy the same relative positions, with the same bandwidth, as when run separately. No significant interactions are observable among the particles tested. Resolution of complex particle mixtures is predictable from data taken on the separate components.
- b. Long-term stability of the apparatus now appears to permit continuous, day-long observation of characteristic fractionation patterns. The data of Figure 4-70 (taken in the order shown, top-to-bottom) were accumulated during an eight-hour working day. A slight bowing is visible top-to-bottom, in the position of given component bands (to the extent of about 0.1 mm). This is consistent for most of the bands and indicates remarkable instrumental stability as well as precision and stability of the band pattern.
- c. The bandwidths for certain components appear generally wider than for others. For example, B. globigii appears consistently wider than E. coli, ultramarine blue wider than Prussian blue. This is believed to reflect actual statistical variation in mobility of particles of a given type. However, minimum bandwidth is determined by the sample volume rate and linear curtain velocity.
- d. Mixed silicates, represented by the ultramarine blue, show a greater deflection than any bacterial particle. The broad "skirt" trailing to the right of the main ultramarine blue band appears to be due to minor inorganic components. This pattern suggested a general capability for separating bacteria from inorganics in airborne particulates, a possibility which needed further testing.

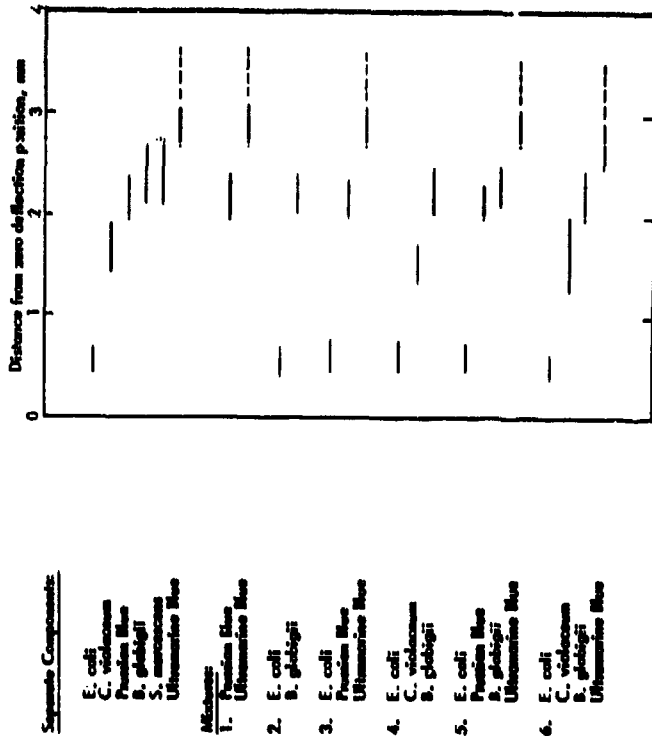
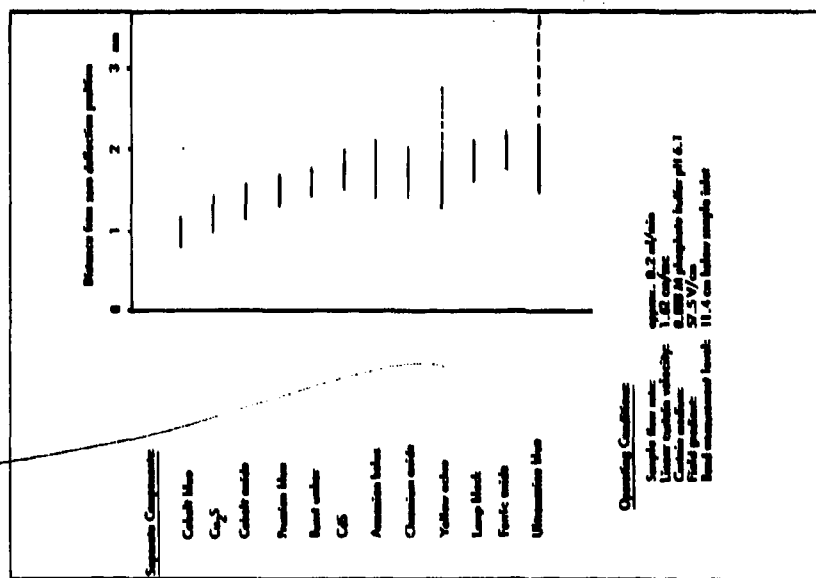


Figure 4-70. Deflection Study of Inorganic Substances,
and Resolution and Pattern Superposition Study
Model D

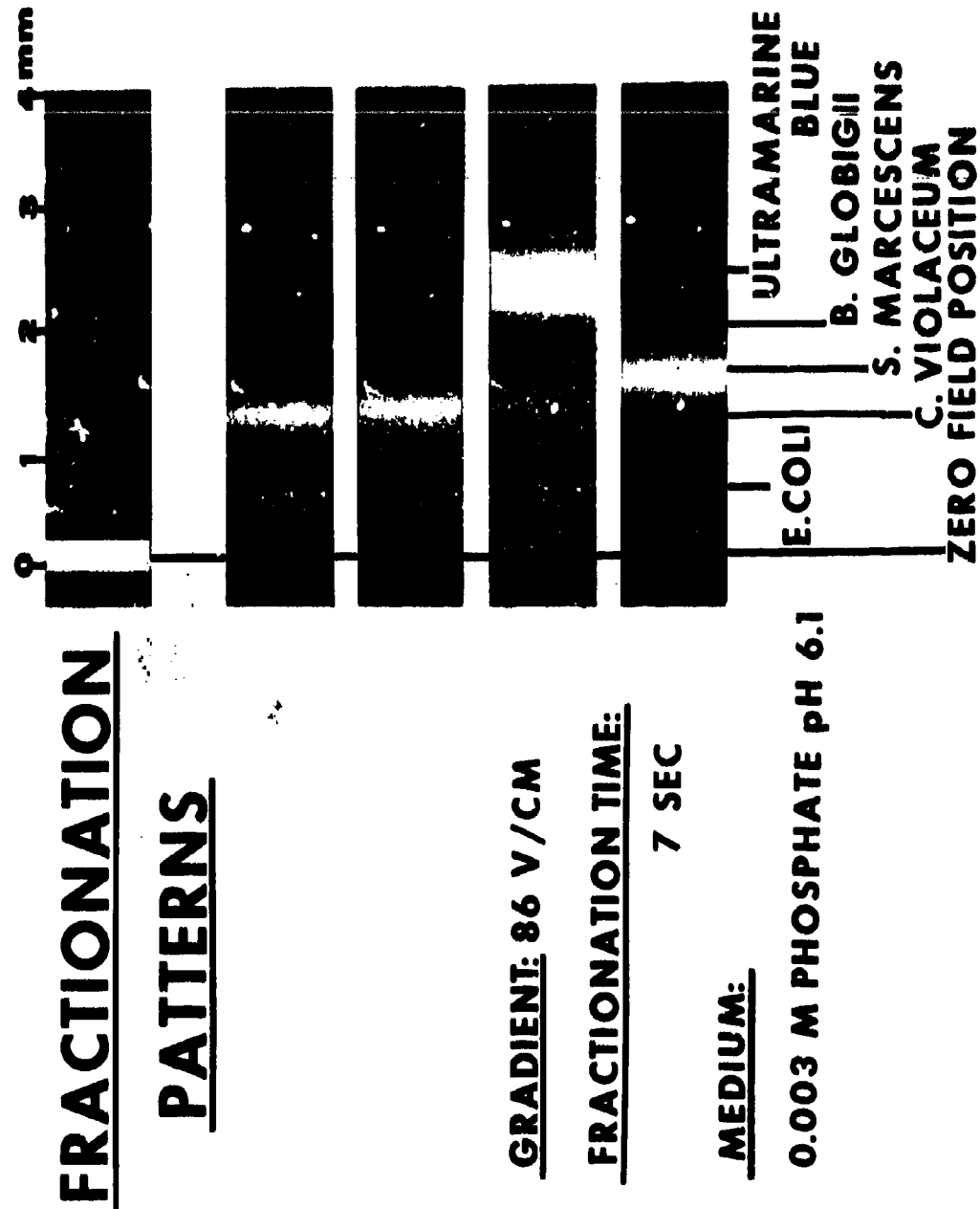


Figure 4-71. Typical Electrophoretic Separations of Mixtures

Accordingly, displacements of additional inorganic particles were measured individually, with results shown in Figure 4-70.

The two charts in Figure 4-70 are seen to be interconsistent, in that both Prussian blue and ultramarine blue give essentially the same deflections on both charts, when conditions are normalized. This is further evidence that these deflections truly reflect a basic property, the particle mobility.

It is interesting to note that the samples, whose composition may somewhat resemble inorganic components in atmospheric dust (umber, bolus, ochre, lamp black), show relatively high displacements, similar to the ultramarine blue. They appear therefore largely distinguishable in mobility from the less negatively charged bacteria examined to date. There is a good possibility that this distinction can be made sharper and more universal by choice of a different pH, composition of electrolyte, use of surfactants, etc. However, this complex question requires much further study at the developmental level.

Five fractions from two soil samples were also examined, after a rough classification. Fractionation and broad banding were evident, but most particles showed high displacements, comparable to those of the other inorganicisms.

The effect of the history of bacterial cultures on their electrophoretic displacement was also studied in the Model D device. Shifts in mobility, broadening, and partial band splitting were observed to occur with aging for periods up to 8 days.

Some other fully resolved separations achieved during earlier development of the apparatus are summarized below. Choice of pH of the medium determined in many cases whether or not a separation could be achieved. Values of pH observed in some early, comparatively crude experiments may require revision. Operating conditions were varied somewhat: for example, applied field gradient, linear film velocity, and transit time of the particle in the field. Typical applied field gradients were from 35 to 50 volt/cm, the linear film velocity 0.30 to 0.45 cm/sec, and transit time 10 to 20 seconds.

Particle Separations

Medium

<u>B. globigii</u> from <u>E. coli</u>	0.003M phosphate, 5.10 pH
<u>C. violaceum</u> from Ultramarine Blue	0.003M phosphate, 6.10 pH
<u>E. coli</u> from Ultramarine Blue	0.003M phosphate, 6.10 pH
Prussian Blue from Ultramarine Blue	0.003M phosphate, 6.10 pH
Ball Clay from <u>S. marcescens</u>	0.003M phosphate, 6.10 pH
<u>E. coli</u> from <u>C. violaceum</u>	0.003M phosphate, 5.00 pH
<u>E. coli</u> from <u>S. marcescens</u>	0.003M phosphate, 5.00 pH
<u>B. globigii</u> from <u>C. violaceum</u>	0.003M phosphate, 6.10 pH
<u>C. violaceum</u> from Prussian Blue from Ultramarine Blue	0.003M phosphate, 6.10 pH
Prussian Blue from Yellow Ochre	0.003M phosphate, 6.10 pH
Ultramarine Blue from o-nitroaniline	0.003M phosphate, 6.10 pH
Carbon Black from Arizona Road Dust	0.003M phosphate, 5.00 pH
Carbon Black from Putnam Clay	0.003M phosphate, 5.00 pH

The separation of excess fluorescent antibody solution from the BG spore-antibody reaction mixture was attempted under various conditions but was not achieved. These experiments suggest that fully stained spores assume the surface properties of the attached antibodies (i.e., the zeta potentials or electrophoretic mobilities appear identical, under the conditions of the experiment).

4.2.1.2.3.4 COUPLED OPERATIONS

As the concluding activity of the program, the Model D electrophoresis device was coupled with a porous electrode electrostatic precipitator collector, and the isolation of bacteria and dust particles nebulized into an air stream was attempted. Satisfactory zoning was not achieved in the first experiments, but is probably achievable under better controlled conditions.

4.2.1.2.4 CONCLUSIONS

Latest experiments with the thin-film microelectrophoresis device justify a recommendation for further investigations at the developmental level. Continuous fractionation of particle mixtures and collection of desired fractions

has been conclusively demonstrated by the Beckman laboratories, at reasonable levels of sample injection rate and dwell time. However, the problem of fractionating aerosols by electrophoresis appears so complex that a thoroughly systematic study will be required.

The ultimate usefulness of continuous electrophoresis in BW detection will depend on the outcome of such studies. To qualify an electrophoretic fractionator as a component of a detection system, it will be necessary to demonstrate effective separation in actual atmospheres, and also additional qualities of stability, reliability, and ease of control.

4.2.1.2.5 REFERENCES

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4.2.1.3 ZONE ELECTROPHORESIS

4.2.1.3.1 SUMMARY

The Beckman laboratories devoted a limited effort to performing zone electrophoresis of bacteria through gels. An apparatus was constructed to operate on a microscope stage under closely controlled conditions, but zoning could not be achieved. Efforts were transferred to continuous thin-film electrophoresis.

4.2.1.3.2 INTRODUCTION

At the inception of the program, zone electrophoresis of bacteria in gels appeared to be a promising novel technique. There was speculation that non-electrophoretic motions of bacteria would be inhibited in a weak gel suspending medium. Further, if electrophoretic zoning could be accomplished in a partially melted gel, the zones could be preserved by solidifying the gel. Many useful adaptations of electrophoresis in gels were visualized. However, the effect had still to be demonstrated in the laboratory; this effort was undertaken by Beckman Instruments.

4.2.1.3.3 STATUS

Repeated attempts to zone bacteria in gels were unsuccessful. An apparatus was constructed on a microscope stage which gave satisfactory control of all experimental conditions. It provided reliable electrical contact, prevented evaporation of water from the thin gel layer, held temperature constant within $\pm 1^{\circ}\text{C}$, and provided heating and cooling as desired. In several gel compositions, including that used most (0.2 percent agar and 2.0 percent gelatin in 0.06M phosphate buffer at 34°C), little or no mobility was observed. (In this system, the strong gel structure (gelatin) melts, while the weak gel structure (agar) persists.) On the other hand, when the same composition was fully melted at 40°C , E. coli gave migration speeds comparable to the values in free liquids.

4.2 CONCLUSIONS

As a result of this trial, the concept of zone electrophoresis of bacteria in gels was abandoned in 1963. Efforts of the Beckman laboratories were shifted to continuous liquid film electrophoresis, as described in another section.

4.2.1.4 ELECTROMAGNETOPHORESIS

4.2.1.4.1 SUMMARY

The electromagnetophoretic migration of various small particles was briefly examined. Movement occurs through an aqueous medium placed in an electromagnetic field, at velocities depending upon particle conductivity. Velocities of different particles several microns in diameter, e.g., pollens and iron powder, indicated conductivity differences. However, separations according to conductivity alone tend to be masked by the influence of particle size.

4.2.1.4.2 INTRODUCTION

A principle considered for separation is that of electromagnetophoresis. In this technique, a particle suspended in a liquid whose electrical conductivity differs from that of the particle experiences a force when a current is passed through the liquid perpendicular to a magnetic field permeating the liquid. The velocity of migration of the particle is given by the relation^(1,2)

$$V = \frac{JB (\sigma'' - \sigma')}{2\sigma'' + \sigma'} \frac{r^2}{3\eta} e_y \quad (1)$$

where

V	= velocity of migration	η	= viscosity
J	= current density	r	= particle radius
B	= magnetic field strength	e_y	= electric field perpendicular to magnetic field
σ'	= conductivity of particle		
σ''	= conductivity of medium		

It is expected that the conductivity of most insoluble background particulates (siliceous materials) will be very low while the conductive background materials (inorganic chlorides, nitrates, sulfates, organic acids, etc.) will generally be soluble in the collecting medium. If the phenomenon is considered only for 1 to 5 μ particles, the larger particles being removed prior to collection by a suitable primary filter, then variation in the conductivity factor $(\sigma'' - \sigma')$ is expected to be much larger than variation in the r^2 term and hence the former is

the controlling function. To be established, however, is the range of conductivities of biological particles.

4.2.1.4.3 STATUS

A series of migration velocities has been determined for particles suspended in a medium of moderate conductivity (1 g CuSO_4 , 46 g sucrose/100 ml aq. solution). A cell 14 x 14 x 12 mm was placed between the poles of a 5000-gauss permanent magnet and migration velocity determined by viewing the particles with a travelling microscope. Some migration velocities follow:

<u>Particle</u>	<u>Voltage Across Cell</u>	<u>Migration Velocity, mm/sec</u>
Ragweed Pollen (19-20 μ)	20	1.0
	25	1.25
	30	1.25
	35	2.0
Paper Mulberry Pollen (12-13 μ)	20	0.29
	25	0.33
	30	0.45
	35	0.55
Carbonyl Iron (5 μ)	20	1.0
	25	1.4
	30	3.3
Arizona Road Dust (0.1-70 μ)	30	0.1

The Arizona road dust appeared to migrate very slowly and sedimentation was much more rapid than migration.

An approximation of the effect of conductivity on the electromagnetophoresis effect can be made by comparing the ratio $V/r^2 e_y$, in Equation (1), assuming that all other terms are constant.

<u>Particle</u>	$\frac{v}{r^2} e_y$ <u>sec⁻¹ volt⁻¹</u>
Paper Mulberry Pollen (12-13 μ)	14.7
Ragweed Pollen (19-20 μ)	18.4
Iron (5 μ)	720

These results indicate a substantially different migration rate for the pollens and the conductive iron when corrected for particle size effects.

4.2.1.4.4 CONCLUSIONS

Work on electromagnetophoresis was discontinued to concentrate effort on developing a continuous electrophoresis technique. Although the method has the disadvantage that particle-size differences may tend to mask separation velocities which are due to conductivity differences, the method is still interesting since reasonably rapid migration of small particles has been demonstrated by a different force than that involved in conventional electrophoresis. The method is worthy of further investigation.

4.2.1.4.5 REFERENCES

- (1) Leenov, D. and Kolin, A., J. Chem. Phys. 22: 683-688, 1954.
- (2) Kolin, A., Science 117: 134-137, 1953.

4.2.1.5 pH-DENSITY GRADIENT ELECTROPHORESIS

4.2.1.5.1 SUMMARY

The combined effects of pH and conductivity (density) gradients have been briefly examined for the selective separation of microorganisms from atmosphere debris. A "focusing" of the organisms into a thin line as predicted by Kolin was observed, but the concept was not examined other than in an initial screening since it was considered, following the screening, not to be sufficiently sensitive, selective, or rapid.

4.2.1.5.2 APPROACH

The use of pH and density (conductivity) gradients for the selective migration of protein materials has been described by Kolin (1,2). In this work, advantage is taken of the fact that proteinaceous material can be either positively or negatively charged, depending on whether the pH is below or above the isoelectric point. By creating and maintaining a pH gradient in an electric field, the particles can thus be made to converge towards a pH zone equal to the isoelectric point. By adding a conductivity gradient (with a dissolved solute such as glucose, thus making a density gradient), it has been found by Kolin that a potential gradient is introduced which tends further to focus the protein fractions into a narrow region or electrophoretic 'line spectrum'. It was of interest to establish whether the sensitivity and selectivity of the method is sufficient to separate bacterial microorganisms from atmospheric background particulates.

4.2.1.5.3 STATUS

A double "U" micro cell was constructed for the evaluation of the pH-density gradient electrophoresis effect and the migration of B. globigii and E. coli was studied. This cell is similar to that described by Kolin, except that it has been made thinner (~3 mm) so that measurements can be made in the field of a microscope. The overall cell dimensions are 5 x 8 cm; the capacity is about 3 ml. Both phase contrast and conventional optics have been used in observing the migration of B. globigii whereas only phase contrast (at 430 to 860 X)

was satisfactory for the smaller E. coli. B. globigii were studied in a cell containing buffer at pH 3.0, made dense by adding 100 g sucrose/100 ml in the lower layer and pH 8.0 buffer in the upper layer. The bacteria migrated in a thin line in about 1 minute in accordance with the principles described by Kolin.

4.2.1.5.4 CONCLUSIONS

The technique of pH-density gradient electrophoresis was dropped after a preliminary examination, to concentrate on electrophoretic separation techniques which were considered to be more sensitive, rapid, and selective. Although migrations were demonstrated, the technique appears to offer little hope for providing a practical separation device.

4.2.1.5.5 REFERENCES

- (1) Kolin, A., J. Chem. Phys. 23: 407-8, 1955.
- (2) Kolin, A., J. Chem. Phys. 22: 1628-29, 1954.

4.2.1.6 ELECTRIC FIELD GRADIENTS

4.2.1.6.1 SUMMARY

In the electric field gradient technique, particles containing a permanent or induced dipole will migrate in strongly non-homogeneous fields. A velocity of migration dependent upon the dielectric properties, particle size, etc., suggests a means for separating atmospheric debris from microorganisms. A number of particles simulating atmospheric background, and the bacterial simulants E. coli and B. globigii, were studied briefly and all migrated rapidly. However, further work was not carried out because other methods were considered more selective and offered a better chance of providing a practical separation device.

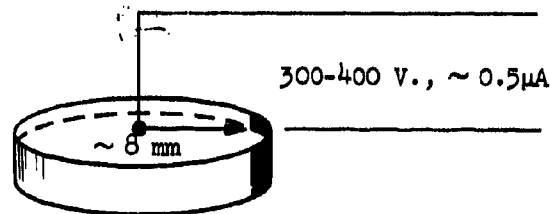
4.2.1.6.2 APPROACH

The electrical field gradient technique, described by Pohl⁽¹⁻⁶⁾, makes use of the polar behavior of materials in a highly divergent electric field. The approach may be stated briefly as follows: any dipole (induced or permanent) will have a finite separation of equal amounts of positive and negative charge. Impressing this material in a highly non-uniform electric field will cause an alignment of the dipole in the field. However, because the field is non-uniform, one end of the dipole will be in a weaker region than the other, and a net force will result, with the dipole being pulled toward the area of greatest field intensity. It may be seen that if the direction of the field is reversed, the field intensity pattern will remain and the particle should move in the same direction. Accordingly, particle motion should take place in either a direct or alternating current field.

The motion of the particle will be related to the difference between the dielectric constant of the particle and that of the medium, to the field strength squared, and to a function of the particle diameter. Pohl points out that the electric field gradient effect is probably not useful with particles of molecular dimensions but is sufficiently sensitive that selective grading of polymer molecules by molecular size should be possible.

4.2.1.6.3 STATUS

Tests have been conducted at Space-General in which small particles were made to migrate in an electric field. A cell of the following type was constructed and utilized in these experiments. Colloidal particles of carbon black were caused to migrate with a velocity of 1 cm/sec or greater in either carbon



tetrachloride or the fluorocarbon fluids FC 43 or FC 75(3M). Interestingly, the carbon particles appeared to be dispersed before extensive migration took place. No migration of 100μ glass beads was noted and the effect of the field on clay particles with diameters <10μ was quite small.

In further tests with suspensions of E. coli, it was determined that the unstained organisms could not be seen in the microcell unless phase contrast optics were employed. Another micro cell was constructed to work within the limited depth of field of the high power phase contrast objective (powers up to 860X have been used; working distance of 43X phase objective: 0.3 mm). In this cell, the liquid column radius is about 3 mm.

It has been found that E. coli suspended in aqueous media migrated rapidly in either a DC or AC field, although electrode bubbling quickly obscured the field when DC was used. The migration of a suspension of lyophilized B. globigii in fluorocarbon FC-43 has also been examined. The particles migrated rapidly, moving about 3.6 mm/min when a 70 volt/mm field at 0.2 microampere was impressed on the cell.

4.2.1.6.4 CONCLUSIONS

Although rapid migrations of small particles, including bacteria, was demonstrated in a highly divergent electrical field, the method was dropped in favor of other techniques which were considered to be more selective and sensitive.

4.2.1.6.5 REFERENCES

- (1) See, e.g. Pohl, H. A., J. Electrochem. Soc., 107: 386-390, 1960
- (2) Pohl, H. A. and Flymle, C. E., J. Electrochem. Soc. 107: 390-396, 1960
- (3) Pohl, H. A. and Schwar, J. P., J. Electrochem. Soc. 107: 383-385, 1960
- (4) Pohl, H. A. Scientific American, 106-116, Dec. 1960
- (5) Pohl, H. A., J. Appl. Phys. 22: 869-71, 1951
- (6) Pohl, H. A., Princeton Univ. Plastics Lab. Tech. Rept., 48B, 1 Dec. 1957, Contract DA 37-039-SC-70154

4.2.1.7 POROUS ELECTRODE ELECTROSTATIC PRECIPITATOR

4.2.1.7.1 SUMMARY

The Metronics porous electrode electrostatic precipitator was developed to provide a universal aerosol collector for BW detection systems. The unit depends on the principle, previously discovered by this group, that operation is most effective when precipitation is accomplished with an electric field parallel to the air flow, rather than with the conventional field normal to air flow. Collection of solids is achieved, without moving mechanical parts, in a dilute aqueous collecting fluid flowing through and over a horizontal porous glass disc anode. Potential gradients of 1 to 2 kv/cm are employed. Two units have been evolved on the present program through several model changes. The first has a capacity of about 1000 l/min, and collects the particulates into 5 ml/min of water. The second operates over the range 20 to 60 l/min air flow and 0.1 to 0.33 ml/min water flow. Both have collected 1.3 μ polystyrene spheres into water at efficiencies exceeding 90 percent, under optimal operating conditions. With microorganisms, collection efficiencies have been lower, and material balances on intake air versus exhaust air and collection water indicate some retention within the collector. In a recent test series, the 1000 l/min collector gave an average balance of 85 percent for 8 runs with FITC-stained BG spores. Means of further improvement are under study.

4.2.1.7.2 INTRODUCTION

The need for a simple, lightweight, and efficient collector for aerosols prompted the investigation by Metronics Associates of a novel design principle. It is an adaptation of an earlier invention by the Stanford Aerosol Laboratory, the predecessor of Metronics Associates. Essentially, particles are precipitated electrostatically on a moving thin film of collection water; the electrostatic field is positioned parallel to the air flow to take advantage of the forward inertia of the particle in enhancing collection⁽¹⁾. By this arrangement, linear flow rates (~ 6 m/sec) can be used which are much lower than for collection by impaction alone. The absence of moving parts within the collector is also an important advantage.

The development of useful devices from this principle raises a number of problems. A major problem is efficient transfer of particulates from air to the minimal liquid stream required by most sensing devices. The geometrical arrangement of parts is critical, and materials of construction must be carefully chosen. Detection of different sizes must be scaled to the needs of various detection systems. Ultimately, coupled operation with detection and other components of total systems must be demonstrated.

4.2.1.7.3 STATUS

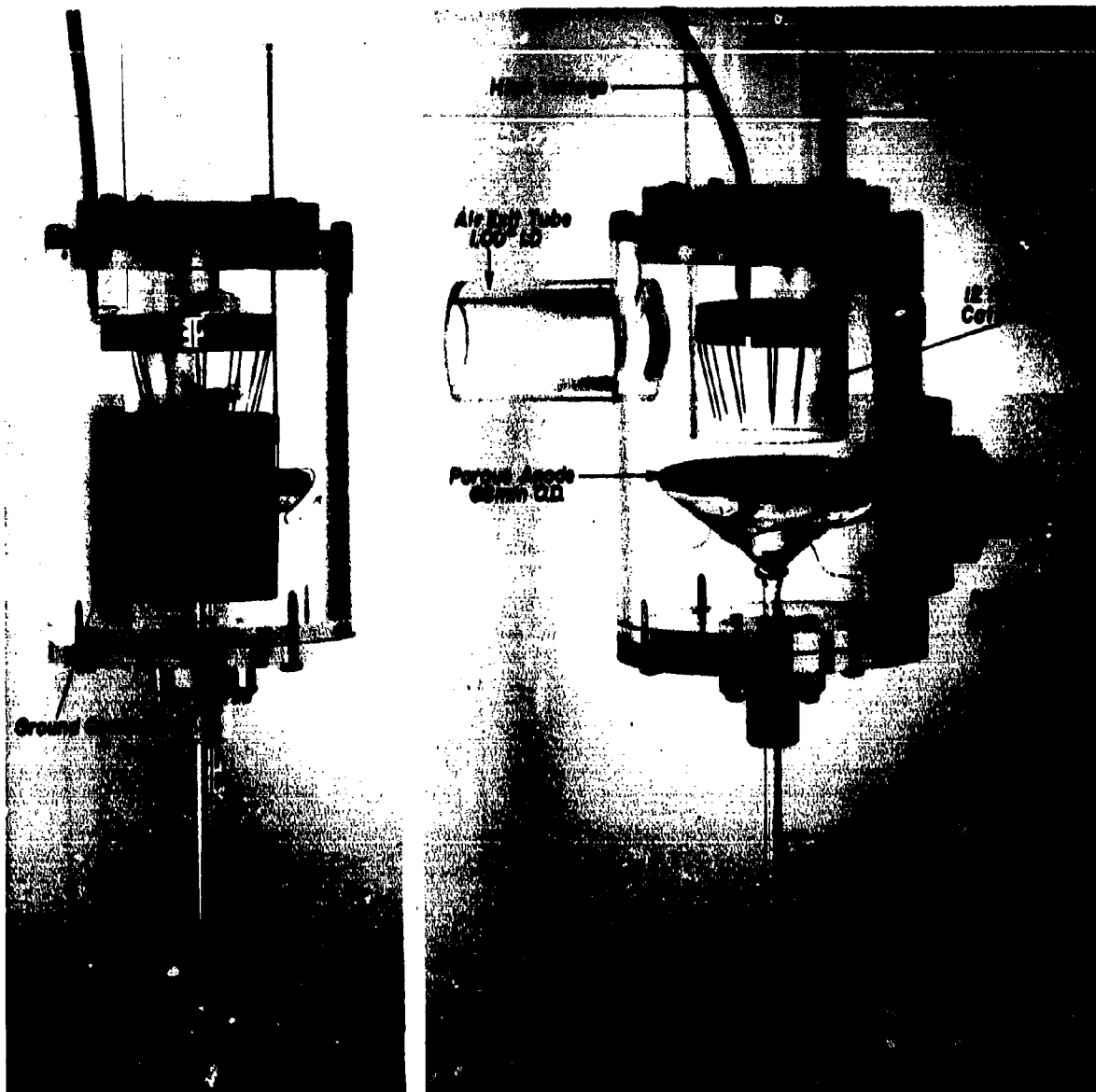
4.2.1.7.3.1 PLAN OF DEVELOPMENT

The porous electrode electrostatic precipitator (PEEP) which embodies the new concept is illustrated in Figure 4-72. Although the figure shows a recently developed model in current use, earlier versions contained the same major features. Air is drawn in (by a blower attached to the air exit tube) through the vertical intake tube. It passes through a corona formed by these electrodes, and over the aqueous fluid film of about 0.2-mm thickness on the porous anode. Aerosolized particles are caught and retained in the fluid, which passes down over the glass cone under the anode, and drips into the drain tube. The air is exhausted through a side exit tube.

Following construction of the first working model, development has proceeded through the design, fabrication, and testing of numerous units. The first unit constructed had an air intake capacity of 1000 l/min, but several units were developed subsequently with both this capacity and with smaller capacities. However, large and small units were not evolved independently; instead ideas were freely adapted back and forth. Table 4-22 is included to clarify this somewhat complex sequence of development, which is discussed in detail in the following sections.

4.2.1.7.3.2 LARGE COLLECTOR DEVELOPMENT

A collector which samples air at a rate of 1000 l/min has direct application to the chemiluminescence detector and other devices. The first model PEEP was designed for this capacity. Thirty-seven needles were used in the cathode assembly, each provided with a protective, 10-megohm resistor.



SGC/870

Figure 4-72. Porous Electrode Electrostatic Precipitator
for 1000 l/min Air Intake, Model MB, Liquid
Flow Rate - 5 ml/min

Table 4-22

CHRONOLOGY OF DEVELOPMENT OF POROUS ELECTRODE
ELECTROSTATIC PRECIPITATOR

<u>Model</u>	<u>Nominal Air Capacity, l/min</u>	<u>Period of Use</u>	<u>Remarks</u>
I	1000	Late 1963, Early 1964	
II	20 to 60	Spring, 1964	
III	20 to 60	To Aug, 1964	Plastic box housing
IV	20 to 60	Aug, 1964 to present	Used on another program
MB	1000	Through 1965	
V	20 to 60	Early 1965	Interim device
VI	20 to 60	Feb, 1965 to present	Used on another program
VII	100 to 200	May, 1965 to present	Used on another program

The porous electrode was a fritted glass (fine grade) disc attached to a glass stem. This assembly was made by removing the upper portion of a regular laboratory Buchner funnel. Conducting paint was applied to the periphery of the disc, after being ground to a smooth edge, and was also applied from the periphery to the base of the glass stem where the ground connection was made.

Sample collecting water, made conductive with sodium chloride (0.4 percent), was introduced at the base of the stem (on the axis) from a reservoir approximately 18 inches above the electrode. With this head, the water flowed through the entire surface of the porous disc at a constant rate of 15 cc/min. At the periphery the water drained over the conducting rim to a rubber retaining ring around the upper end of the glass stem. The water then drained through a hole in the retaining ring into an outer tube.

When spacings of the needle tips and the central tube bottoms were respectively 1.0 and 1.3 cm above the anode, the 23 kv could be applied without arc-over. The measured current was 1 to 1.5 ma. As expected, both the maximum voltage and current drain depended on the electrode spacing.

The efficiency of the precipitator was evaluated early, using first-version techniques for purposes of reference, previous to development of improvements. The particle removal efficiency was determined by measuring the particle concentration at the porous electrode precipitator air outlet side-arm with and without voltage applied to the electrodes. All tests were conducted with naturally occurring atmospheric particles. Particle concentrations were measured with the Royco Counter. Typical results obtained at 1000 l/min air flow are:

<u>Particle Removal Efficiency (%)</u>		
$\frac{1.0 \mu}{90}$	$\frac{0.8 \mu}{88}$	$\frac{0.5 \mu}{70}$

When the airflow was reduced to 500 l/min, the 0.5 μ collection efficiency was increased to 85 percent.

Following an improvement in the (grounded) anode connection by connecting directly to a fine wire fastened across the diameter of the disc, additional particle removal tests were performed. (The change was made necessary because connection below the disc appeared to create an electric potential which lodged collected particles in the frit.) All measurements were made at 1000 l/min air flow rate and 10 to 15 cc of water flow per minute using 1.3 μ polystyrene beads. The results confirm those reported for room air particles, viz., 70 percent removal efficiency at 0.5 μ and 90 percent at 1.0 μ , and verify the absence of objectionable side-effects. Thus, both room air particles and polystyrene beads in the micron and submicron sizes are effectively removed by the porous electrode electrostatic precipitator.

4.2.1.7.3.3 SMALL COLLECTOR DEVELOPMENT

A porous electrode electrostatic precipitator for application to a miniaturized collection-detection system was also constructed and operated. This miniature unit is based on the same operating principles as the 1000 l/min unit described previously but is smaller in size and differs in certain other details of construction. A comparison of major features is given below.

<u>Feature</u>	<u>Larger Unit</u>	<u>Miniature Unit</u>
Nominal air flow rate, l/min	1000	22-60
Maximum water flow rate, cc/min	15	0.33
Porous electrode diameter (nominal), mm	90	20
Outside tube ID, inches	4.5	1.25
Outside tube length, inches	10	4.5
Air intake tube ID, inches	2.25	0.5
Intake air speed, m/sec	6.4	6.2 (60 l/min)
Number of electrodes	37	7
Nominal voltage, KV	20-25	15-20
Nominal current, ma	1.5	0.2

At 50 l/min the linear velocity of the air stream in the miniature unit is comparable to that of the 1000 l/min device.

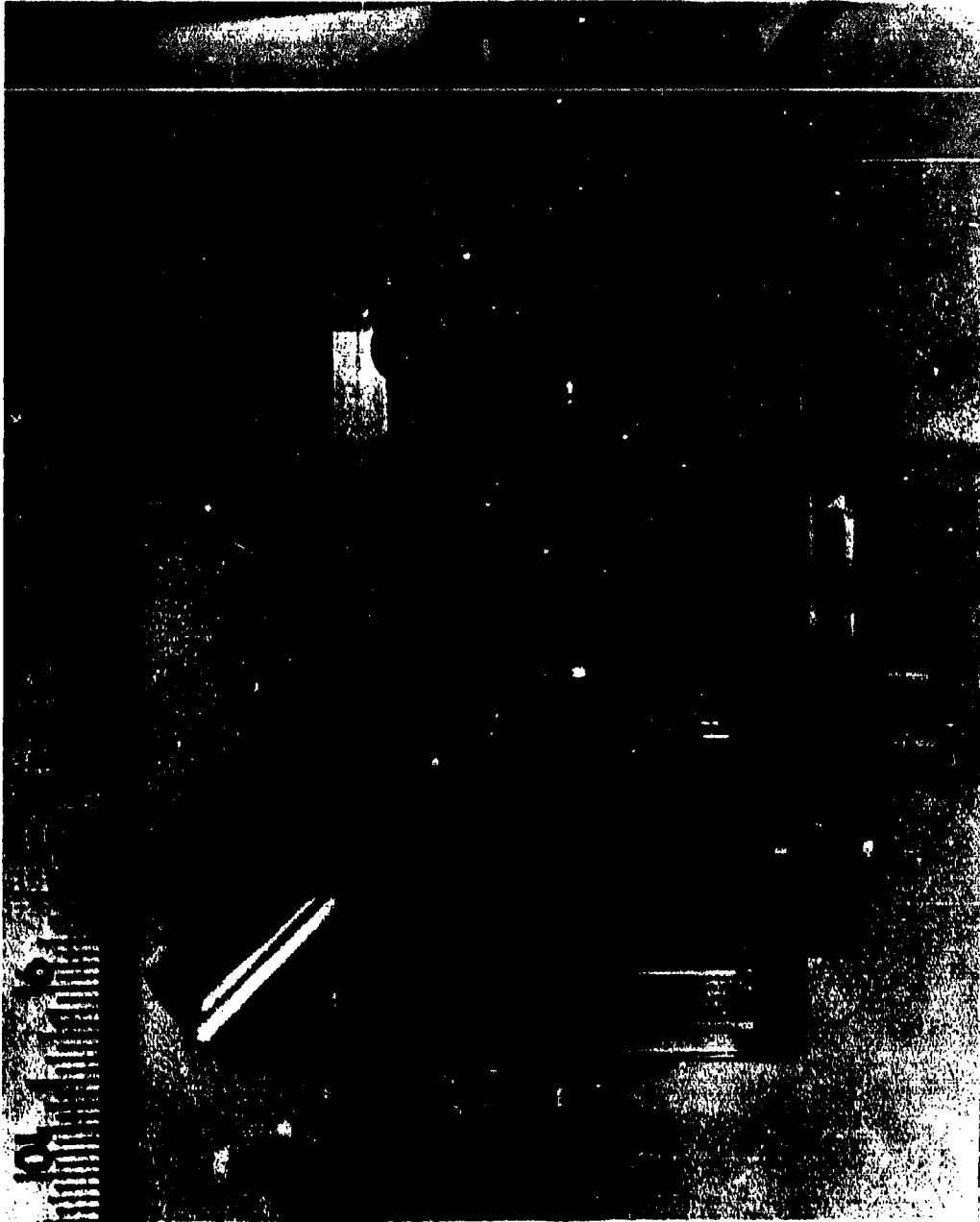
Initial tests of mechanical characteristics showed that without the addition of a wetting agent the water flow was erratic, as might be expected, since the surface tension is significant compared with the forces available from the slight differences in water heads used. With 0.5 cc of Photo-Flo (Eastman Kodak Company) per liter the water stream is easily controlled even at rates as small as 0.1 cc/min. Dwell time of suspended particles was found to be about 8 seconds, at a fluid flow rate of 0.33 ml/min.

Testing of the first generation miniature collector with aerosols revealed one serious problem. While 85 percent or more of the particles (polystyrene latex) are removed consistently from the air, the particles in the water may amount to no more than 45 percent of the input. The difference between the 45 percent collected in water and the > 85 percent removal from the air stream appears to represent material deposited in the collector. Deposited material is evident on the walls of the inlet tube and near the anode on the container walls.

To solve this problem, development was continued by constructing and revising several models and by changing the operating conditions. Sites of deposition were located with tracer particles, and eliminated. Particle removal was increased by increasing the corona current. To some extent recovery was improved by use of a capillary drain tube, which interrupts the exit fluid with a steady succession of air bubbles, and prevents deposition at the upper water surface. Other changes were found to improve water flow and electrical stability.

Results of this experience were embodied in an advanced model, PEEP-IV (Figure 4-73). Major specifications are summarized below:

Case:	Plastic 1/4 inch thick; exterior dimensions 2-1/4 x 2-1/4 x 4 inches including removable top and bottom plates.
Air Inlet Tube:	Glass, nominal OD 22 mm, nominal ID 19 mm. Held with O-ring assembly on upper plate.



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Figure 4-73. Advanced Model, Porous Electrode
Electrostatic Precipitator

Water Drain: Glass, nominal 5 mm OD; top end flared to collect overflow from anode; bottom end beveled and slotted. Held in bottom plate with O-ring assembly.

Cathode: Six stainless steel surgical needles soldered to 7 mm wide split brass ring held by friction in air intake tube.

Anode: 30 mm diameter coarse grade fritted disc attached to glass fixture. Anode supported by glass water intake tube held in front plate with O-ring assembly.

At 50 l/min airflow through the air intake, the pressure drop within the precipitator case is 0.12 inches water.

Data for the collection efficiency of PEEP-IV in water are shown in Figure 4-74. These were taken at a nominal airflow rate of 50 l/min, using the 1.30 μ polystyrene latex beads. The collecting fluid was the dilute saline solution + Photo-Flo described earlier.

The following conclusions can be drawn from these results.

- The average efficiency of collection in water is 86.5 percent with a standard deviation of 7.13.
- There is no significant change in collection efficiency over the voltage range used, viz., 12.5 to 18.8 kv.
- Approximately half of the variance in the efficiency data results from uncertainty in assessment. In each trial, 500 to 800 particles were counted in the collected samples which, on the basis of an assumed Poisson distribution give an expected variance of 0.0033, i.e., approximately the observed variance.
- A grounded shield around the back and two sides of the precipitator, used in two runs, does not alter the collection efficiency.

The miniature porous electrode electrostatic precipitator has been shown efficient for the collection and concentration of biological particulates, as well as the non-biological particles described previously. One run in which BG spores were nebulized into an airstream, which was sampled into the collector, gave the following record:

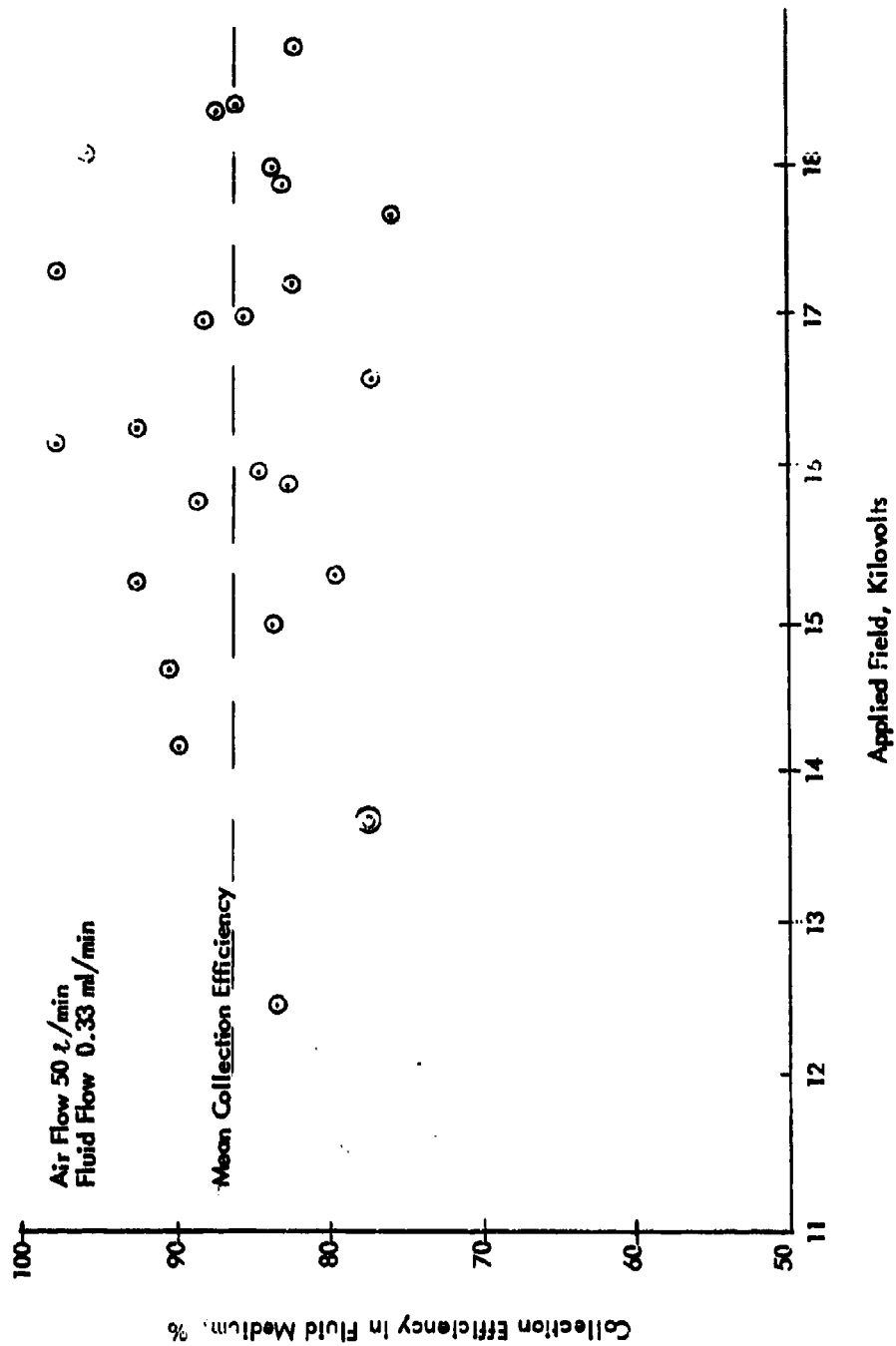


Figure 4-74. Efficiency of Collection of 1.3μ
Polystyrene Latex with Porous Electrode
Electrostatic Collector

	<u>Total BG</u>	<u>BG/l of air</u>
Air Entering Precipitator:	7.03×10^4	291
Air Leaving Precipitator:	5.19×10^3	21.5
Collection Water		
Viable bacteria:	5.43×10^4	224
FAST-stained bacteria:	6.34×10^4	262
Collection Efficiency		
Basis viable count:	77.2%	
Basis FAST-stained:	90.2%	
Balance		
Basis viable count:	84.6%	(BG in air leaving + collection water vs BG in air entering)
Basis FAST-stained:	97.6%	
Precipitator Conditions		
Air flow:	40.2 l/min	Potential: 11,200 volts
Collection water flow:	0.25 ml/min	Current: 0.1 ma

A further important conclusion was demonstrated when fluorescent staining activity was retained in organisms collected in the porous-electrode electrostatic precipitator, even though viability was not preserved. This result was obtained when S. marcescens aerosols were successfully collected and brightly stained with good recovery, although the viable count of this experiment indicated kill. Actually, the stainable cells in the collection fluid exceeded the viable cell input, indicating the presence of a substantial number of non-viable cells in the latter.

At this point, further studies of the small unit were transferred to the FAST and Partichrome development programs.

4.2.1.7.3.4 NEW LARGE COLLECTOR

As a basis for the design of an advanced 1000 l/min unit, a series of experiments were conducted on the existent small collector described above to determine the effects of air flow rate, number of needle cathodes, etc., on collection. Specifically, tests were completed with the unit shown in Figure 4-73, using both a range of air flow rates and from one to six cathode needles. All

tests were conducted with 1.30 μ polystyrene latex beads. Particle collection efficiency in the water (containing 4.0 gm NaCl + 0.5 cc Photo-Flo/liter) was determined in the usual manner. Results are summarized in Table 4-23, together with comparative data from ten tests previously run under standard conditions.

The results shown in Table 4-23 suggest that in scaling up the flow rate to 1000 l/min the critical dimensions of the large precipitator should be based on the following considerations:

- a. The collection efficiency of the 50 l/min collector is not significantly lowered if the intake air speed is increased by a factor of four, i.e., the flow rate is raised from 50 to 200 l/min.
- b. Reducing the number of cathode needles from six to three does not lower the collection efficiency even at 200 l/min. A single needle gives satisfactory collection efficiency at 50 l/min but not at 96 l/min. Accordingly, in scaling from 200 to 1000 l/min, the number of needles should be increased from three to approximately fifteen.
- c. A water-air ratio of approximately 5 ml per 1000 liters of air appears to be satisfactory as regards collection efficiency.
- d. The coarse grade fritted anode disc is satisfactory. Its diameter should be approximately 50 percent larger than that of the air intake tube.
- e. The theoretical minimum anode-air intake tube spacing for unrestricted air flow is $D/4$ where D is the intake tube diameter. For the 50 l/min unit the theoretical spacing is 4.75 mm; the actual spacing used is 4.3 mm. Similar spacing limitations apply to the large precipitator.

Components for duplicate 1000 l/min precipitators, designated Model MB, were fabricated using standard size tubing most closely meeting the criteria outlined above. Their critical dimensions are as follows: 44-mm inner-diameter air intake tube, and 68-mm anode diameter. At 1000 l/min air intake, the linear speed is 11 meters/sec.

In each precipitator, the coarse fritted disc for the anode is sintered into a glass tube rather than being held by epoxy cement as in the 50 l/min unit tested. Water enters the anode from the bottom through a 20 mm OD

Table 4-23

COLLECTION EFFICIENCY TESTS FOR DESIGN OF 1000 l/MIN PRECIPITATOR
TESTS WITH NOMINAL 50 l/MIN COLLECTOR

Air intake tube: 19 mm ID
Anode: Coarse grade fritted disc 30 mm OD
Anode-air intake tube spacing: 4.3 mm

Air Flow l/min	Water Flow cc/min	No. Cathode Needles	Volts KV	Current ma	Water Collection Efficiency, %	Intake Air Speed m/sec	Water-Air Ratio cc/1000 l
200	0.80	3	19.6	0.28	88.1	11.8	4.0
96	0.50	3	19.2	0.24	96.9	5.65	5.2
96	0.50	6	18.2	0.20	95.5	5.65	5.2
50	0.33	1	20.0	0.095	83.9	2.94	6.6
96	0.50	1	20.0	0.11	33.6	5.65	5.2
50*	0.33	6	15-18	0.07-0.14	88.0 ±6.5	2.94	6.6

* Average of ten tests under standard conditions.

glass tube which also supports the anode. A rubber retaining ring on the supporting tube catches the water flow from the anode surface and directs the flow into a glass water-drain tube. Two platinum wire ground connections are attached to opposite sides of the anode surface. Components fit in a 4-inch-diameter Lucite tube for test purposes. Anode-air intake tube spacing is adjustable.

A 12-needle cathode was provided. The stainless steel surgical needles are soldered to a thin brass ring which slides inside the air intake tube; cathode-anode spacing is adjustable. Provision has been made to include 3 to 6 additional needles if necessary. This model, designated MB, is shown in Figure 4-72.

Model MB was first tested for mechanical and electrical performance without collecting particles. At 5 ml/min water flow the anode surface is wetted uniformly and the water flows smoothly off the periphery of the fritted disc. There is no indicating of irregularities on the water surface at 1000 l/min air flow with 20 kv applied voltage. Air was collected and water was admitted continuously with a spacing of 10.0 mm between the glass air inlet tube and the anode and 14.7 mm from the tips of the cathode needles to the anode. The air inlet-anode spacing approximated the theoretical minimum of one-fourth of the inlet tube diameter (44 mm). At 1000 l/min air flow the pressure drop across the unit was 0.42-inch water.

Typical voltage current relations for the standard electrode spacings, 1000 l/min air flow, and 5.0 ml/min liquid flow were as follows:

<u>Applied Voltage, kv</u>	<u>Current, ma</u>
2.0	0.0
4.0	0.0001
6.0	0.013
8.0	0.057
10	0.110
12	0.165
14	0.250
16	0.370
18	0.410
20	0.500

In general, the electrical and mechanical operating characteristics of the precipitator appear to be satisfactory.

The efficiency of collection in water was measured in the Metronics Laboratories under the same conditions with the following results:

Particle Concentration: From 66 to 3000/liter nominal

Sampling Time: 15 minutes nominal

Some data on the efficiency of collection in the liquid effluent follow:

<u>Voltage,</u> <u>kv</u>	<u>Current,</u> <u>ma</u>	<u>Efficiency,</u> <u>%</u>	<u>Notes</u>
20.0	0.50	76.6	A
18.6	0.50	98.4	B
20.0	0.50	93.8	B
16.0	0.40	95.7	B
19.0	0.50	65.5	B,C
20.0	0.50	95.2	B,D
20.0	0.50	84.5	B,E

A - Particle concentration 66 particles/liter nominal

B - Particle concentration 3000 particles/liter nominal

C - Test run on unit with an un-firepolished air inlet

D - Test run on Serial No. 1 with a firepolished air inlet tube
(unit kept at Metronics)

E - Test run on unit prior to shipment to SGC (Serial No. 2)

The high-air volume range of operating conditions was also briefly explored. For this purpose, the spacing between air inlet tube and anode was increased to 14.7 mm, while maintaining the cathode needle spacing unchanged at 14.7 mm. Air could be sampled at flow rates exceeding 2000 l/min, with some increase in the total number of particles collected, but with some sacrifice of percentage collected.

In tests following delivery to Space-General, good liquid flow characteristics were obtained when phosphate-buffered saline containing 0.25 percent Tween 80 was substituted for the saline-Photo-Flo solution used at Metronics.

Most recent tests of the Model MB large collector at SGC have demonstrated its effectiveness in collecting actual bacteria. FITC-stained BG spores were used as the test organism. The collector was connected directly behind a nebulizing train; the entering and exhaust airstreams were sampled through membrane filters, and a portion of collection fluid was also filtered. Organisms deposited on the filters were counted under the microscope.

Test results are compiled in Table 4-24. The average collection efficiency is 55 percent. Material balances show that approximately 15 percent of the organisms are not accounted for, and have presumably accumulated on the walls of the collector. (Both collection efficiency and balance fall much lower if the fluid flow rate is decreased much below 5 ml/min.) These results are comparable to those recently obtained at Metronics with the small collector on other programs. Although inferior to values obtained with polystyrene spheres, they are certainly adequate. It should also be recalled that the total number of particles sampled may be increased (though at the expense of percentage efficiency) by increasing the air sampling rate beyond the design value. Nevertheless, the long-time accumulation of organisms in collectors is undesirable, and will be studied systematically in future efforts. Radioactive-tagged organisms will be used to locate the sites of accumulation.

In other recent experiments, Model MB collectors have been applied to coupled operation with the chemiluminescence detector and the liquid partition separator, as described in the appropriate sections.

4.2.1.7.4 CONCLUSIONS

Performance of the porous electrode electrostatic precipitator has proved very encouraging. It is recommended for use in detection systems which require an aqueous suspension of aerosols, and should find a wide application. Further development is also desirable, and has been planned for the program continuation. In particular, there is a need to eliminate particle hold-up, to decrease collection fluid requirements, and to assure compatibility with other system components.

4.2.1.7.5 REFERENCES

- (1) U.S. Patent 2, 868, 318, January 13, 1959.

Table 4-24

**EFFICIENCY TESTS OF POROUS ELECTRODE ELECTROSTATIC
PRECIPITATOR, MODEL MB**

FITC Stained *B. globigii* (spores)
1000 l/min Air Sampling Rate
Liquid Flow 5 ml/min

Run No.	Collector Fluid	Sampling Location	Bacterial Conc. #/ml	% Recovery	Conditions
1	PBS + 0.05% Photo-Flo	Entry Collector Exhaust	6.0×10^7 3.9×10^7 0.87×10^7	64 14 78% Balance	19.6 KV 0.42 ma.
2	PBS + 0.05% Photo-Flo	Entry Collector Exhaust	1.4×10^8 9.7×10^7 1.4×10^7	68 14 82% Balance	19.2 KV 0.40 ma.
3	Water + 0.05% Photo-Flo	Entry Collector Exhaust	2.7×10^4 1.1×10^4 1.1×10^4	39 39 78% Balance	18.8 KV 0.39 - 0.40 ma.
4	Water + 0.05% Photo-Flo	Entry Collector Exhaust	1.6×10^4 6.9×10^3 6.7×10^3	45 43 88% Balance	18.4 KV 0.38 ma.
5	Water + 0.05% Photo-Flo	Entry Collector Exhaust	1.5×10^4 9.0×10^3 5.2×10^3	60 34 94% Balance	20 KV 0.52 ma.
6	Water + 0.05% Photo-Flo	Entry Collector Exhaust	5.6×10^5 4.3×10^5 2.3×10^5	77 42 119% Balance	20 KV 0.50 ma.
7	0.4% Saline	Entry Collector Exhaust	2.7×10^4 8.8×10^3 6.0×10^3	33 20 53% Balance	20 KV 0.45 ma.
8	0.4% Saline	Entry Collector Exhaust	2.8×10^4 1.4×10^4 1.0×10^4	50 38 88% Balance	20 KV 0.49 ma.

% Collector Recovery, Average Value 55 ± 14 (σ), Average Balance 85%

4.2.1.8 ELECTROSTATIC SPRAY COLLECTOR

4.2.1.8.1 SUMMARY

Promising results were shown in preliminary tests of a new electrostatic spray collector. This device uses for collection a fine spray produced by running the collecting water through a hollow electrode connected to a high voltage source. In the initial test, a collection efficiency of 39 percent was obtained for 3 μ latex with 45 l/min air flow and 0.5 ml/min water flow. The device is of interest because the particles (e.g. fragile vegetative cells) might be collected in the charged water droplets, without charging the bacteria directly in the high voltage field.

4.2.1.8.2 INTRODUCTION

Under proper conditions a fine liquid spray can be produced by running water through a hollow electrode connected to a high voltage source. If particle removal can be achieved by this principle, a novel collector might be developed having certain advantages over electrostatic units now being evaluated on this contract and elsewhere. One advantage, for example, is that the particles might be collected by charged droplets and hence could be less subject to damage than if the particles (for example, fragile bacterial vegetative cells) were charged directly by the corona discharge.

4.2.1.8.3 STATUS

Exploratory experiments were conducted by Metronics Associates to determine if an electrostatic spray could be used to remove particles from a moving air stream and retain them in the collected spray. Water was sprayed from an 18-gage hypodermic needle (anode) centrally placed at the entrance of a 1-inch diameter vertical metal tube 6 inches long. A potential of 20 kv DC at about 0.2 ma was impressed across the cell. Air containing 3.0 μ polystyrene latex beads was passed through the tube at 45 l/min and the collected spray was removed at the downstream end of the tube. The results show that electrostatic sprays can be maintained at water flow rates of 0.5 ml/min, and that the nature

of the spray depends upon the applied voltage, polarity, geometrical configuration and conductivity of the water. Collection efficiencies in the water as high as 39 percent were obtained in the limited preliminary experiments; factors controlling collection efficiency have not been determined.

4.2.1.8.4 CONCLUSIONS

Since it is evident that significant particle collection can be obtained with the electrostatic spray collector, it is desirable to examine this principle further, especially to establish whether the principle can be used for a novel collector that is less damaging to bacterial particles than the usual electrostatic collector. The factors controlling collection efficiency must be identified and suitable values established. The collection efficiency as related to process variables and the condition of collected cells (damage to vegetative cells, viability, etc.) must be established.

4.2.2 INERTIAL METHODS

4.2.2.1 DENSITY GRADIENT CENTRIFUGATION

4.2.2.1.1 SUMMARY

The separation of bacterial fractions from atmospheric debris by the technique of density gradient centrifugation was briefly examined in Beckman laboratories. Successful separations of bacteria from airborne background particulates were made, but the separations were not deemed sufficiently rapid for practical use. The research was therefore discontinued in favor of other methods considered more practical, rapid, and selective.

4.2.2.1.2 INTRODUCTION

It is desirable to investigate the practicability of separation by density gradient centrifugation. The technique should be applied both to fractionating mixed bacteria and to separating bacteria from other airborne particulate matter. This examination was undertaken by Beckman Instruments, Inc.

Density gradient centrifugation has been widely used in separation of dissolved macromolecules and suspended particles, including some of biological origin. Although the literature records applications to DNA, viruses, mitochondria and other materials⁽¹⁾, applications to bacteria are not common. However, for the present program the technique offers a new approach to fractionation based on density differences, and backed by much experience and a high development of instrumentation. Certain separations are especially challenging, such as elimination of fluorescent background particles, or removal of non-pathogens from pathogens.

In adapting this technique to the present program, bacterial suspensions were exposed to high centrifugal force in a liquid medium having a density gradient. Both single species and mixtures were tested. Detection and identification of zoning was accomplished by plating small aliquots taken at various levels in the centrifuged material. An attempt was also made to obtain zoning in a gellable medium.

4.2.2.1.3 STATUS

Bacterial suspensions were successfully fractionated in a sucrose density gradient ranging from 1.0 + to 1.2 g/ml. Not only were single species collected in specific density zones, but some separation of species in mixed suspensions was accomplished. Bacteria were separated from large quantities of airborne background particulates. In a gellable (agar) gradient medium, bacterial zoning or fractionation was not observed.

As an example, results are given below which were obtained on centrifuging suspensions of E. coli (Tube No. 1), E. coli + B. globigii (Tube No. 2) and B. subtilis + S. marcescens (Tube No. 3). The 5-ml tubes contained identical portions of sucrose solution, giving a density range (d_4^{20}) of 1.0 to 1.154. Tubes were centrifuged at 38,000 rpm for 150 minutes (room temperature) in a Spinco Model L-HT preparative ultracentrifuge, with an SW-39 swinging bucket rotor. Contents of each tube were removed in successive layers with a sterile pipette, and were identified by culturing or microscopic examination as follows:

Tube No. 1: E. coli in pipette fractions 19 to 24 (from a total of 34), at density zone 1.09 - 1.113.

Tube No. 2: B. globigii in pipette fractions 13 to 17 and E. coli in 20 to 22 (total 32), approximately at zones 1.065 - 1.085 and 1.10 - 1.11 respectively, (Growth of B. globigii in fractions 13 to 17 and in the control sample was very slight).

Tube No. 3: S. marcescens in pipette fractions 15 to 18 and B. subtilis in 31 to 34 (total 34), approximately at zones 1.071 - 1.085 and 1.146 - 1.16, respectively.

4.2.2.1.4 CONCLUSIONS

Although separations of bacteria from atmospheric background particulates was achieved in this limited study, the investigation was discontinued. The separations required too great a period of time in a complex device to justify further effort.

4.2.2.1.5 REFERENCES

- (1) An Introduction to Density Gradient Centrifugation, Spinco Division, Beckman Instruments Inc., Technical Review No. 1 (1960).

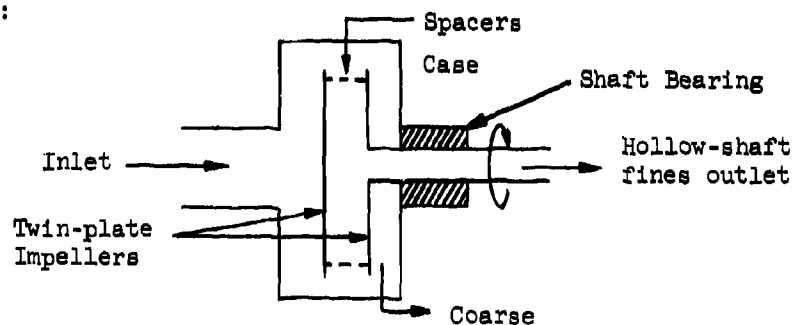
4.2.2.2 DOUBLE PLATE IMPELLER CENTRIFUGAL SEPARATOR

4.2.2.2.1 SUMMARY

A centrifugal separator based on a double-plate impeller, which was thought to provide efficient separation of large (>5 to 8μ) particles from bacterial aerosols has been described. A device of this type was constructed and evaluated, but was found unsuitable for use in a practical collection sub-system. The sharpness of separation is no better than in the simpler open-cell foam filter; operation of the device is very complex because of the interdependence of several variables; and the holdup appears significant for small quantities of particulates. Further work on this technique was stopped in order that more promising techniques might be studied.

4.2.2.2.2 INTRODUCTION

There are a variety of separators in which the classification of particulates depends on the inertia of the particle, this factor being strongly dependent on the particle size. Theoretically then, efficient particle size separation should be achieved in a device based on the inertia of the particle in a gas or liquid stream. However, in practice, sharp separation is not achieved because the turbulence of the flow through the device produces a mixing action whereby the separations deviate somewhat from the theoretical. A new type of centrifugal classifier has been described which promises more efficient production of sharply defined fractions of particulates in the micron range. This device is the double-plate impeller centrifugal classifier described by Godridge, *et. al.*⁽¹⁾. It was the objective of this study to evaluate this device for the effective separation of large background particles (>5 to 8μ) from bacterial aerosols. The unique principle, as described by Godridge, is depicted below:



The gas stream containing the particulates enters the inlet tube and flows radially outward between the case and the first rotating disc and then radially inward between the two rotating discs to the axial outlet. The rotation of the discs imparts a spin to the air stream and hence to the particles. This centrifuging action provides a barrier to the entry of particles between the spinning discs so that only those smaller than a given size are able to pass with the gas to the outlet. The coarse (oversize) fractions circulate in the annular space between the rotating discs and the case, where the dispersion and circulation aids in providing further separation of fine particles which may remain in the oversize fraction. The coarse particles are intermittently drawn off from the collection zone inside the case. The smaller particles (in the present application, the biological-containing gas, from which coarse inorganic and atmospheric contaminants has been removed) flow out through the hollow shaft and into a suitable collector or concentrator. The quality of separation described by Godridge was excellent. In his work, the cut size ranged from 4 to 26 μ diameter with air flows ranging up to about 500 l/min, with a 4-inch diameter impeller. The scale-up of this device to handle 1000 to 10,000 l/min appeared readily achievable.

4.2.2.2.3 STATUS

A double-plate impeller centrifugal separator was designed and constructed at Metronics on another program and made available to this contract. This device employed 6-inch diameter plates in the impeller, slightly larger than the 4-inch impellers used in the original classifier described by Godridge. The evaluation of the double-plate separator yielded the following observations.*

- a. The sharpness of separation of the double-plate separator is not significantly different from that of the simpler cyclone or open-cell foam filter.

* The observations are based largely on the results made available to this program from a more extensive study with a similar device. These studies, which were not a part of this contract, involved separation of 1 to 30 μ quartz, zinc cadmium sulfide fluorescent particles, glass beads, tungsten, dolomite, feldspar, and several clays.

- b. In its present form, the device is very complex to operate because of the interdependence of several operating variables. For example, the pressure drop through the system is dependent on the rotational speed of the double-plate impeller; for each separation condition of size and material required, there appears to be an optimum rotor speed and inlet air flow rate. In addition, the coarse fraction must be removed from the collector housing at regular intervals to maintain uniform distribution.
- c. For small quantities of particulate matter, such as exist in ambient atmosphere, there is a significant holdup of particulates in the device.

On the basis of these experiences, work on the double-plate impeller centrifugal separator has been stopped in order that more promising techniques, such as the foam filter, might be studied further.

4.2.2.2.4 CONCLUSIONS

It was concluded from these studies that the double-plate separator was not suitable for the intended use as a part of a biological aerosol collecting, concentrating, and separating system. It appears that much simpler approaches, such as the open-cell foam filter, are now able to satisfy the requirements for an efficient primary separator to remove the large (> 5 to 8μ) particles of atmospheric debris from the biological pathogen sample. Further studies with the double-plate separator are not recommended with sensing systems as presently envisioned.

4.2.2.2.5 REFERENCES

- (1) Godridge, A.M., Badzioch, S. and Hawsley, P.G.W. "A Particle Size Classifier for Preparing Graded Subsieve Fractions", J. Sci. Inst. 39: 611-13, 1962.

4.2.2.3

INVESTIGATION OF PRIMARY SEPARATORS

4.2.2.3.1

SUMMARY

Four types of devices have been shown effective for use as a primary separator in a collection subsystem to remove efficiently the approximately 50 percent by mass of atmospheric background larger than 5μ . Of the principles tested, impingement on the fibers of open cell polyurethane foam (commercial Scott Foam) appears most practical. Separation in a 50-liter/min air stream can be achieved with a pad 1.5 inches in diameter and 0.5 inch thick of the 60 pore/inch foam. The filter will retain about 90 percent of the 5.6μ material of density 1.5, while holding only 10 percent of the 2.6μ material. The pressure drop across such a filter is only 0.07 inch of water, and the capacity appears sufficient for several days of continuous operation. The cut size can be varied over reasonably wide ranges by changing the pore size and face velocity.

The May pre-impinger also appeared satisfactory for a small primary separator. Both the May pre-impinger and open-cell foam appear superior to a standard cyclone or a tortuous path of tubing.

4.2.2.3.2

INTRODUCTION

The need for a primary separator which will effectively remove the background particles larger than about 5μ is evident. The examination of a number of the atmospheric particulates collected in the background study has revealed a substantial number of large particles, in some cases in excess of 50μ in maximum dimensions. The further examination of these large particles under lighting conditions such as used in the FAST system reveals that a substantial number of the particles fluoresce, with blues (predominantly), reds, and orange and green particles being visible. It has been shown also that more than one-half of the particulates in the atmosphere on a mass basis are larger than about 5μ (Section 4.3), while it is also known that particulates $< 5\mu$ in diameter are most likely to be inhaled. A primary separator which can efficiently remove these large particles while not removing the microorganisms in the aerosol will thus contribute substantially to reducing the ratio of background particulates to pathogens to be detected.

A consideration of the principles available for separation suggests that the inertia of the particle may offer the best means for separation. Since the background particles to be separated are large relative to the pathogen to be detected and, in general, may be more dense, the atmospheric contaminants will have somewhat greater inertia than the biological particle. It was on this basis that Metronics conducted a study of several types of primary separators. These studies have generally been made in the 30 to 60 l/min flow rate range although the principles are readily extended to other flow rate ranges of interest.

The preferred classifier would be one with low pressure drop, sharp size classification, and small dimensions, and would be maintenance-free, at least over 24-hour operating periods.

4.2.2.3.3

STATUS

In consideration of the principles outlined, the following classifiers were examined in the Metronics laboratories.

A glass cyclone⁽¹⁾

Open-cell foam of several pore size openings*

The May Pre-Impinger⁽²⁾

A straight-through plastic tube in a tortuous path

4.2.2.3.3.1

CYCLONE CLASSIFIER

A qualitative test was conducted in which an outside air sample was drawn first through a glass 2-1/2 inch diameter cyclone, and a fraction containing small particles was drawn through a 25 mm, 0.8 μ pore size, black Millipore filter. This cyclone is a special design, in which all dimensions are scaled in definite proportions to the principal diameter. Air was drawn through the cyclone at the rate of 50 l/minute and air was sampled by the filter at 15 l/minute.

* Scott Industrial Foam - Chester, Pa.

Both filter samples were examined microscopically at 200X under ultra-violet illumination. There was a distinct difference in the appearance of the two filters indicating that many of the larger particles, 20 to 50 μ , had been stopped by the cyclone.

Classifier performance may be rated by measuring the cut-size for the particles separated. The other important characteristic of a classifier is the sharpness of size separation. The more nearly vertical is the curve on a plot of efficiency of retention versus particle diameter, the sharper is the particle size separation.

In this case and in future reference, cut-size is defined as the particle diameter at which 50 percent of the particles are retained by the classifier and 50 percent allowed to pass. The usual equation relating the cut size, the dimensions of the cyclone, and parameters involving particle density and flow rate are given by Perry⁽¹⁾.

The cut-size was measured quantitatively at a sufficiently high flow rate so that particles could be sized and counted with the Royco Particle Counter. For this purpose an aerosol of ground feldspar was generated in a test chamber and particles withdrawn through the cyclone at controlled flow rates until a rate was found at which a cut-size could be measured with the Royco Particle Counter. The quantitative measurement of cut-size with the Royco Particle Counter was limited to diameters less than 5 μ . It was found that the 2-1/2 inch cyclone had a cut-size for feldspar of 2.75 μ at 266 l/min flow rate of air. The shape of the curve showing the retention of various sized particles by the cyclone was then developed as shown in Figure 4-75; the comparison with some other separators is shown in the same figure. With these data, it was then possible to calculate the cut-size (D) of a cyclone for various flow rates. The equation for the cyclone configuration used reduces to:

$$D = 19.1 \times 10^{-4} D_c^{3/2} (F\rho)^{-1/2}$$

where

$$D_c = \text{diameter of cyclone (cm)}$$

$$F = \text{air flow rate (cm}^3\text{/sec)} = VD_c^2/8, \text{ for this particular configuration}$$

$$\rho = \text{density of aerosol particle (g/cm}^3\text{)}$$

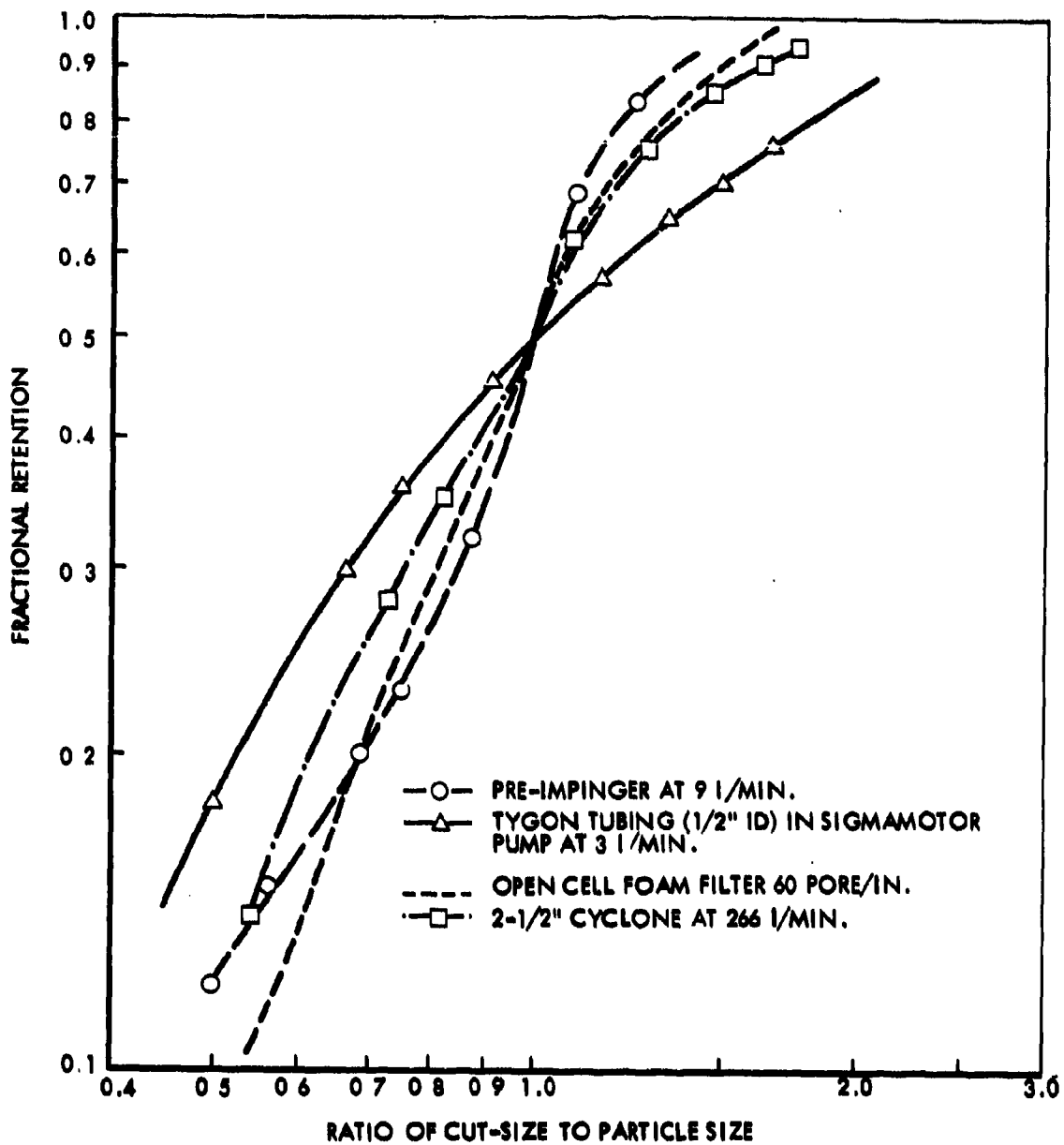


Figure 4-75. Retention of Feldspar by Open Cell Foam Filter, Pre-Impinger, 2-1/2-Inch-Diameter Cyclone and Tygon Tube Classifier

The constant 19.1×10^{-4} probably applies only to the particular cyclone used, but it is indicative of the relationship between cut-size, density, flow rate and cyclone size.

4.2.2.3.3.2

OPEN-CELL FOAM CLASSIFIER

Open cellular polyurethane material is obtainable in a range of pore sizes and thicknesses as Scott Foam, in which all cells within the foam are intercommunicating. Thus, the passage of air through such a foam would be approximately equivalent to the use of a multi-stage ribbon type impaction classifier in which fine particles streamline around the fibers and larger particles are collected by impaction and are retained on the fibers.

As in the case of the cyclone, a qualitative test was made on outside air. The air was drawn through a pad of 20 pore/inch Scott Foam, 1 inch thick and 2-1/8 inch diameter, at the rate of 50 l/min. An inspection of the Millipore filter samples of air that had passed through the foam and air that had been collected directly showed that qualitatively the foam was comparable to the 2-1/2 inch cyclone.

A series of tests was then run to determine the cut-size of the open-cell foam over a range of pore sizes, pad thicknesses, and flow rates. The cut-sizes of several foam grades and conditions used are shown in the following tabulation.

CUT SIZE OF SCOTT FOAM FOR FELDSPAR

<u>Grade</u> <u>(pores per in.)</u>	<u>Thickness</u> <u>(inches)</u>	<u>Face Velocity</u> <u>(ft/sec)</u>	<u>Cut-Size</u> <u>(microns)</u>	<u>Pressure Drop</u> <u>(inches water)</u>
60	1.0	4.8	2.0	---
80	1.0	2.4	2.75	0.660
60	2.0	2.4	2.75	---
60	1.0	2.4	3.0	0.215
80	1.0	1.2	3.5	0.300
80	1.0	0.6	4.0	0.145
60	1.0	1.2	4.0	0.090
60	0.5	2.4	4.0	---

The experience with the foam filter suggested that the material would serve at very low pressure drops and would have reasonable life between replacements in a continuously operating detecting system. Some indication of the life of these filters has been obtained.

From the previous experiments, it appeared that a 0.5 inch thickness of 60 pore-per-inch foam would be satisfactory for the removal of $> 5\mu$ particles, at air flow rates of 20 to 50 l/min. Data for this filter are shown in Figure 4-75. The previous experiments with feldspar (density 2.5 g/ml) indicated that the cut-size (50 percent retention) was 4.0μ at a face velocity of 2.4 ft/sec. Since the retention should be proportional to ρd^2 , where ρ is the particle density and d the diameter, examination of the curves indicate that 90 percent of the 5.6μ material of density 1.5 and 10 percent of the 2.6μ particles are retained.

A filter assembly was constructed to determine how the pressure drop across the filter varied with exposure time to the atmosphere. A 1-1/2 inch diameter pad of the 60 pore/inch pad, lightly coated with vaseline*, was operated at 50 l/minute. The initial pressure drop was 0.070 inch of water. After running a total of 60 hours (40 in outside air (Palo Alto), and 20 hours in normal-laboratory air), the pressure drop increased by less than 0.015 inch. Therefore, it appears that under normal atmospheric conditions a filter of this type can be run for several days without undue pressure change resulting from pore clogging.

Data were also obtained for the separation of large, low-density particles with the open cell foam filter. In this experiment, aerosols preceding and following the filters were sampled with Rotorod samplers. The aerosols used were paper mulberry pollen (12μ diameter), "Morgan" wheat rust spores (10μ), and Lycopodium fern spores (28μ). Each of these materials is approximately mono-disperse, but the particles are not completely spherical nor are the densities known exactly. For this reason the Stokes diameter of each material was measured by settling tests in the chamber. Some data indicating percent removal by a 1/2 inch thick 10 pore/inch foam filter follow:

* Dipped in a 5 percent solution of vaseline in CCl_4 and squeezed dry, to increase retention efficiency.

	Percent Removal at Face Velocity, ft/sec					
	<u>0.5</u>	<u>1.0</u>	<u>1.5</u>	<u>2.0</u>	<u>2.5</u>	<u>3.0</u>
Paper Mulberry Pollen (12 μ)	-	21	42	63	72	-
Morgan Rust Spores (10 μ)	60	78	85	-	-	-
Lycopodium Fern Spores (28 μ)	72	83	87	-	-	-

Initial results indicated that the filter should be given an adhesive coating so that particles would remain after impaction (i.e., particle retention by the uncoated foam filter did not show a reasonable correlation with changes in air flow rate and other operating conditions). However, when the foam was coated with vaseline as described previously, reasonable separations were achieved.

4.2.2.3.3

THE MAY PRE-IMPINGER

The May Pre-Impinger, a selective aerosol sampler, was designed to retain particles that would normally be retained in human nasal passages, and to pass particles that are retained by the lungs. It consists of a small vessel partly filled with water, in which the path of the entering aerosol passes over the water surface. It is sometimes used by connecting ahead of a standard impinger in a sampling train. The cut-size of the Pre-Impinger was designed at 4 μ , giving approximately the size range designed for the classifier in the present studies. The shape of the curve of impinger efficiency and its cut-size were verified experimentally with the Royco Particle Counter, as shown in Figure 4-75.

4.2.2.3.4

TORTUOUS PATH TUBING CLASSIFIER

Passage of an aerosol through any type of tortuous path tends to produce particle size classification. It seemed reasonable to expect that the passage of an aerosol through a Sigma Motor Pump, in which flexible tubing is alternately squeezed and released to produce a peristaltic pumping action, might result in an acceptable size classification. If this could be accomplished, it would have the further advantage of a collection system capable of operating under pressure and thereby minimizing some of the problems of transferring water out of the collector. An aerosol of feldspar was pumped through the 1/2 inch ID Tygon tube in a Sigma Motor Pump at 3 l/minute. A significant classification resulted, as shown in Figure 4-75.

4.2.2.3.4

CONCLUSIONS

The investigation of primary separators indicated that the open cell polyurethane foam filter functions as an effective, compact, inexpensive, and highly efficient filter operating at very low pressure drops. As such, its use as a primary separator in detection systems can be strongly recommended. The cyclone classifier and May Pre-Impinger were also effective primary separators but are not as compact, inexpensive, or effective at low pressure drops as the foam filter. The tortuous path of tubing was also effective in removing particles but is not as attractive a primary separator as the open cell foam.

4.2.2.3.5

REFERENCES

- (1) Perry, J., ed., Chemical Engineers Handbook, 3rd ed. McGraw-Hill, p. 1024.
- (2) May, K. R. and Druett, H. A., Brit. J. Ind. Med., 10: 142,151, 1953.

4.2.3

COMBINED FORCE EFFECTS

4.2.3.1

LIQUID PARTITION

4.2.3.1.1

SUMMARY

A continuous liquid partition separator was developed and operated successfully. The operating principle is the opposing distribution of bacteria and background particles between immiscible phases of certain aqueous polymer solutions. This behavior was shown to be general for a large number of species of bacteria. A continuous centrifuge was adapted for rapid disengagement of liquid phases, which pass out as two separate effluents. Total feed rates (sample + polymeric medium) of about 10 to 20 ml/min are attainable. The separating medium was a 2-phase solution of dextran and polyvinyl alcohol. With bacteria alone, more than 90 percent were retained in the dextran-rich effluent under continuous, steady-state conditions. For a binary mixture of bacteria and dust, favorable operating conditions gave a retention of > 90 percent bacteria and removal of > 90 percent dust. Additional runs with P^{32} -tagged bacteriophage showed the technique to be adaptable to virus separation. The separator was also coupled with a porous electrode electrostatic precipitator to demonstrate continuous collection and separation of aerosolized BG spores.

An improved centrifugal separator was constructed, and will be operated on a successor program. It is designed to be a more efficient developmental tool than the original separator, having advantages of simplicity, compactness, minimal maintenance, short dwell time, and ease of change of operating variables.

4.2.3.1.2

INTRODUCTION

The objective of research on liquid partition is to develop a process for selectively separating microorganisms from atmospheric background particles, or for performing other separations of biological materials. The operation is based on a principle which has been investigated in recent years by workers in Sweden^(1,2). Fine particles of different kinds are observed to

partition in characteristic ways between pairs of immiscible aqueous polymer solutions. A number of polymers whose solutions form conjugate immiscible pairs are suggested in the cited references. Some examples are dextran/polyethylene glycol, dextran/methyl cellulose, and sodium dextran sulfate/polyethylene glycol. These systems have proved their effectiveness in segregating and preserving biological materials such as bacteria, yeast cells, erythrocytes and viruses. The desired material concentrates in one phase, while the contaminants to be eliminated are retained in the other.

For particles in the size range of interest, the pattern of distribution is largely determined by interfacial energies, and is relatively independent of density and shape factors. Although the bacteria and other particles are suspended and not dissolved, the effect is analogous to partition of solutes between two immiscible solvents, in that distribution coefficients are constant over a range of concentrations. Therefore, separation methods may be evolved which are analogous to the well-understood techniques of liquid-liquid extraction. Moreover, distribution coefficients for suspended particles are ordinarily high, and this circumstance favors ease of separation in a small number of stages.

However, in its applications reported to date in the literature, liquid partition has remained a tool for laboratory research and not a process operation.

Certain preliminary steps were considered essential for development of a continuous process based on liquid partition. The first step was a choice, by means of test tube experiments, of the most effective system of polymer solutions. The choice had to be largely empirical, except for guidance from the literature, because of the great difficulties of measuring interfacial energies to provide a basis of prediction.

This was to be followed by quantitative confirmations of selectivity by measurements of distribution coefficients. The aforementioned tests are concerned only with distribution equilibria, and it may also be advantageous to find new means of increasing the rates of separation. Finally, a continuous flow system must be designed, constructed, and evaluated.

4.2.3.1.3

STATUS

4.2.3.1.3.1

LABORATORY EXPERIMENTAL WORK

The first step in the development of a process is selection of the desired phase system by means of test tube experiments. The solvent systems formed by water with organic or non-aqueous liquids were first considered. However, the harshness of the solvents towards bacterial cells and the affinity of all particles for the aqueous phase forced a solution to be sought among all-aqueous systems. The aforementioned conjugate pairs of aqueous polymeric solutions seemed to offer better possibilities of separating all types of particles.

Examination of many water-soluble polymers in many combinations and at different concentrations, volumes, and temperatures led to the present system. This system showed an ability to segregate bacteria in one phase and typical contaminants (road dust, clay, carbon black, puffball spores) in the other phase at room temperature. The effective system is made up of immiscible solutions of polyvinyl alcohol and dextran, of selected molecular weights. Results of determination of the partition of fine particles in this system by quantitative measurements are given in Table 4-25.

The polymer system used for the separations made thereafter was composed of 3 percent PVA 72-60 and 10 percent dextran, D-250. The sample of particulates was added to this mixture and centrifuged for two minutes at 300 x g. The cells mainly separated into the lower (dextran-rich) phase.

Other experiments showed that the distribution coefficient of organisms remains roughly constant over a range of concentration of several log cycles, and is not greatly affected by the presence of other particulates. This behavior is important for continuous processing.

As a next step in process development, the long time interval for disengagement of the mixed, viscous layers was largely eliminated by use of centrifugation. This reduced the time taken for separation of test-tube samples from more than one hour to two minutes.

Table 4-25

SPECIFIC PARTITIONING OF PARTICLES

	Preferred Phase	Distribution Ratio	Determination
<u>E. coli</u> (P ³² -labelled) S/6	lower	11	P ³² count
<u>Chromobacterium violaceum</u> (CV)	lower	33	plate count
BG spores	lower	71	plate count
<u>Pseudomonas aeruginosa</u>	lower	15	plate count
<u>Micrococcus citreus</u>	lower	7	plate count
BG vegetative	lower	92, 81	plate count
BG in presence of concentrated ARD	lower	39, 54	plate count
<u>Sarcina lutea</u>	lower	12	plate count
Mixed bacteria from soil sample	lower	10	plate count
Zinc cadmium sulfide tracer	upper	15	microscopic
Arizona road dust (ARD)	upper	5	plate count
ARD in presence of concentrated CV	upper	3.9	plate count
Rodgers clay (80% 0.1 to 6 μ)	upper		
Carbon black (0.47 μ avg)	upper		
Puffball spores (4 to 5 μ)	upper		
<u>B. subtilis</u>	lower		
<u>Neisseria perflava</u>	lower		
<u>B. mycoides</u>	lower		
<u>Neisseria flavescens</u>	lower		
<u>Pediococcus cerevis</u>	lower		
<u>Pseudomonas fluorescens</u>	lower		
<u>B. cereus</u>	lower		
<u>B. megatherium</u>	lower		
<u>Asperigillus niger</u> (mold)	upper		
<u>Serratia marcescens</u>	lower		
<u>Mycobacterium phlei</u>	lower		
<u>Staphylococcus aureus</u>	lower		
<u>Saccharomyces cerevisiae</u>	lower		
<u>E. coli</u> B/5	lower		
<u>E. coli</u> O26 B6	lower		

It was now necessary to transfer from batch to continuous operation. A remodeled version of a Sorvall Model SS-1-KSB-1 continuous centrifuge was used as the separator (Figure 4-76). The feed line and the two effluent lines passed through the hollow shaft to separate rotating chambers, from which they led to the offset test tube (within the centrifuge rotor) in which the liquid phases separated under a force field of approximately $300 \times g$. Experience with the first version showed contamination of the separated effluent phases by the feed, due to leakage along the shaft between chambers. Accordingly the device was revised by inserting rotary seals between chambers, and the dextran effluent chamber was changed to a direct line with no hold-up volume, and some other parts of the shaft assembly were also modified.

As the operation was used and tested, other mechanical improvements were incorporated. A simple change in the entrance line leading into the offset test tube appeared to be most effective. The open end of the line was plugged, and small holes were drilled in the cylindrical surface near the end, thereby introducing the feed as several fine lateral sprays instead of large falling drops.

In another useful change, the electric motor was rewired and a speed regulator was procured, giving very stable operation in a speed range of 500 to 5000 rpm. A final change was decided when effluent purities were improved by substitution of a new axial feed distributor in the centrifuge. The new part has a downspout approximately 3 mm longer than the old, which prevents leakage from occurring between the influent and the dextran effluent.

Although the modified Sorvall centrifuge was used in all continuous operations of the present program, a new centrifuge has been provided for future use. Increasing experience has focused attention on the ways in which the original centrifuge is unsuitable for an intensive test program. The large number of parts requires excessive time for assembly between runs, and leads to frequent delays for maintenance and replacement. More flexibility is needed in varying the operating parameters. With the test-tube-shaped separating chamber positioned in a solid rotor, it is not possible to experiment freely with the size and shape of the phase-separating zone to increase efficiency. Similarly, there are difficulties in varying residence time. It would be highly desirable, in fact, to provide a transparent separating zone so that the process of phase equilibration could be observed.

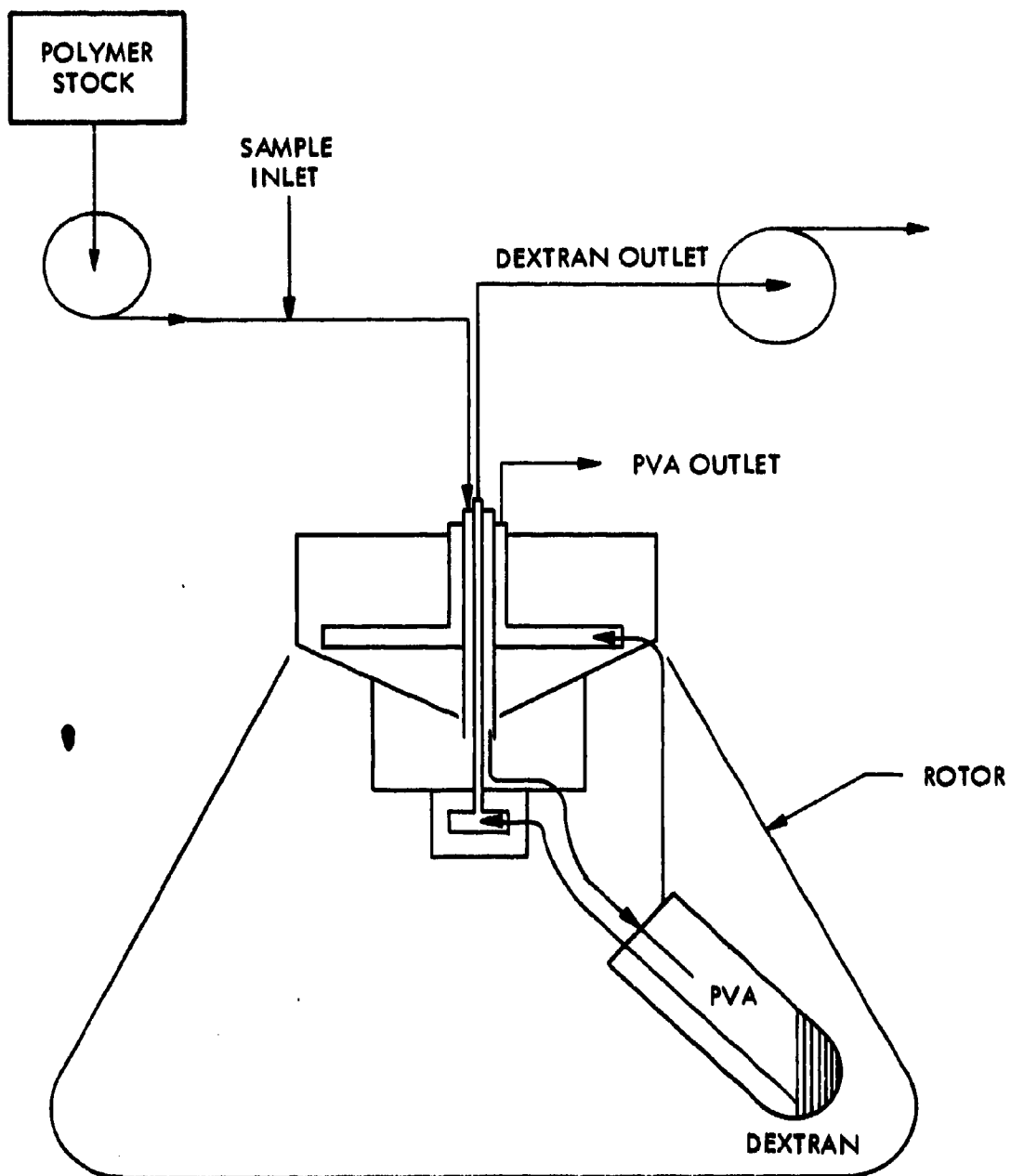


Figure 4-76. Continuous Separation in Remodeled Sorvall Centrifuge

The new centrifuge was designed to satisfy these needs which have become so evident. A drawing is shown in Figure 4-77. Aside from the drive motor and frame, the unit consists essentially of (1) a rotating portion, and (2) a stationary portion. The rotating portion is built up from transparent discs of polymeric materials bolted together. The separating zone is a disc-shaped volume (of any desired size) machined out of the lower disc. The layers above act as gaskets and provide channels for the flow of the incoming and effluent streams. A flat, stainless steel part is bolted on the top center of these layers. It also contains holes drilled through for liquid flow, and bearing surfaces for contact with the stationary portion. The cylindrical stationary portion, in turn, is a Teflon distributor block. A series of concentric O-rings is cut in the bottom, providing separated channels for flow between the distribution block and the rotor.

The new partition centrifuge design will operate with system parameters similar to the modified Sorvall unit: flow rates 2 to 20 ml/min and centrifuge force fields of 200 to 500 x g. However, the designed holdup volume can be smaller than 25 ml in the new design. This can reduce the hold-up time normally obtained with current operating conditions from 5 minutes to less than 2 minutes.

Experimentation with various pumping arrangements using the modified Sorvall led to the choice of constant-displacement pumping of the influent (polymer mixture and sample) and of the upper (FVA-rich) effluent, combined with free flow of the lower (dextran-rich) effluent. Both the influent and the FVA-rich effluent are now pumped with Sigmamotor T-8 peristaltic pumps, which maintain constant flow rates for long periods of operation. Smaller pumps of similar capacity may be provided for the new centrifuge.

4.2.3.1.2.3

ASSAY METHODS

Accurate distribution ratios and therefore controlled assay tests needed to be developed for characterizing the effluent from the liquid partition separator. The tests had to be reliable but rapid, and measure concentrations of both bacteria and background particulates.

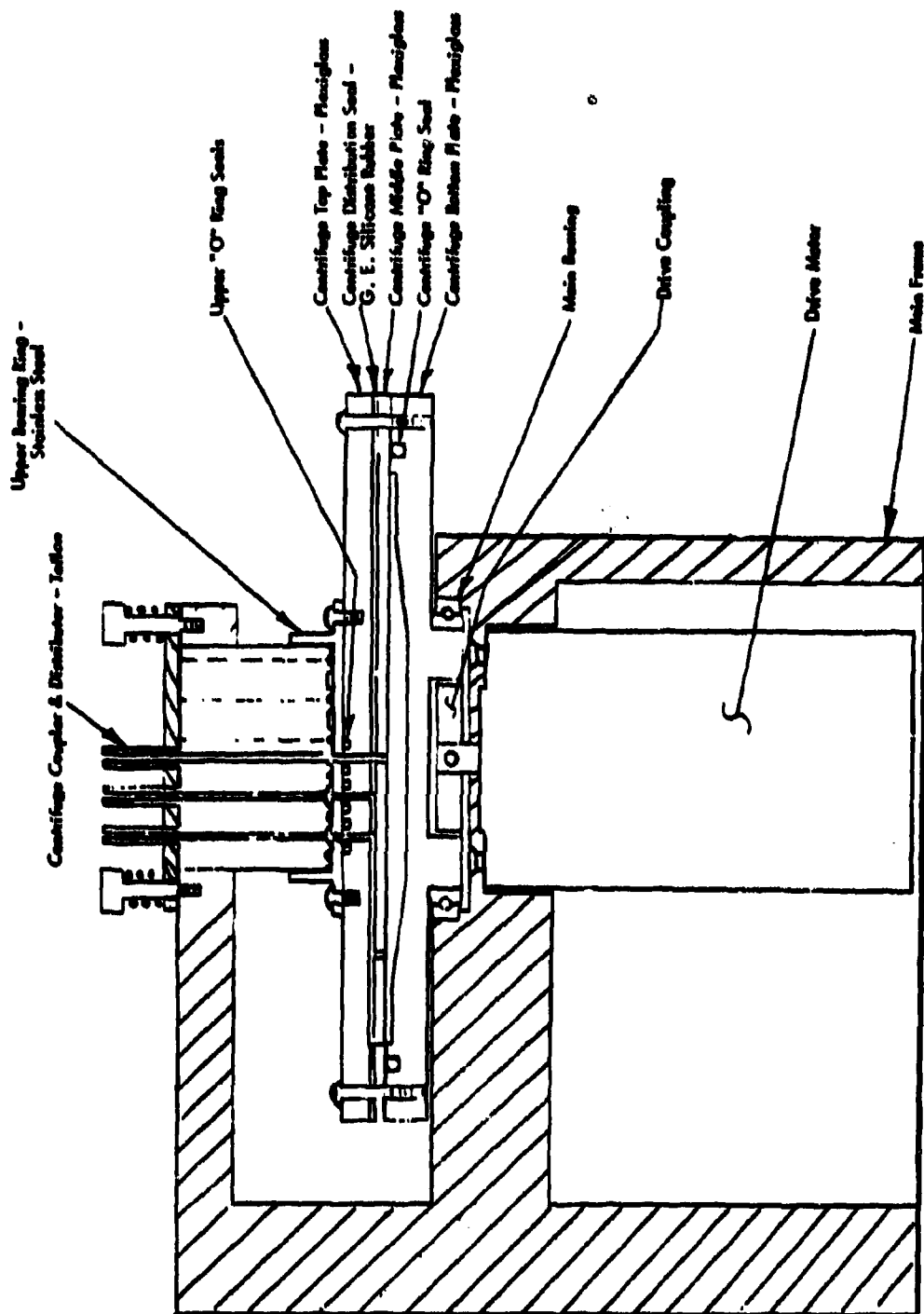


Figure 4-77. New Design of Centrifugal Separator for Liquid Partition

The assay methods considered included various techniques of counting microscopically. Staining or tagging bacteria with colored or fluorescent dyes or with radioisotopes can facilitate counting. Methods depending on colorimetry or turbidimetry were also used.

Extraction of aqueous suspensions of Chromobacterium violaceum (CV) with ether was found to transfer the color completely with an absorption peak at 525 mμ. The cellular mass and extraneous materials remained in the aqueous layer. This colorimetric method was standardized by correlating absorbance of the ether extracted color with total cell counts using both microscopic and viable cell plate counts. However, pigmentation changes in the strain of CV necessitated a change to the direct counting methods. These direct counting techniques demonstrated improved counting reliability due to the use of phase microscopy. An actual count has the advantage of being a direct measurement, independent of mutation, growth phase, and other biological irregularities.

A Breed-type counting method was adapted to monitor the continuous separations of BG from Arizona Road Dust. In this method, particles are counted against a known-area reticle (grid). Various stained organisms were examined for ease of counting. These included Serratia marcescens, E. coli and BG vegetative cells, all stained with methylene blue and crystal violet, and BG spores stained with carbofuchsin. All stained organisms were observed to partition normally in the dextran-EVA medium. It was found, however, that unstained BG spores were clearly distinguishable from background when viewed with the following phase contrast microscopy set-up:

Leitz Phase Contrast and brightfield condenser, PHACO
N.A. 0.90 with revolving disc, including built-in
annular diaphragms on dovetail carrier. Also the
achromatic dry phase objective PHACO 40X/0.65.

A different counting method evolved from the PEEP-Partitioning hookup runs. Because of the high amount of contamination and debris in the air which was being pulled into the PEEP, FITC-tagged BG spores were used as test organisms. The counting method involved filtering a known sample aliquot (0.1 ml) through a 0.45 μ black Millipore disc and counting organisms in a deposit area measured with a microscope stage micrometer.

Through an intensive test program continuous liquid partition was developed to the point of capability for coupled operation with an aerosol collector. Clean separations of microorganisms and contaminants were performed. The modified Sorvall centrifuge was used throughout these operations.

Partition of Bacteria Alone - Runs with bacteria-alone showed the high degree of segregation possible with a component having a large distribution ratio. Results from one of many similar runs are recorded in Figure 4-78. Chromobacterium violaceum was used as the test organism because its strong coloration made visual observation possible. The figure shows that, under steady-state operation, 90 percent or higher of the bacteria were recovered in the dextran phase, which represented only 1/4 of the total effluent. This result was calculated entirely from the distribution of bacteria between effluents.

Partition of Bacteria and Background Simulant - Under conditions utilizing all of the mechanical improvements in centrifugal separation, several runs were made which efficiently separated bacterial cells from a background simulant. For example, a test solution of 2 ml of a 0.5 mg/ml Arizona road dust solution and 5 ml of a 9.0×10^8 cells/ml B. globigii spore suspension was added to the standard polymer system of 400 ml of 3 percent polyvinyl alcohol 72-60 and 200 ml of 10 percent dextran D-250. This mixture was continuously separated over a period of 35 minutes into two effluent streams. Fractions of the two effluents were assayed microscopically with a phase microscope. On the average, 94 percent of the spores were retained in the dextran effluent, and 96 percent of the dust was removed in the PVA effluent. Complete results are presented in Figure 4-79.

Continuous separation of organisms from actual dirt was demonstrated with a mixed dust and topsoil sample. This sample was mixed with a 10 ml solution of B. globigii spore suspension of 10^6 spores/ml and added to the standard polymer system. This mixture was continuously separated into two effluent streams. The two effluents were then assayed microscopically, using a Breed-type counting method in correlation with counts from Millipore-filtered samples. The average values of 74 percent spores retained and 79 percent dirt discarded, referred to the dextran effluent, were reasonably good.

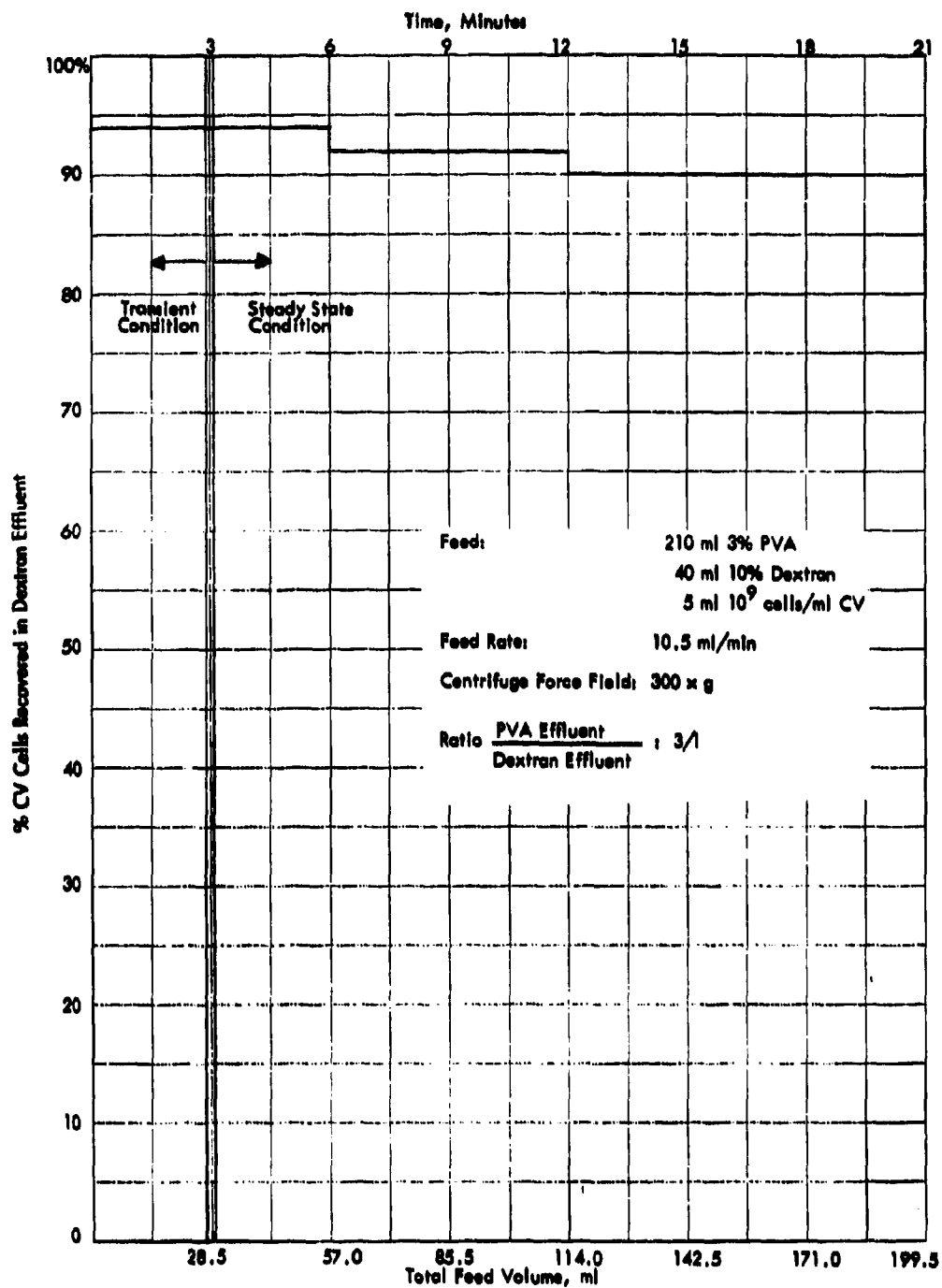


Figure 4-78. Liquid Partition of Chromobacterium violaceum

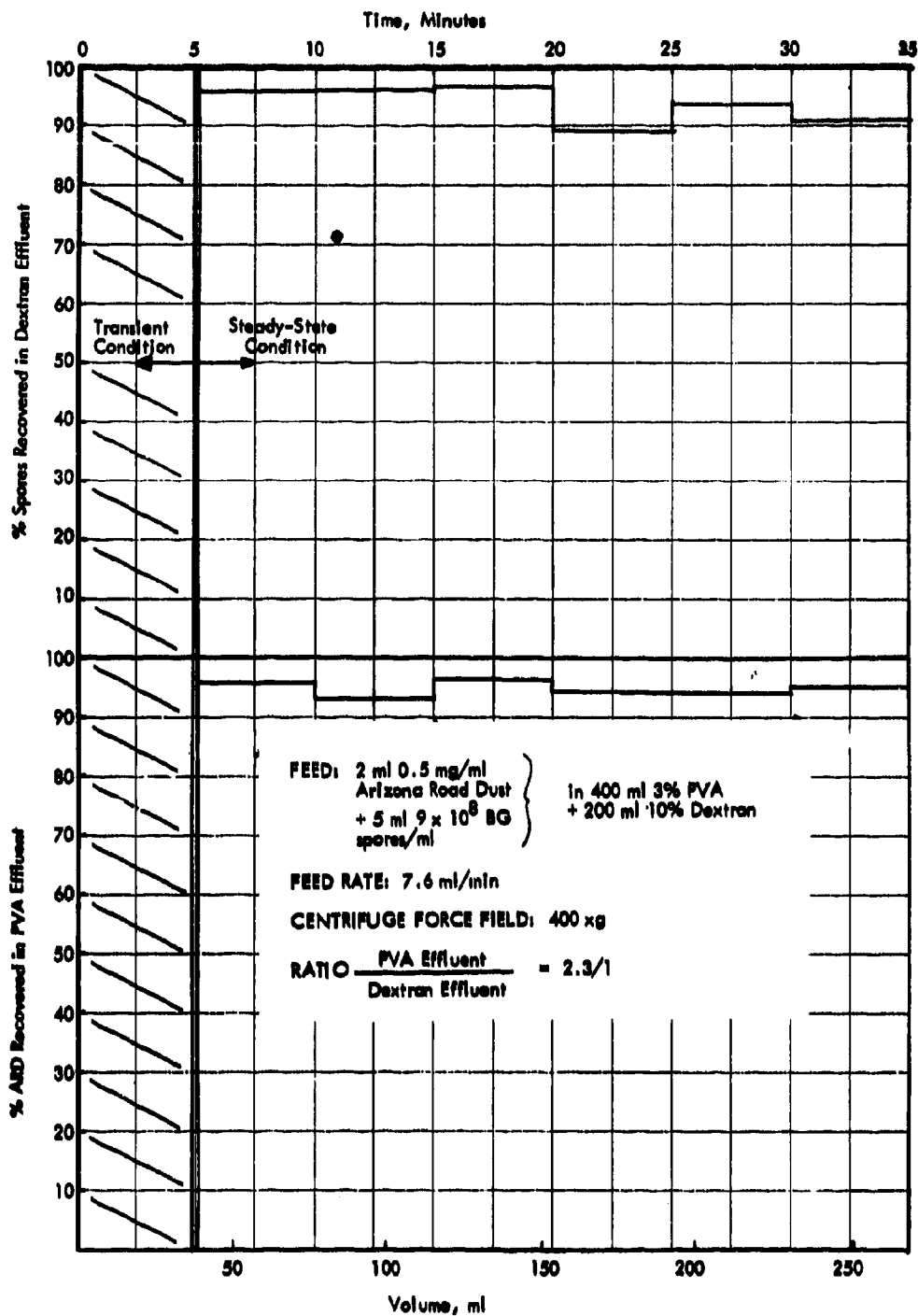


Figure 4-79. Improved Liquid Partition of B. globigii Spores and Arizona Road Dust

The recent separations cited above represent a marked improvement over earlier runs. This is due to an increase in degree of liquid phase purity in the effluents, which resulted from lengthening by 3 mm the downspout in the axial feed distributor of the centrifuge. The advantage gained may be summarized in tabular form as follows:

Liquid Phase Purity

<u>Distributor Downspout</u>	<u>Dextran-Rich Effluent</u>	<u>PVA-Rich Effluent</u>	
Short	58-95%	78-94%	Averages for 9 runs
Long	100%	89-94%	Averages for 5 runs

Partition of Virus - The separation of virus using partitioning would be a quick and mild method of purification or concentration of the viral suspension. Several continuous liquid partition runs using radioactive bacteriophage have indicated good separation into the dextran effluent. The influent solution for the latest run was prepared by mixing a suspension of P^{32} -tagged phage into a new medium. This was a mixed polymer solution of methyl cellulose (Dow Methocel MC 400) and dextran (Pharmacia D-250). The mixture contained approximately 3×10^6 phage/ml. The volume ratio of MC-rich phase to dextran-rich phase was 1.8/1.0 at equilibrium. The effluents were separated continuously in the usual way, using a ratio of methyl cellulose to dextran effluents of 2/1 to assure phase purity of the latter. Fractions were surveyed by drying 0.1 ml aliquots on a planchet and counting in a gas-flow proportional counter. As a control, a sample of the mixed influent was separated and 0.1 ml aliquots of each phase were counted in the same manner.

	<u>Average Counts per Minute</u>	<u>Apparent Distribution Ratio</u>	<u>Apparent % Phage Recovery</u>
From Continuous Run, MC-rich Effluent:	1,437		
Dextran-rich Effluent:	14,835	11/1	84
From Feed Equilibration, MC-rich Phase:	750		
Dextran-rich Phase:	15,700	22/1	-
(background gives 34 cpm)			

The actual recoveries may be much better than these figures suggest, because of the presence in the phage preparation of radioactive contaminants which are soluble in the Methocel phase.

PEEP Hook-Up - The final stage of development included several runs connecting the porous electrode electrostatic precipitating collector (PEEP) with the liquid partition separator. These runs gave evidence of good separation of the FITC-stained BG spores into the dextran phase. A concentration of these spores (1.25×10^8) was nebulized through a train to the PEEP, in which they were collected into a PBS-NaCl liquid medium and transferred to the separator influent. At this point the collected fluid was mixed with the two aqueous polymer solutions and introduced into the continuous separator.

Samples for testing were taken at the upstream port (just prior to entering PEEP), at the downstream port (exhaust from PEEP), at the collector tube, and at the two polymer effluent lines. The data from the latest of the runs are given in Table 4-26.

The overall operation of collector and separator showed good material balance of the total amount of sample organism (BG spores) used. The recovery through the PEEP was 82 percent while the recovery through the separator was 87 percent. The separator effluents showed high percentages of phase purity. One run (not shown) delivered a 100 percent pure dextran effluent while the run described in Table 4-26 averaged 88 percent purity. The PVA effluents averaged an 88 percent purity throughout.

The distribution ratio of the stained spores at the peak of the nebulization pulse was 26.4. Therefore, the feasibility of a continuous separation subsystem was bolstered by the latest runs. A simple addition of a detection indicator (FITC or S^{35} -tagged antisera or other) creates a compact operation of collection, tagging, and separation. Previous work had demonstrated successful tagging with the FITC antisera in the polymer mixture.

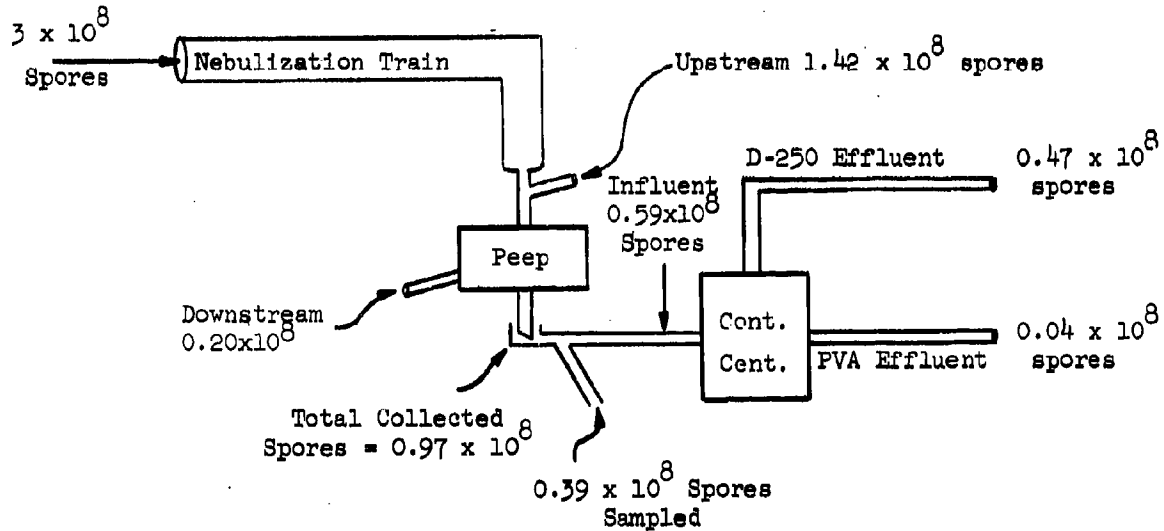
4.2.3.1.4

CONCLUSIONS

The principle of a two-phase, water-soluble polymer partitioning system has been well established by intensive test tube research. Bacterial cells have consistently demonstrated distribution ratio preferences of

Table 4-26

PEEP - PARTITION HOOK-UP DATA



% Recovery (Material balance)	$\frac{\text{collector} + \text{downstream}}{\text{upstream}} = \frac{9.70 \times 10^7 + 1.95 \times 10^7}{1.42 \times 10^8} = 82\%$
PEEP Efficiency	$\frac{\text{collector}}{\text{collector} + \text{downstream}} = \frac{9.70 \times 10^7}{9.70 \times 10^7 + 1.95 \times 10^7} = 83\%$
PEEP Collection Efficiency	$\frac{\text{collector}}{\text{upstream}} = \frac{9.70 \times 10^7}{1.42 \times 10^8} = 68\%$
Separator Efficiency (Material balance)	$\frac{\text{total separator effluent}}{\text{separator influent from collector}} = \frac{5.14 \times 10^7}{5.92 \times 10^7} = 87\%$

Separation Efficiencies

	FR-1		FR-2		FR-3		FR-4		FR-5	
	PVA	D	PVA	D	PVA	D	PVA	D	PVA	D
Phase Purity %	100.0	100.0	92.3	100.0	89.1	95.8	83.8	85.2	83.8	75.0
Distribution Ratio	-		8.4		13.8		26.4		18.4	

(Note: maximum nebulization for 5 minutes, in total operating time of 24 minutes)

up to 100 to 1 for the dextran-rich phase. The rapid, continuous segregation of both bacteria and viruses in single liquid phases has been demonstrated, as well as the elimination of background simulants and of actual dirt. To give this research the broadest possible base, phase equilibria in other systems of water-soluble polymers should be thoroughly investigated. Other aspects of increasing separation rates include additives such as surface-active agents, buffers, and salts. The "salting down" of bacteria into the proper phase has been experimentally achieved as shown in Albertsson's work, and to some extent at Space-General.

In future plans, liquid partition breadboard components will be developed to provide an effective module for detection breadboards. For example, in a breadboard system using a Coulter Counter equipped with a multi-channel pulse height analyzer for detection, liquid partitioning might be used in a series of several stages of separation to remove all but a minute fraction of non-bacterial background particles.

In addition, the outlook for separation of viruses appears to be very good on the basis of much research evidence. A wide variety of viruses have been separated, including ECHO, polio, tobacco mosaic, phage, and vaccinia. Research on applications to other biological separations is also warranted. Liquid partition separations of arbovirus from tissue, separations of erythrocytes, DNA, chlorella, and algal protein have all been performed. Some of these topics have direct applications to BW detection, such as the separation of phage or labelled antibody from bacterial fragments, isolation of ECHO virus from monkey kidney culture growth medium, fractionating antigens, and separating and concentrating virus. Further uses should appear, especially after the possibilities of rapid continuous separation have been demonstrated. These are clear indications of the course of future development.

4.2.3.1.5

REFERENCES

- (1) Tiselius, A., et al, Science 141: 135, June 1963
- (2) Albertsson, P. A., Partition of Small Particles and Macromolecules, Wiley, New York, 1960

4.2.3.2 CONCENTRATION OF PARTICLES IN FOAM

4.2.3.2.1 SUMMARY

A limited feasibility study indicates promise for a method of concentration/separation involving the collection of particulates in an aqueous foam. In tests with 1.3 μ polystyrene latex, 90 percent of the particles were found in the 25 percent of the liquid which was foamed. However, with BG spores, the ratio of concentration in foam to concentration in residual liquid was 1.7 to 3.2 in the initial experiment. A new method of producing foam electrolytically was presented. This may lead to a compact, easily controllable foam separator.

4.2.3.2.2 INTRODUCTION

A limited study was made by Metronics of the feasibility of concentrating particulates in foam. This technique was of interest for at least two reasons. First, the technique may offer a way of concentrating the effluent from the collector still further, thus allowing a smaller collector and perhaps a more compact system. This concept is of special concern in those sensing systems which are concentration-dependent and now require large-volume air samplers. Further, the collection of particulates in foam may be sufficiently selective to serve as a means of separating the biological pathogens from the interfering atmospheric background particulates.

Since the process of flotation (not to be confused with density-dependent flotation) is related to the surface free energy of the particulates at the air-water interface of the foam, significant separation of bacterial and background atmosphere particles is expected. It is on the basis of these considerations that the limited feasibility study was initiated.

4.2.3.2.3 STATUS

The concentration of particles on the basis of their surface free energy characteristics was suggested by an observation in the Metronics laboratory during an investigation of particle holdup in collectors. It was noted

that iron oxide particles were especially prone to collection on transfer surfaces. This tendency was attributed to the small size of iron oxide, and possibly surface characteristics. However, when ground ilmenite ore, classified to 0.3 to 10 μ in diameter, was used in place of the iron oxide, a somewhat different behavior was noted. Practically none of the ilmenite was retained on the walls of the collection system but it was held almost quantitatively in the outlet tube of the water separator in a thin film of water.

It was further observed that hydrophobic particles tend to migrate from the surface of a liquid to the walls of the container if the contact angle between the wall and liquid is 90° or greater. At less than 90° contact angle the reverse is true. Thus, hydrophobic particles on the surface of water contained by a hydrophobic surface, such as a Tygon or a glass tube with an oily surface, will readily transfer from the water surface to the container wall. The possibility of development of a method of separating particles of different kinds based upon their tendency to be selectively retained at the air-water interface was suggested by the results.

The concentration of particles at an air-water interface caused by bubbles and foam was then illustrated at Metronics by a simple experiment. A known concentration of 1.3 μ polystyrene beads, in a buffered solution containing Photo-Flo (Eastman), was foamed with gas. The batch test apparatus consisted of a Pyrex funnel with a medium porosity fritted glass disc sealed into the conical section of the funnel. The test cell capacity was 50 ml above the fritted disc. A horizontal plastic collar was fitted around the top of the funnel to serve as a collector to facilitate collection of liquid from the broken foam and the suspended particles contained therein. Nitrogen gas was introduced through the funnel stem at a pressure sufficiently low to provide gentle rather than vigorous aeration. Approximately 25 percent of the liquid was formed into foam which was later collected and allowed to settle. The settled foam and the liquid from which it originated were both filtered and the particles contained in each were counted. Approximately 90 percent of the particles in the original liquid were found in the 25 percent portion of the liquid that had been converted to foam. On the basis of these tests, a similar foaming experiment was conducted with BG spores to determine the partitioning of this biological microorganism.

However, in contrast to the earlier results with polystyrene latex, viable BG spores concentrated only moderately in an aqueous foam. The spores were suspended in an aqueous solution containing Photo-Flo surface-active agent, which was then foamed until 20 percent had been removed as foam. Concentrations of bacteria per ml in the original liquid were determined by plate counts. Control tests indicated negligible blanks in each case in the absence of spores. The counts showed the following distribution:

Run	<u>Foam</u>		<u>Residual Liquid</u>			<u>Balance on Concentrations of Original Liquid</u>	
	<u>Concentration</u>	<u>Volume % of Total</u>	<u>Concentration</u>	<u>Volume % of Total</u>	<u>Distribution Ratio</u>	<u>Calculated</u>	<u>Measured</u>
1	17.4x10 ⁵ 20.1x10 ⁵	20	5.9x10 ⁵	80	3.2	8.4x10 ⁵	9.4x10 ⁵
2	17.3x10 ⁵ 16.5x10 ⁵	20	10.0x10 ⁵	80	1.7	11.4x10 ⁵	9.4x10 ⁵
3	14.7x10 ⁵ 18.2x10 ⁵	20	9.1x10 ⁵	80	1.8	10.6x10 ⁵	9.4x10 ⁵

The distribution ratio (i.e. ratio of concentration in foam to concentration in residual liquid) of 2 to 3 is low compared to the value of approximately 27 previously observed for polystyrene latex, and to the apparent distribution of iron oxide powder.

A further discovery in the Metronics laboratory appears to indicate a method for producing a fine foam, which should be highly effective for separation. An aqueous solution was allowed to drip into a 5.5-mm ID glass tube fitted with platinum electrodes spaced 3 mm apart. When a potential of 5 volts AC (60 cycle) was applied across the electrodes, bubble formation was negligible. However, with 8 volts DC (current 5 ma), a substantial stream of fine bubbles of hydrogen formed at the negative electrode. Within two minutes, a 4 mm layer of stable foam developed on the surface of the meniscus. At 15 volts DC, the surface foam developed in approximately half the time.

If there is a tendency for particles to concentrate on the foam surface as suggested by earlier experiments, electrolytic foam production is attractive for several reasons:

- a. The amount of foam is readily controlled by varying the applied voltage.
- b. The hydrogen bubbles are much smaller than bubbles produced pneumatically, hence greater interfacial surface is obtained with a given volume of gas.
- c. Foam is easily produced in a small volume of liquid, i.e., a few drops; hence foam separation might be achieved without need for accumulating a large volume of liquid, with accompanying reduction in response time.
- d. Even though small, the bubbles form quickly, rise to the liquid surface, and provide an interface within the liquid as well as on its surface.

4.2.3.2.4 CONCLUSIONS

Although a highly effective separation of fine polystyrene latex was demonstrated, initial tests with B. globigii spores was not as promising. Further tests with other foaming agents and conditions may demonstrate that this technique is effective. However, the effort was shifted to other techniques in which more effective separations had been demonstrated.

4.2.4 CONCENTRATION BY FILTRATION

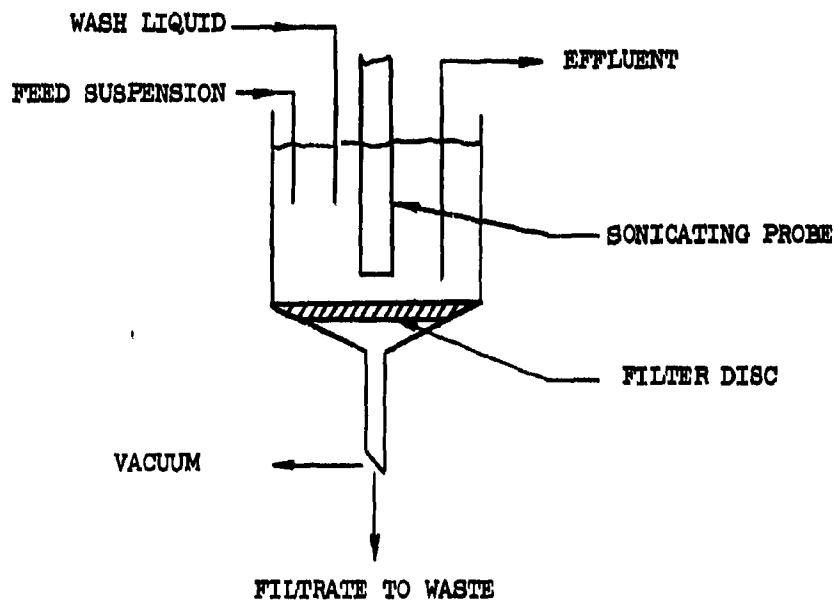
4.2.4.1 SONICATING CONCENTRATOR-WASHER

4.2.4.1.1 SUMMARY

A new method for concentrating or washing suspended particulates, which depends on simultaneous filtration and sonication of the suspension, has been demonstrated. Tests showed a quantitative recovery of BG spores; 41 counts per field were recovered out of 42 possible, after washing with 3125 volumes of liquid. Other tests showed, qualitatively, a retention of complexes of BG spores in a liquid concentrate, while unreacted antibodies were either filtered out or held on the filter. In any case, an easy scheme of separation is provided.

4.2.4.1.2 INTRODUCTION

Concentration and washing of suspended particulates may be effectively conducted in a very simple unit under development at Space-General Corporation. The principle is illustrated below:



In this device a suspension of fine particles is filtered while being agitated lightly by a sonicating probe. The particles do not lodge in or on the filter, but remain suspended and are concentrated in the small volume of unfiltered water. The suspension may be washed repeatedly if desired, without loss of particles. Obviously, unwanted solutes are removed.

4.2.4.1.3 STATUS

The validity of the underlying concept was confirmed by a simple experiment. A filtration apparatus incorporating a 0.8 μ Millipore disc was assembled. The probe of a Biosonik laboratory sonicator was inserted into the filter funnel with a few millimeters clearance between the tip of the probe and the filter disc. The test sample was a suspension of BG spores in water. In operation, the funnel was filled with 10 ml of suspension, and the sonicator energy was increased until the liquid in contact with the filter disc was seen to be strongly agitated. The frequency was 10,000 cps. A small measured aliquot of the suspension was withdrawn in a hypodermic syringe for analysis, and the remaining liquid was filtered to a volume of 2 ml. The volume was then made up to 10 ml, and the cycle of sampling, filtration, and make-up was repeated several times. Each sample was assayed by filtering through a clean disc, followed by a microscopic count of the spores retained in a measured fraction of the total filtration area.

Results from one test illustrate the close agreement between measured values and predicted values (allowing for the volume of sample withdrawn):

<u>Wash Volumes</u>	<u>Count</u>	
	<u>Actual</u>	<u>Predicted</u>
Original suspension	44.3	--
5	39	44
25	40	43.5
125	44	43
625	47	42.5
3125	41	42

Even after washing with several thousand volumes of liquid, substantially all spores remain in suspension. The number lost by attachment to the filter surface is immeasurably small.

At the time of conclusion of this program, experiments were under way to test the separation by this principle of complexed BG spores from unreacted specific antibody (S^{35} -labelled). Such a separation could greatly enhance the sensitivity of detection with tagged or stained antibodies. Preliminary results indicated that, as expected, spores tend to remain suspended, while antibody is in part filtered out and in part adsorbed on the membrane.

4.2.4.1.4 CONCLUSIONS

This separation principle will be developed further in the successor program. A continuous unit for filtering and washing fine particles will be constructed and subjected to thorough trial. Applications to the chemiluminescence detector system, the radioactive antibody detector, and other types of detector will be examined.

4.2.4.2 VERTICAL FILTER-CONCENTRATOR

4.2.4.2.1 SUMMARY

Metronics Associates demonstrated a concentrating device which removed water by continuous filtration along a vertical membrane strip. The recovery of suspended particles and liquid concentration factors varied widely with operating conditions. This approach to the problem of concentration was discontinued in favor of other approaches.

4.2.4.2.2 INTRODUCTION

Initial experiments at Metronics Associates suggested the possibility that a liquid suspension passing over a vertical filter might be concentrated by removing water through the filter while the particles are carried down the front surface of the filter in the residual flow. This behavior is of interest because of the many requirements for particle concentration in BW detection systems. A thorough experimental demonstration was considered desirable before considering recommendations for development.

4.2.4.2.3 STATUS

In the Metronics test device, the concentrating zone contains a 0.8 μ Millipore filter held against a 3/8 inch thick plastic block by a metal plate and Teflon gasket. Both the plate and gasket have a 1 cm² triangular opening (0.5 cm x 4 cm). This opening coincides with a similarly shaped cavity 1/4 inch deep cut into the plastic block. Two tubes for filling and draining enter the cavity through the edge of the block, one at the side near the cavity top, the other at the bottom. A 50 mesh wire screen supports the filter across the cavity opening.

In operation, the rear cavity is filled with water, and the top tube is sealed. A pressure drop is maintained inward from the filter surface by the tendency of the cavity to drain. However, surface tension in the filter pores is sufficient to prevent water from draining out of the cavity after the apparatus has been filled, even though the head is as much as 30 cm.

The suspension to be concentrated is delivered to the top of the filter through a glass feed tube held approximately one millimeter from the filter surface. At the bottom, a sanded glass rod in contact with the apex picks up the liquid stream which then flows down the rod and drops off at its outer end. The amount of liquid removed from the stream can be controlled by adjusting the liquid head, i.e., by raising or lowering the cavity drain. If less than approximately 70 percent of the water is removed, the entire surface of the filter is covered with a film of water.

A total of 35 bead-recovery measurements were made using variants of this system. Test suspensions consisted of 1.3μ polystyrene latex beads in salt/Photo-Flo solution (roughly 10^5 beads/ml). Three major types of filter material were tested: (a) the 0.8μ Millipore, (b) the silver membrane filter, and (c) the Nuclepore filter with 1.0μ holes. Whereas 99 percent recovery of beads was initially achieved at a concentration factor of 6.6 with the 0.8μ Millipore membrane, performance after operation for 1 hour was badly impaired. High values of both recovery and concentration were not obtained simultaneously under any other conditions.

There was no case in which all of the experimental problems were overcome at one and the same time. These problems and the measures to be taken for solving them are listed below:

- a. Non-uniform coverage of filter surface by the water film - Improvements have been obtained by the effect of ultrasonic vibration and by the use of a cover plate; improvement by tilting the filter more than about 30° toward a horizontal position leads to lowered bead recovery.
- b. Decrease in filter permeability due to plugging - Improvement was obtained by the effect of ultrasonic vibration and by using freshly filtered solutions; the area of the filter may also be increased.
- c. Lack of adequate flow control in effluent solution - Adequate flow through the filter can be maintained by use of a constant-flow peristaltic pump operating at 4.5 ml/min , rather than by reliance on a constant hydrostatic head; a second pump, operating at 5.0 ml/min , may be used to feed in the reservoir solution.

- d. Low Bead Recovery - Improvement should result by optimizing the ultrasonic power and frequency, by selecting the optimum geometry for the system, and by use of clean tubing and filtered and sterilized reservoir solution.

4.2.4.2.4 CONCLUSIONS

Development of this approach to concentration was terminated, to focus efforts on other approaches which appear more promising. The sonicating washer-concentrator (described in another section) and low-effluent versions of the porous electrode electrostatic precipitator will be studied in the future program.

4.3.1

SUMMARY

The particles present in a normal atmosphere should not interfere with the detection of biological pathogens. A limited study of the characteristics of atmospheric particulates has contributed to an understanding of this need, particularly as applied to detection techniques such as the fluorescent antibody sensing technique (FAST).

Over half of the particulates by weight are larger than 5μ , the usual upper limit of size for oral infection, thus providing a basis for useful size separation of aerosol particles entering a detector. Further, over 50 percent of the particles were shown to be water soluble on 4-second contact. Since Space-General collection schemes have been based on liquid collection-concentration-separation, it is clear that a large number of particles will be removed by solution on collection. A study of fluorescing properties revealed that, while about 30 particles/l of air will glow, the majority are very dim. The colors are predominately blue, with lesser quantities of orange, red, green, and yellow particles, and many are quite large. The particles which are of a color, brightness, and size potentially to interfere with the FAST technique appear to number substantially less than 1 per liter. The number of viable organisms sampled per liter of air ranged from 0.01 to 0.25 and were mainly spore-forming, gram-positive rods.

4.3.2

INTRODUCTION

A knowledge of the background atmosphere that may be experienced by the detection system is important for establishing the limitations and suitable operating conditions for any sensor. A review of the literature indicated that the available information on size distribution of atmospheric particulates was meager and often inconsistent. Although extensive data exist on the weight-loading of particulates in the atmosphere, e.g., the comprehensive results of the National Air Sampling Network, few data exist for the background characterization necessary for proper operation of a detection system. It is with the objective of supplying data needed in characterizing the background from which

the sensor must discriminate that the program to be described was established. To be described will be these preliminary efforts to establish the total particulate background, the microorganism content of the atmosphere, and some properties of the background particulates.

4.3.3 STATUS

In consideration of the requirements of the program, the following items have been investigated.

- Particle-Size Distribution of Atmospheric Particulates
- A National Network for Obtaining Samples
- Solubility Characteristics of Atmospheric Particulates
- Fluorescence of Atmospheric Particles
- A Microspectrofluorimeter for Studying Particle Fluorescence
- Microorganisms in the Atmosphere

4.3.3.1 PARTICLE-SIZE DISTRIBUTION OF ATMOSPHERIC PARTICULATES

Two general problems exist in attempting to describe the particle-size distribution of atmospheric particulates. The first is concerned with measurement techniques. Although some workers claim the upper size limit of atmospheric particles is as low as 2μ , this is considered incorrect since particles in the 50 to 100μ size range account for most of the "nuisance-type" dust which settles on exposed surfaces. Data will be shown which demonstrate that, although the large particles are not significant in number, their contribution to the mass loading of particulates in the atmosphere is significant. The confusion in the literature appears related to the methods used for measurement.

The second problem area is that of the description of the particle size data.

Junge⁽¹⁾ shows that the size distribution applicable to "clean" outdoor atmosphere can be represented by a straight line between the limits 0.1

to 10μ where $\log \frac{dN}{d \log d}$ is plotted against $\log d$. The Air Force Logistics Command (2,3) uses a different presentation ($\log N(d_1)$ vs $\log d_1$), where $N(d_1)$ is the number of particles of diameter d_1 and greater) and reports a linear correlation in the log-log plot between 0.1 and 100μ . These data apply to outdoor air as well as "clean rooms."

Data reported by the above two methods can be presented in a more convenient generalized form to show the total mass between arbitrary particle sizes d_1 and d_2 . Thus:

$$M(d_1 d_2) = \frac{\pi \rho}{6} N(d_1) d_1^3 \left\{ \frac{\beta}{3-\beta} \left[\left(\frac{d_2}{d_1} \right)^{3-\beta} - 1 \right] \right\} \quad (1)$$

where

$M(d_1 d_2)$ = total mass of particulate material per unit volume of air with diameters between d_1 and d_2

ρ = average density of particles d_1 and d_2

$N(d_1)$ = number of particles per unit volume of air equal to or greater in diameter than d_1

β = constant

For Junge's data $\beta = 3$; for Air Force data $\beta = 2$.

When $\beta = 3$, Equation (1) is indeterminate in the form given above but reduces to

$$M(d_1 d_2) = \frac{\pi \rho}{6} N(d_1) d_1^3 3 \ln \frac{d_2}{d_1} \quad (2)$$

It may be noted that if the particle size distribution is linear in a log-log plot by either of the two methods referred to above, a limited

extrapolation to sizes larger than measured is justified. Obviously, Equation (1) can be applied to the extrapolated estimate.

This method has been applied to the representation of data from four different sources. These representations are shown in Figure 4-80. In this figure, the data and transformations from original to a comparative form are as follows.

- Curve 1. Mumma⁽⁴⁾. Plotted directly from the referenced journal.
- Curve 2. Junge⁽¹⁾. Obtained from Junges' plot of $\frac{dN \text{ (part/cm}^3\text{)}}{d \log r}$ vs $\log r$ by the conversion $N(r) \text{ (part/l)} = \frac{0.4343}{r} \times 10^3 \frac{dN}{d \log r} \text{ (part/cm}^3\text{)}$. The number of radius r , $N(r)$, was plotted at the appropriate diameter.
- Curve 3. Austin^(2,3). Obtained from the Air Force Curve of $N \text{ (part/ft}^3\text{)}$ vs diameter, D , by the conversion $N(D) \text{ (part/l)} = 3.53 \times 10^{-2} \frac{N(\Delta \log N)}{D(\Delta \log D)}$
- Curve 4. Metronics. Mean concentration measurements at Metronics Associates in Palo Alto, California, based on a total of approximately 1200 10-minute samples counted by a Royco Particle Counter at a flow rate of 300 cc/min. Curve obtained from $N \text{ (part/m}^3\text{)}$ with diameter D by the conversion

$$N(D) \text{ (part/l)} = 10^{-3} \frac{N(\Delta \log N)}{D(\Delta \log D)} .$$

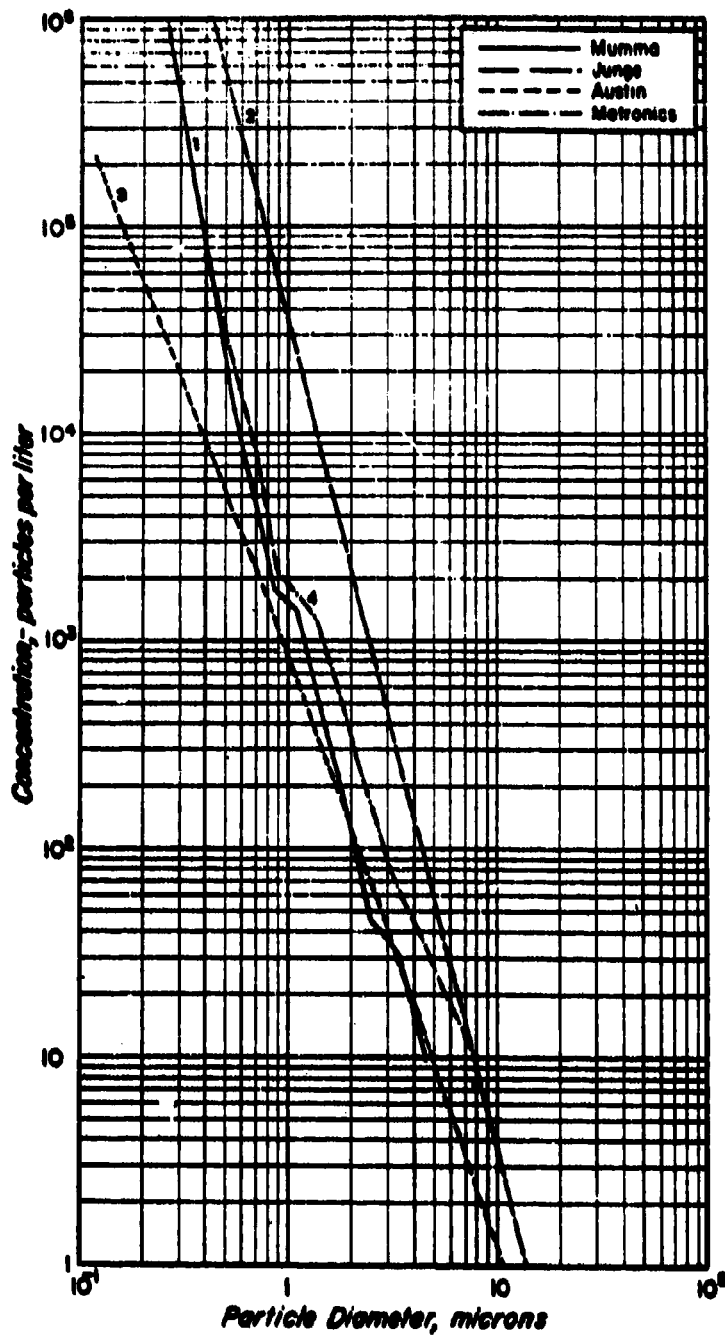


Figure 4-80. Correlation of Particle-Size Distribution from Different Sources

To extend the observations to larger particle sizes than are recorded by the Royco Particle Counter, a Rotorod sampler was operated at Metronics in the same atmosphere as the Royco Particle Counter. It was allowed to run for four hours, at the end of which time the particles were weighed and counted in two size ranges. The slope of the curve changes on extension to particles as large as 80 μ . Optical measurements revealed the presence of the 80 μ particles, but not in as large numbers as a straight line plot would require.

Using a linear extrapolation, to 20 μ , of the Metronics Royco data in Figure 4-8Q together with Equation (1), the weight distribution of particles between 0.3 and 20 μ and 5 to 20 μ was calculated with the results as shown below, assuming density 2 g/cc.

<u>Period</u>	<u>β</u>	<u>MASS OF PARTICLES</u>		<u>RATIO OF MASS</u> <u>5-20μ/0.3-20μ</u>
		<u>5μ to 20μ</u>	<u>0.3μ to 20μ</u>	
1730 Aug 20				
to	1.75	42.6 $\mu\text{g}/\text{m}^3$	52.4 $\mu\text{g}/\text{m}^3$	0.813
0905 Aug 21, 1963				
2100 Aug 16				
to	2.13	87.4 $\mu\text{g}/\text{m}^3$	123 $\mu\text{g}/\text{m}^3$	0.710
2200 Aug 17, 1963				

These calculations indicate that most of the mass of the atmospheric particles between 0.3 and 20 μ lies in the 5 to 20 μ size range, provided the extrapolation to 20 μ is justified. In one case where Rotorod counts were obtained in conjunction with the Royco sample, the extrapolated Royco curve gives about 50 percent more particles at 20 μ than the curve projected to include the observed large particles. This means the estimated mass to 20 μ may be somewhat high. On the other hand, all particles larger than 20 μ are neglected in the comparison of mass above and below 5 μ and their aggregate mass undoubtedly exceeds the over-estimate made at 20 μ . Thus, the deduction that most of the mass of atmospheric particles lies above 5 μ appears to be valid.

Considering the varied nature of the origin of particles in the atmosphere and the varied meteorological and terrain conditions to which they

are subjected, it is not surprising that the slopes of the log-log distribution curves are not identical for all locations or time periods. Obviously, more data are needed to verify the general applicability of the types of curves examined to date. However, even in their present form, the curves have been useful in appraising the need for a particle size separator in the sampling subsystem. On the basis of the results, a separator having a 5 μ cut-off appeared desirable.

4.3.3.2 A NATIONAL NETWORK FOR OBTAINING ATMOSPHERIC SAMPLES

Since it was the objective of the program of studies on the atmospheric background to attempt to correlate properties of particulates with potential interference with biological sensors, and to attempt to devise means to reduce potential interferences, it was clearly necessary to obtain samples representative of particle size effects. While the samples obtained by the National Air Sampling Network of the U. S. Public Health Service are useful for obtaining weight loading information on the atmosphere, the form in which they are collected do not make them useful for particle size characterizations. Accordingly, a limited program was set up by Metronics with the cooperation of the U. S. Public Health Service* to obtain samples in ten sites throughout the country. Particulate samples were collected on a routine basis at these stations on Millipore filters, for microscopic examination for total particulate concentration, particle-size distribution, solubility, fluorescence, etc.

Ten sampling stations were activated on 21 October 1963. One station was located in each of the following cities: Gainesville (Fla.), New Orleans, Washington, D. C., Cincinnati, Chicago, Helena, Salt Lake City, Palo Alto (Metronics), El Monte (Space-General), and San Francisco. The sampler at each station has been located at an urban or residential site to obtain outdoor air samples.

*Dr. E. C. Tabor, Chief, National Air Sampling Network, assisted in selecting the stations. His valuable suggestions and wholehearted cooperation are gratefully acknowledged.

All stations used 25 mm AA type membrane filters. Filters were first examined at Space-General to make certain the surface was free of fluorescent particulate material. The filters were then forwarded to Metronics where they were loaded in magnetic type holders. The filters were then air-mailed to each station in sets of four in returnable mailers containing molded polyurethane foam pads to protect the assembled holders. After exposure, the holders (with filter in place) were returned to Metronics. The filters were mounted on glass slides and forwarded to Space-General for assessment; the holders were cleaned and reloaded and sent back to the stations. In view of the observed contamination resulting from filter handling in the laboratory, special precautions were taken during the loading and mounting procedures.

Initial samples collected at the ten sites throughout the country were made using 25 mm type AA Millipore filters (pore size 0.8μ) and sampling at a rate of 13 to 15 l/min. These samples represented air volumes as large as 16,000 liters, and revealed that the deposition was so great that the filters appeared to be completely covered with particulates. Therefore, new critical orifices were installed in the filters so that the sampling rate was reduced to 3 to 5 l/min. Samples then represented about 2000 liters of air; the deposition was sufficiently spread out so that examination of individual particles could be carried out.

The particulate collection program was initially established to collect six samples per week (3 daytime, 3 nighttime) at each site. However, on January 15, 1964, the sampling was reduced to two samples per week (one day sample, one night sample). The sampling stations continued operation until August 31, 1964, giving a period of about 10 months, so that seasonal variations in particulates could be examined. Some data from these samples will be discussed in later sections.

4.3.3.3 SOLUBILITY CHARACTERISTICS OF ATMOSPHERIC PARTICULATES

Information relating the solubility of atmospheric particulates in solvents which may be used as collection fluids is important in designing the most appropriate collection system. The approach generally applied at Space-General has been to design the collection, concentration, separation

subsystem with a liquid collecting phase. This system not only permits a substantial concentration of the biological aerosol in a form compatible with the sensor, but may remove a large portion of the background particulates by dissolving them in the collecting fluid. It was therefore important to establish the solubility of background material, and the effect of the undissolved material on fluorescence, etc.

In preliminary studies, the extraction of the particulates with a variety of solvents was examined. Samples collected on glass fiber filters with the Gelman High Volume Sampler, each loaded with solids from about 200,000 liter of air (~20 mg samples), were treated in micro-soxhlet extraction units. Preliminary results from refluxing with solvents for about four hours indicated 12 percent by weight extracted by benzene, 15 percent by CCl_4 , about 40 percent by acetone, and 60 percent by water. These results show that a considerable quantity of the background particulates (probably organic acids, chlorides, nitrates, and sulfates) will be separated by a water collection system.

In further studies, an examination was made of the particle size distribution of particulates and the solubility characteristics, in water, as a function of particle diameter.

These results have suggested that particle-size distribution is not affected by even intensive washing. In one experiment a sample was collected on a 25 mm membrane filter (0.45 μ pore size). The sample represents a four-hour daytime sample at 15 l/min (ca 3600 l). The entire sample was then washed with 125 ml of water. The water was in contact with the sample for about 15 minutes. The size distribution of fluorescent particles in the sample under UV excitation conditions (UGI, BG38 filters) and of a parallel sample which was not washed, follows.

<u>Diameter, μ</u>	<u>Unwashed, % at Diameter</u>	<u>Washed, % at Diameter</u>	<u>% Removed by Washing</u>
< 2	47	45	73
2 to 5	33	41	67
5 to 10	16	10	84
> 10	4	4	<u>75</u>
Weighted Average			72.9

In another experiment, a small part of the membrane filter from a sample collected in a manner similar to the first experiment was placed on a vacuum stage on the microscope and the sample was washed with 1 ml of water. The contact time was about 4 seconds. The size distribution of fluorescent particles in the sample before and after washing follows.

<u>Diameter, μ</u>	<u>Before Washing, % at Diameter</u>	<u>After Washing, % at Diameter</u>	<u>% Removed by Washing</u>
< 2	37	41	51
2 to 5	32	33	55
5 to 10	15	10	72
> 10	16	17	<u>55</u>
Weighted Average			56.3

The data from both of these runs appear to indicate that the fluorescent particles are washed from the filter in a manner not dependent on diameter. Secondly, it is important to note that the fluorescence can be substantially reduced by washing out soluble fluorescent particles.

4.3.3.4 FLUORESCENCE OF ATMOSPHERIC BACKGROUND

Important to the operation of any pathogen sensing technique, such as FAST, is an understanding of the nature of the fluorescent particles in the atmosphere. A large number of the filters collected from the ten sampling sites across the country (see Section 4.3.3.2) have been examined microscopically for fluorescence. Several conclusions are apparent. Although a number of particles are seen to glow, the preponderance are seen to be blue, with a much lesser number of red, orange, green, and yellow particles. Furthermore, most particles will glow much less brightly than a fluorescent-antibody tagged organism, although a very few particles will be seen to be as bright. An example of the number of particles which can be seen to fluoresce under extreme conditions is shown in the following tabulation. In this table, all particles which could be discerned on the film were counted. In this analysis, samples were illuminated with an HBO-200 mercury lamp with UGI and BG38 filters; a UV filter was used over the eyepiece. The Leitz microscope with Ultrapak illumination was used. Photographs were made on Polaroid film with a 1-minute exposure.

PARTICLE-SIZE DISTRIBUTION OF FLUORESCENT PARTICLES FROM AIR

City	Sample	Fluorescent Particles/ Liter Air	Fluorescent Particles, %, of Size, μ			
			0-2	2-5	5-10	>10
Chicago	CHI-16	20	32	49	13	6
Cincinnati	CVG-25	31	52	41	7	0
Gainesville, Fla.	GNV-16	25	24	43	19	14
El Monte	LAX-44	38	28	40	21	11
Helena, Mont.	HLN-21	30	16	20	44	20
New Orleans	MSY-21	28	27	56	12	5
Palo Alto, Calif.	PAO-36	42	11	34	42	13
Salt Lake City	SLC-15	21	25	43	25	7
San Francisco	SFO-15	33	35	48	15	2
Washington, D. C.	DCA-13	46	27	33	34	6
Average		31.4	27.7	40.7	23.2	8.4

Several conclusions are evident from an analysis of these data. In all cases, fluorescent particles were clearly evident, although one-tenth or less of the particles counted are as bright as FA-stained organisms. On the average, about one-third of the particles are larger than 5μ and can be eliminated by suitably operated primary separators (such as the cyclone used in the FAST breadboard). Wide variations are shown from city to city, as is seen in the table, the percentage larger than 5μ ranging from 7 percent in Cincinnati to 64 percent in Helena, Montana. Examination of a number of filters suggests that particles having the brightness, color, and size potentially to interfere with the FAST technique appear to be substantially less than 1 per liter of air.

In another experiment, Scott Foam filters were placed over the membrane filters to determine whether the removal of the larger particulates resulted in a significant reduction in the fluorescing particles. The results of experiments, in which 3150 liters of day-time air were sampled, follow.

<u>Mesh Size of Foam Separator</u>	<u>Particles/l Passing Foam</u>		<u>% Particles Fluorescing</u>
	<u>Tungsten Light</u>	<u>UV Light</u>	
60	680	140	20.6
80	597	118	19.7
110	158	38.6	24.4

These results indicate that, with increasing fineness of the foam, there is a gradual decrease in the particles passing the foam separation, in the expected manner. However, the percentage of particles fluorescing does not appear to change significantly over the range studied.

4.3.3.5 MICROSPPECTROFLUORIMETER

The need for studies of fluorescent spectra was recognized early in the program. In the previous sections, investigations were reported on the number, size, brightness, and color of particulates collected in El Monte. The observation of numbers of fluorescent particles lent urgency to the development of an instrument for quantitative characterization. In addition, the fluorescent spectra of bacteria stained with antibodies conjugated to various dyes were of particular interest.

On the basis of this need, the monochromatic microspectrofluorimeter was developed by Beckman Instruments. The instrument could be used to obtain action and emission spectra of individual fluorescent particles on a microscope stage, as well as simple absorption spectra. Results of studies with this instrument were expected to lead to techniques for maximizing specificity and signal brightness in the detection of BW agents. The design is based on the principle of adapting special entrance and exit monochromators to a B & L microscope. The instrument had the following characteristics:

- a. Prism monochromators in both excitation and fluorescence channels. High resolution selection of wavelengths and spectral bandwidths are therefore readily made in both channels.
- b. Illumination of only a very small area ($\sim 1\mu$ diameter). This permits examination of individual particles.

- c. Capability of a line scan, which permits the sequential examination of many μ areas in a relatively short time.

In this instrument, the object plane of the microscope is illuminated with a spot approximately μ in diameter. The wavelength in the spot can be selected by two knobs on the source monochromator, controlling the center wavelength and the bandwidth, respectively. A torsional galvanometer mirror mounted at the microscope entrance pupil sweeps the μ spot across the object plane of the microscope. The galvanometer mirror can be driven at any frequency up to 3000 cycles per second. However, the most probable driving wave shape for the galvanometer was a sawtooth wave of 100 cycles per second or less.

The same objective lens is used both to illuminate the sample and to observe its fluorescence (or transmittance). The fluorescent energy emitted by the object passes through the objective and through a second monochromator enroute to the photomultiplier detector. There is no ocular in the exit optics of the microscope; instead a Littrow prism is inserted following the image plane of the objective. Following the Littrow prism is first a focusing mirror, next an exit slit, and finally an end-on photomultiplier. The focusing mirror re-images the image plane onto the exit slit. The particular wavelengths which pass through the slits to the detector are determined by the angular position of the Littrow prism and the slit width. Each has a control knob. The optics transmit in the range from 340 to >700 m μ .

A 50 percent reflecting aluminized mirror is used as a beam splitter to separate the source optics from the detector optics. This mirror reflects or transmits uniformly nearly all wavelengths. It is placed between the objective lens and its image plane.

The photomultiplier has an S-20 cathode surface to insure maximum response through the whole visible wavelength region.

A series of systematic comparisons were made of the relative intensity of emission of background particles and FA-stained BG particles at a series of excitation wavelengths. In some experiments, a comparison of a spectrum obtained with 435 m μ (bandwidth 35 m μ) excitation was made with data at 365 m μ excitation. The lower wavelength region was chosen to test the brightness of emission when the very bright line of the mercury spectrum at this wavelength

is used for excitation. At the 365 mμ excitation, spectra were obtained at 25 mμ and 45 mμ bandwidth.

FA-stained BG particles were excited at both 435 m μ, 35 mμ bandwidth, and at 365 mμ , 45 mμ bandwidth. Both excitations gave an increase in emission from 500 to 600 m μ, in line with data presented by Nairn⁽⁵⁾. However, when the bandwidth was reduced to 25 mμ the increased excitation due to FA-stained BG fluorescence was not evident.

In other curves, data were obtained comparing the tape background under conditions of (1) "dry" objective and (2) glycerin immersion. The very pronounced "darkening" of the tape with liquid immersion by about tenfold is clearly shown. A reduced emission from the background particle by about threefold is also shown.

The spectra for the background particles in Figure 4-81, are for particles selected to have a particularly bright image. It is thus evident that some background particles may be brighter than the FA-stained bacteria. However, only a few very bright particles of the type shown in Figure 4-81 can be found on a filter representing ~2000 liters of air, substantiating the statement of Section 4.3.3.4 that there will be less than one interfering background particle/1 air.

4.3.3.6 MICROORGANISMS IN THE ATMOSPHERE

Microorganisms normally present in the atmosphere may provide a background or introduce cross-reactions. A knowledge of their number, size, and type is important to the establishment of any type of biological or physical sensing method.

A useful method for the collection and characterization of particulate microorganisms is the Andersen sampler⁽⁶⁾. This device impinges the organisms on a series of agar plates and gives a rough particle size distribution of the viable organisms.

The first few collections made with this device showed a characteristic distribution of organisms. This led to the tentative hypothesis that most organisms present in the atmosphere (at least in daytime) are spore-formers. This hypothesis should be examined further.

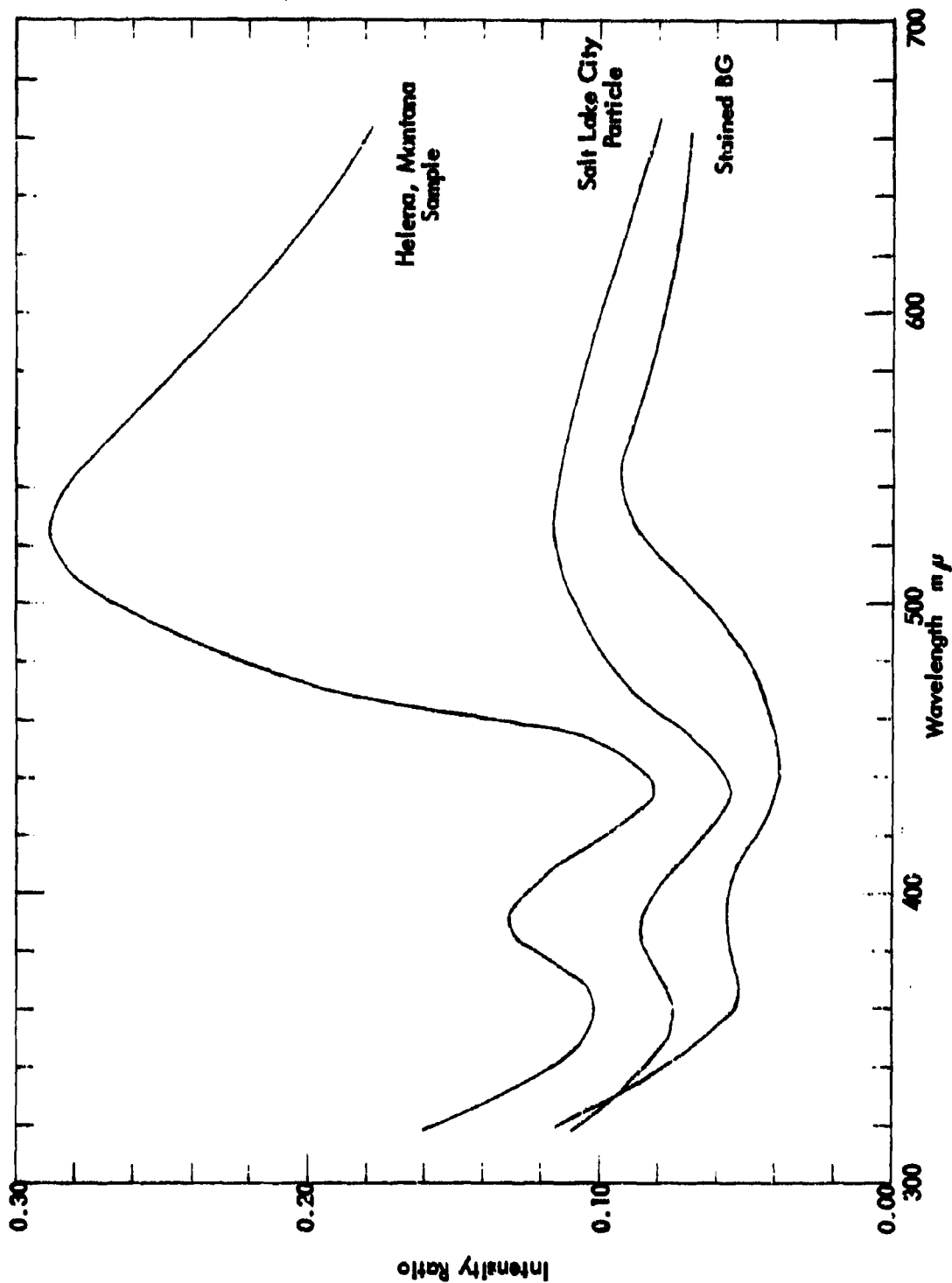


Figure 4-81. Emission Spectra of Particles in Microspectrofluorimeter Excitation at 365 mμ, 45 mμ Bandwidth

Tests showed that sampling for no longer than 1 hour at 1 cfm will give a sufficient sample for colony plate counts by the usual methods. Even with these short times, sufficient organisms may be present to give overgrowth. It was generally noted in samples collected in El Monte that the preponderance of colonies was found on the plates representing larger than 1 micron organisms. The data from one series are illustrative:

<u>Plate</u>	<u>Particle Size, Microns</u>	<u>Colonies</u>
1	> 8.2	76
2	5.0 to 10.4	70
3	3.0 to 6.0	27
4	2.0 to 3.5	202
5	1.0 to 2.0	44
6	< 1.0	10

This series represents a 1-hour sample at 28 liter/min. The high count on plate 4 appears atypical; generally, the count is reasonably constant throughout the first four plates with somewhat less on plate 5 and a sharp drop-off on plate 6. The count of viable organisms was generally in the range of 0.01 to 0.25 per liter of air.

Several of the plates were shipped from El Monte to Palo Alto and returned to determine whether the agar strength was sufficient for shipment and to establish whether contamination might occur on the additional handling. Samples containing both 1.8 and 3.0 percent agar were successfully shipped without special handling (conventional air mail) and no rupture of the agar was noted. Covered agar plates, shipped as blanks along with the Andersen samples, showed no colonies. It thus appears that Andersen samples can be collected at stations throughout the country and shipped to a central laboratory for analysis.

Several interesting conclusions were shown in the results of the characterization of isolates prepared from some of the Andersen plates. These data are shown in Table 4-27. The bacterial isolates appeared to be mainly spore-forming, gram-positive rods. Of those bacteria which were not spore-forming rods, the majority were streptomyces and micrococci. Among the spore formers, there was a considerable variation from day to day in the kind of organism, i.e.,

Table 4-27

CHARACTERIZATION OF ISOLATES FROM
ATMOSPHERIC SAMPLES COLLECTED AT EL MONTE
WITH AN ANDERSEN SAMPLER

<u>Sample and Plate No.</u>	<u>Organisms, Characteristics</u>	<u>Colonies per Plate</u>
I. Sept. 19, 1963, One hour, 4:30 - 5:30 PM Plate No. 2	(Varieties)* Gram + rods** (6) Gram - rods Gram + cocci (2) Molds (1) (<u>A. niger</u>) Streptomyces	8 0 2 3 <u>0</u> 13
Plate No. 5	Gram + rods (2) Gram - rods Gram + cocci (2) Molds (1) Streptomyces	2 0 5 1 <u>0</u> 8
II. Sept. 20, 1963, Two hours, 3:00 - 5:00 PM Plate No. 5	Gram + rods (4) Gram - rods (1) Gram + cocci (1) Molds (1) (<u>Penicillium</u>) Streptomyces (1)	57(35,9,1,12) 7 1 1 <u>1</u> 67
III. Sept. 24, 1963, One hour, 10:30 AM to 11:30 AM Plate No. 5	Gram + rods (5) Gram - rods (2) Gram + cocci (2) Molds Streptomyces (1)	20 5 3 0 <u>74</u> 102

* Based on colony characteristics

** Spores detected in cultures of Gram + rods after 7 days incubation.

whether molds, streptomyces, or bacteria. The molds tended to collect mainly on the plates representing larger sizes.

4.3.4 CONCLUSIONS

A number of important conclusions are apparent from the limited studies on atmospheric background reported in the preceding paragraphs. First, the mass of particles in the air larger than 5μ (the usually stated upper limit for oral infection) is 50 percent or greater of the total weight of particles in the atmosphere, and a suitable primary separator eliminating these particles will substantially reduce the amount of these potentially interfering particles. Secondly, the Space-General technique of collecting particles in an aqueous medium was shown to dissolve 50 percent or more of the particles in as little as a 4-second contact time, again substantially reducing potential interferences. The fluorescence of particles was studied and it was found that, although a large number of particles glow to some extent in fluorescent lighting conditions, the number of particles which glow brightly with the same color as fluorescein-antibody tagged organisms is certainly substantially less than 1 per liter of air. The viable organism count of 0.01 to 0.25 per liter of air, mainly of spore-forming, gram-positive rods, appears to offer limited interference to a pathogen sensor with a 1 organism per liter of air sensitivity.

The studies illustrate the necessity of a concurrent study of interfering background, an assessment of the extent of interference, and means of reducing it, which should be an important part of the evaluation of any sensing technique.

4.3.5 REFERENCES

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Appendix A

A.1 FLUORESCENCE TITER OF B. GLOBIGII SPORES ON SLIDES

1. Prepare a suspension of 5×10^7 BG spores per ml in distilled water. An optical density of .54 at 525μ approximates this concentration of spores. Dilute this suspension 1/10 with distilled water. Spread .02 ml aliquots of the 1/10 dilution over 1 cm^2 areas of carefully cleaned slides of the approximate thickness for dark field work. Air dry and heat fix.
2. Centrifuge fluorescent conjugate in non-heparinized microhematocrit tubes at 12,000 RPM for four minutes. Break-off the sealed ends of the tubes to remove sediment and expel the antiserum into a common container (small test tube or vial). Prepare 0, 1/5, 1/10, 1/25, 1/50, 1/75, 1/100 and 1/200 dilutions of the centrifuged conjugate.
3. Stain smears for 2 minutes. Rinse with 0.5M, pH 9.6 sodium bicarbonate buffer as follows: Hold the slide at a slight angle from the horizontal and allow four 2-ml aliquots to flow over the smear. Place the slide on the staining rack, flood the surface with buffer, and allow to stand for two minutes. Then repeat intermittent rinse.
4. Conditions for observation and photography are as follows:
 - a. Light source - HBO 200 lamp
 - b. Filters - UG1, BG38, and the equivalent of a Leitz UV absorbing filter.
 - c. Lenses and condenser - Substage cardioid oil immersion dark-field condenser, N A. 1.2; periplanatic 15x ocular (visual), 10x periplanatic ocular (photographic), with 1.2x tube factor; or 12.5x compensated ocular with 1x tube factor.
 - d. Photography - Polaroid 3000 speed film, Type 47, Polaroid 110B camera, set at infinite focus and f-stop 4.7. Note that proper centration and focusing of the condenser are essential.

A.2 FLUORESCENCE TITER OF *B. globigii* SPORES IN SUSPENSIONS

1. Prepare 0, 1/2.5, 1/5, 1/12.5, 1/25, 1/37.5, 1/50, and 1/100 dilutions of FITC rabbit anti-*B. globigii* globulin in pH 7.2 Difco FA phosphate buffer, centrifuge for 10 minutes at 12,000 RPM and pass through a Swinny filter containing a millipore disc of 0.45 microns pore size. The final dilution of the antiserum will be twice that indicated above.
2. Prepare a 1/10 dilution of a suspension of approximately 5×10^7 spores per ml (optical density 0.54 at 525 μ) in pH 7.2 buffer. The spores should be prepared exactly according to the method of Dr. Pital; however, to avoid excessive loss of antigen, it is advisable to centrifuge at not more than 1800 RPM and to perform not more than three washings.
3. React 0.2 ml aliquots of antigen and antibody at 25°C in 10 x 75 mm test tubes for two minutes. While staining is in progress, pretreat a 1 cm² area of black millipore tape (.45 micron pores) with 1 ml of a solution of 0.1 percent gelatin in pH 9.6 0.1M bicarbonate buffer. Draw the pretreatment solution through the tape until a thin film remains on the upper surface. (This solution must be made with care to avoid gelatin crystals. Dissolve gelatin completely in a small volume of boiling distilled water and add to buffer. Do not heat the buffer.) Immediately filter the stained BG spore suspension, and rinse immediately with 0.5M pH 9.6 bicarbonate buffer. Follow the rinse with 0.25 ml pH 9.6 buffered glycerol saline.
4. Examine under 500x magnification, using an above-stage condenser and Leitz UV absorbing, UG1, and BG 38 filters. Components of the optical system must be equivalent to the following: Condenser: Leitz "Ultropak" mirror condenser; objective: "Ultropak" 50x, 0.65 N.A.; ocular: Leitz "Periplan" GF 10x wide field. During observation, it is well to be conscious of the effects of overexposure (more than 10 seconds) of black millipore tape and of stained spores to the incident illumination. For higher power use an "Ultropak" 100x oil immersion objective, N.A. 1.0.

A.3

RECOMMENDED PROCEDURE FOR PREPARATION OF FLUORESCENT
ANTISERA (HYLAND LABORATORIES)

1. Dilute bulk serum 1/2 with saline. The titer of the serum should be at least 1:2560.
2. Add an equal volume of saturated ammonium sulfate, and continuously mix for 1/2 hour at room temperature. Hold overnight at 5°.
3. Centrifuge and wash twice with 50% saturated ammonium sulfate (3 to 4 volumes of wash to precipitate). Reconstitute in a minimum volume of distilled water.
4. Dialyze against tap water for 4-5 hours, then dialyze against 0.01M phosphate buffer, pH 7.1, at +5° with adequate changes until the NH_4^+ ion is no longer detected by Nessler's reagent. Dialysis takes about two days.
5. Protein is determined by the biuret method and the purified fraction examined for absence of albumin by electrophoresis on cellulose acetate. Normally, protein should be 5-6% after dialysis. (500 ml serum yield 100 ml of 5 to 6% pure gamma globulin.)

Conjugation:

1. BBL Crystalline FITC is added directly to protein solution at a ratio of 1 mg FITC to 60 mg protein, although this is not critical. The range is 1/50 to 1/80.
2. Hold a minimum of 20 hours at 5°, and pass through G-25 coarse Sephadex, which must be prepared carefully to remove fines by washing, settling and decanting.
3. The typical recovery after passage through Sephadex is one half the volume of the original raw bulk serum. The protein concentration is routinely between 2 and 3%.
4. ϵ - amino caproic acid, 0.05%, and sodium azide, 0.1%, are added.

A.4 RABBIT IMMUNIZATION SCHEDULES FOR SERRATIA MARCESCENS AND B. GLOBIGII

First Week:

Monday	2 ml IM, Freund's adjuvants
Wednesday	1 ml IV
Friday	1 ml IV

Second Week:

Same as First Week

Third Week:

Friday	1 ml IV

Bleeding:

9, 12, and 13 days after last injection

Antigen

5×10^8 to 1×10^9 spores
or vegetative cells per ml

IV = intravenous, IM = intramuscular

FINAL REPORT ON THE RESEARCH PROGRAM ON
BW DETECTION

Volume II
MANAGEMENT AND FINANCIAL SUMMARY

TASK II OF RESEARCH PROGRAM ON BW DETECTION

March 1966

Prepared for
U.S. ARMY BIOLOGICAL CENTER (PROVISIONAL)
Fort Detrick
Frederick, Maryland

on
CONTRACT DA 18-064-AMC-137(A)

by
SPACE-GENERAL CORPORATION
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Section 1

INTRODUCTION

This is Volume II of two volumes of the Final Report on the Research Program on BW Detection conducted by Space-General during the period May 1963 through October 1965. The report is provided in accordance with the requirements of Contract DA 18-064-AMC-137(A).

Volume I presents a detailed technical review of the exploratory research and development program which was conducted to demonstrate the feasibility of a number of carefully selected concepts for utility in BW detection. It describes the research activities which were performed, the work accomplishments, and the evaluations which were made concerning the utility of each of forty-four technical concepts.

This volume (Volume II) of the report includes a discussion of the overall management plan, schedules, responsibilities, and assignments utilized in meeting the objectives of the program. It also presents a detailed breakdown of the costs incurred, and the manpower expenditures. The latter items have been classified in terms of the technical tasks performed (Table 3-4) and in terms of the contracting organizations involved (Financial Chart No. 2).

Section 2

SUMMARY

2.1 MANAGEMENT

The organization and management plan for fulfilling the program objectives in conducting an effective study of new concepts for utilization in BW detection has been carried out as originally proposed.

Forty-four carefully selected concepts have been examined for utility in various aspects of BW detection. Five of these have been partially examined in an initial phase of breadboard evaluation.

Extensive and effective participation by Corporate Management has contributed significantly to the successful completion of all portions of this program.

Management interest in the Research Program on BW Detection at Space-General and the active roles of the Corporate Officers are readily evidenced, in a formal sense, by the more than 131 special and management-attended meetings which were held directly concerning the program. The contributions of Mr. F. H. Lehan, President of Space-General, Dr. J. C. Fletcher, former Board Chairman of Space-General and currently President, University of Utah, Dr. J. E. Froehlich, Executive Vice-President of Space-General, Mr. C. E. Roth, Jr., Vice-President and General Manager of Space-General and Dr. A. L. Antonio, former General Manager of Space-General and currently Group Vice-President of Aerojet-General in their numerous contacts and exchanges of ideas with project personnel have represented a very tangible form of management interest. Dr. E. Mishuck, the initial Program Manager, has continued to operate closely with the program team in his post as Senior Division Manager, Chemical and Biological Operations.

2.2 FINANCIAL

The Research Program on BW Detection has been carried out for a period of approximately thirty-two months within the budgetary limits established to meet USABC requirements.

Section 3

MANAGEMENT

3.1 PROGRAM ORGANIZATION

The special program organization formed within Space-General Corporation to conduct the Research Program on BW detection was designed to meet all requirements for effective management. Specifically, Space-General's interest was demonstrated by assignment of the senior Corporate Officer as a direct participant. Figure 3-1 shows the organization of program management. Authority and responsibility were clearly defined, both within Space-General Corporation and among the participating subcontractors and consultants. Direct lines of authority and communication were established, leading to rapid response to any necessary redirection of the program. In addition, excellent communication has been maintained throughout the program between the Fort Detrick Project Officer and the Space-General Program Manager, as well as between the Contract Administrator counterparts. The program flexibility was evidenced on three separate occasions when, during no-cost extensions, work was continued at reduced levels of effort and scope.

Space-General's recognition of the need for top calibre scientists on a research program of this type was indicated by the 2.5/1 ratio of professional scientists to technicians and support personnel during the program. Similarly, an overall professional-to-support ratio of 2.4/1 has been maintained at the supporting organizations. The continuity of technical effort has been maintained throughout the program through the full-time utilization of personnel originally selected to participate on the program.

3.2 ASSOCIATE AND SUBCONTRACTORS

The team approach originally proposed by Space-General Corporation was maintained throughout the performance of the program. Minimal changes were necessary in the subcontractor team during the course of the program, and all were made within the framework of dropping less promising approaches while seeking

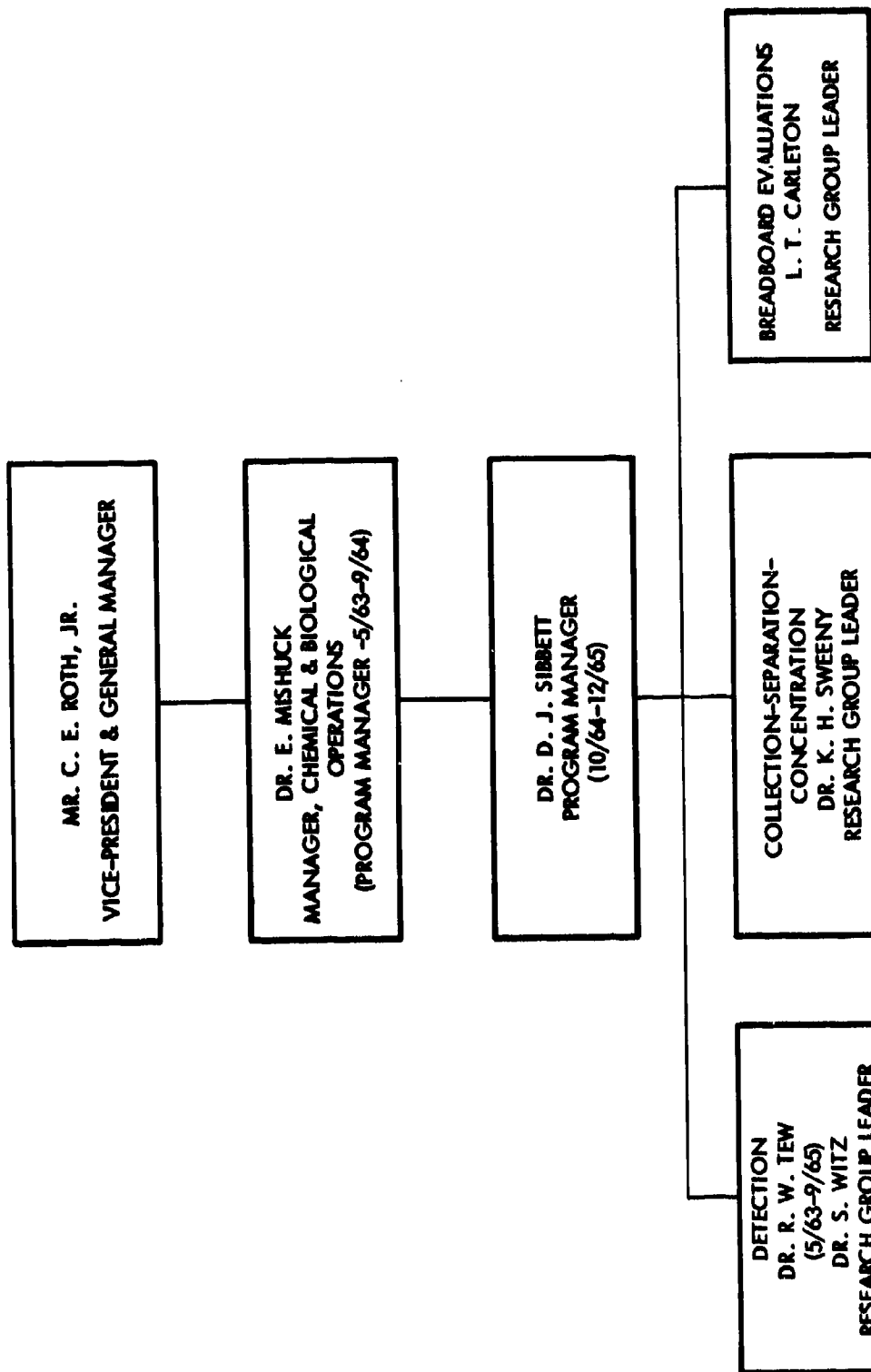


Figure 3-1. Program Organization

and emphasizing more promising techniques. The Space-General team, comprised of organizations of diverse and supplementary skills and capabilities, included the following enterprises:

Space-General Corporation	El Monte, California
Beckman Instruments, Inc.	Fullerton, California
Metronics Associates, Inc.	Palo Alto, California
Hyland Laboratories	Los Angeles, California
University of California	Los Angeles, California
University of Utah	Salt Lake City, Utah

The initial choice of associate and subcontractors was confirmed by performance. Beckman Instruments and Metronics Associates made important advances in key areas. Hyland Laboratories contributed valuably in production of antisera, and in supporting research directed toward improving the biological supplies. The University of Utah, through its Department of Microbiology, provided significant contributions to the research on virus detection techniques. The University of California at Los Angeles strongly defined virus purity problems associated with detection. In addition, less significant efforts were contributed by: Bio-Sciences Laboratories, Varian Associates, and International Nuclear and Chemical Corporation.

3.3 AREAS OF RESPONSIBILITY AND ASSIGNMENTS

The responsibilities and assignments on the research program on BW detection were established and defined according to the major tasks to be performed and the experience and areas of specialization of the participating members of the Space-General team. Space-General was responsible for overall control and coordination of the research effort. In this capacity, Space-General Corporation conducted in-house research on new detection concepts based on biological, chemical, and physical properties and reactions; in aerosol sampling techniques; and instrumentation development of promising detection techniques, components for detection systems, and combinations of operational components.

Beckman Instruments was responsible for research on separation techniques based on microelectrophoresis, on pH matrix methods of detection, on microspectrofluorimetry, on bioelectrochemical methods, and on various centri-

fugation procedures. Metronics Associates was responsible for investigations of aerosol sampling, and background characterization, and participated in research leading to the development of collection, separation, and concentration devices. Hyland Laboratories provided high-quality antisera and other biological reagents and performed studies on improved methods of conjugating fluorescent dyes with immunoglobulins. The University of Utah supported the research at Space-General Corporation in the area of virus detection techniques. UCLA studied detection problems related to virus purity.

Principal tasks performed and the responsible contractors are summarized in Table 3-1.

Table 3-1

RESEARCH TASKS & COGNIZANT CONTRACTORS

Space-General Corporation	Beckman Instruments	Metronics Associates	Hyland Laboratories
Chemiluminescence Breadboard Development Bacteriophage Breadboard Development Subsystems Breadboard Development Immunofluorescence Hypersensitivity Virus Reactions Bacteriophage Enzyme Reactions Magnetically-Stabilized Electrophoresis Electromagnetophoresis Liquid Partition Thin-Film Microelectrophoresis Sample Collection	Thin-Film Microelectrophoresis Microspectrofluorimetry Density Gradient Centrifugation pH Matrix Bioelectrochemical Methods (Magna)	Electrostatic Precipitators Electrostatic Spray Collection Background Characterization Double Plate Impellers Foam Filters Foam Flotation New Principles of Collection, Concentration and Separation of Aerosols	Immunological Supplies
University of Utah	Varian Associates	International Chemical and Nuclear Corporation	UCLA
Viruses (Group A Arboviruses)	EPR	Radioisotopic Techniques	Viruses and Purification

In its capacity of having overall cognizance of the research effort, Space-General has utilized the combined talents of its technical advisors and consultants to guide the research efforts through their specialized skills in multidisciplinary evaluation of research progress. Throughout the program, a constant search was conducted for nationally-known scientists specializing in related fields who could contribute to the program on a consulting basis. These consultants were utilized in both individual meetings and special brainstorming sessions to seek and evaluate novel approaches to BW detection.

A list of the individuals who contributed to the BW research program is compiled in Table 3-2, together with their areas of specialization.

Although Dr. J. C. Fletcher in his position as President of the University of Utah could not function as a consultant, he continued his constructive and enthusiastic efforts after his departure from Space-General for Salt Lake City. His many contributions were exemplified by his chairmanship of the meeting on "Rapid Quantitation Problems in Virology", on April 30 and May 1, 1965.

Table 3-2
CONSULTANTS

Name	Affiliation	Specialization
M. S. Blois	Stanford University	Biophysics, Free Radical Reactions
D. H. Campbell	Cal. Tech.	Immunochemistry
R. Donovick	Squibb Institute for Medical Research	Microbiology
H. W. Dorris	Private Consultant	Air Sampling
R. Edgar	Cal. Tech.	Viruses, Genetics
H. Eyring	Univ. of Utah	Biophysical Chemistry, Kinetics
D. Glaser	Univ. of Calif. (Berkeley)	Biophysics
J. S. Garvey	Cal. Tech.	Biochemistry, Immunochemistry
S. Golomb	Univ. of Southern Calif.	Information Theory
B. W. Grunbaum	Univ. of Calif. (Berkeley)	Environmental Physiology, Biochemistry
H. Halvorson	Univ. of Wisconsin	Bacteriology
E. M. Heimlich	Cedars of Lebanon Hospital	Immunology
A. Kolin	U.C.L.A.	Electrophoresis
A. F. Langlykke	Squibb Institute for Medical Research	Biochemistry
A. G. Marr	Univ. of Calif. (Davis)	Microbiology
G. Middlebrook	Univ. of Maryland	Immunology, Medicine
M. J. Pickett	U.C.L.A.	Bacteriology
A. F. Rasmussen	U.C.L.A.	Virology
C. E. Schwerdt	Stanford University	Biochemistry, Virology
S. Shankman	Pasadena Foundation for Medical Research	Centrifugation

In addition, consultations with the following scientists have been utilized during the program:

P. Berg	Stanford University	Biochemistry, Nucleic Acids
R. F. Chaiken	Private Consultant	Biophysics, Physical Methods
J. J. Holland	Univ. of Calif. (Irvine)	Virology
W. L. Nyborg	Univ. of Vermont	Biophysics, Sonics
M. J. Vinograd	Cal. Tech.	Centrifugation, Nucleic Acids

A successful research program must be conducted within a flexible and dynamic framework. Yet rigorous planning and scheduling must be maintained to provide constant program control. Technical progress and accomplishments dictated the course of the efforts, promising approaches were emphasized, new ideas were introduced, and approaches showing little or no promise were eliminated.

Space-General's flexibility in carrying out the research effort within a dynamic framework is readily evidenced by examining Figure 3-2 and Table 3-3. Figure 3-2 and accompanying legend summarize the progression of tasks on the research effort. Table 3-3 indicates the research concepts investigated on the program, as well as their disposition.

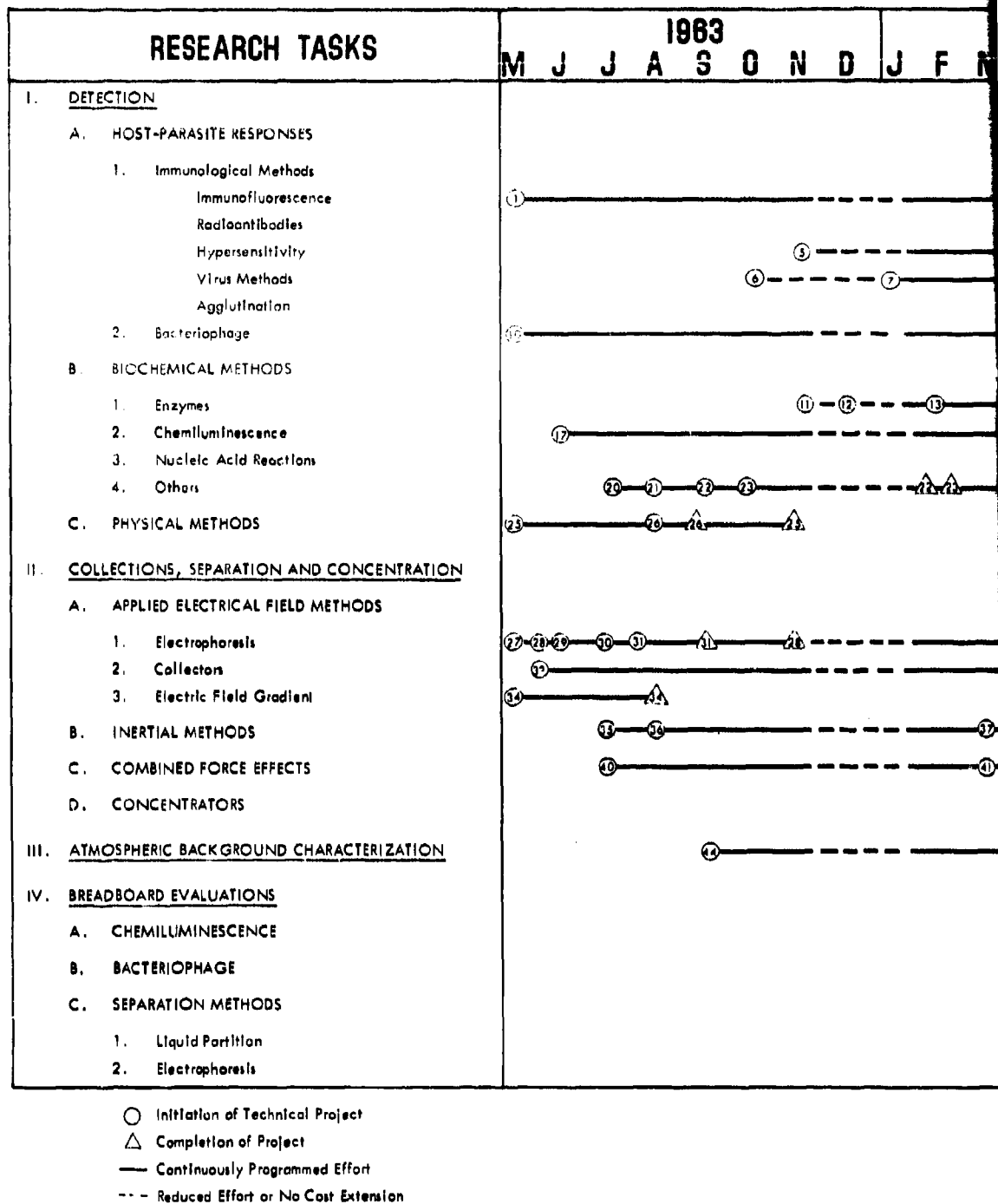


Figure 3-2. Schedule, Research

Legend for Figure 3-2

Identification of Research and Development Efforts

1. FITC Staining Methods
2. Methods Utilizing Labelled Polymer
3. Spinning Disc Research
4. Radioantibodies
5. Hypersensitivity
6. Virus Detection Utilizing Synthetic Particles
7. Virus Purification and Detection (UCLA)
8. Rapid Detection of Group A Arboviruses (U. of Utah)
9. Agglutination
10. Bacteriophage
11. L-Glutamate Dehydrogenase
12. Fermentation Matrix
13. Alkaline Phosphatase
14. C^{14} -Glucose Fermentation
15. Cytochrome c Reductase
16. Transaminase
17. Chemiluminescence
18. Hemin Staining Methods
19. Nucleic Acid Reactions
20. NH_3 Detection by Electron Capture
21. Quenching of Luminescence
22. Quenching of Fluorescence
23. Phosphorescence of Proteins
24. Sialoresponsin Formation
25. Bioelectrochemical Methods
26. EPR
27. Electromagnetophoresis
28. Zone Electrophoresis
29. Thin-Film Electrophoresis
30. Magnetically Stabilized Electrophoresis
31. pH - Density Gradient Electrophoresis
32. Porous Electrode Electrostatic Precipitators
33. Electrostatic Spray Collectors
34. Electric Field Gradient Separation
35. Double Plate Impeller Separator
36. Density Gradient Centrifugation
37. Open Cell Foam Classifier
38. May Pre-Impinger
39. Cyclone Classifier
40. Liquid Partition
41. Concentration in Foams
42. Sonicating Washer-Concentrator
43. Vertical Filter-Concentrator
44. Atmospheric Background Properties

Table 3-3

DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Aided</u>
I. DETECTION			
A. Immunofluorescence			
1. FITC Staining	Provides methods for detecting all pathogens		
2. Labelled Polymer Methods		Promising, but initial trial showed little advantage	Promising, selective collection method
3. Spinning Disc Techniques			
B. Hypersensitivity	Sensitive, specific; readily capable of instrumentation		
C. Bacteriophage	Good basis for breadboard, for bacterial agents		
D. Chemiluminescence			Iron Porphyrins occur widely; detection principle is simple, sensitive; breadboarded
E. Enzymatic Methods			
1. L-glutamate dehydrogenase reaction		Fluorescence intensity too low	
2. Alkaline Phosphatase Hydrolysis	Enzyme is widely distributed; potentially sensitive method		

Table 3-3 (Continued)

DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Aided</u>
3. C ¹⁴ -labeled Glucose Fermentation		Time lag in response of resting cells	
4. Transaminases			Widely distributed in microorganisms; involves metabolic processes
5. pH Fermentation Matrix		May detect bacteria via fermentative capabilities; complex method	
6. Cytochrome-c Reductase		Widespread enzyme; questionable sensitivity	
F. Other Biochemical Methods			
1. NH ₃ Detection by Electron Capture		Poisoning of detector prevents long-term operation	
2. Quenching of Luminescence by S-H groups		Other components of aerosols may give false positives	
3. Quenching of Eosin-Y Fluorescence by Protein		Operational time too long	
4. Phosphorescence of Proteins		Desired sensitivity not attainable	
5. Hemin Staining			Selective staining; increases ability of chemiluminescence to detect viral agents; sensitive response
6. Sialoresponsin Formation			Unique host-parasite response

Table 3-3 (Continued)
DISPOSITION OF TASKS ON EW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
G. Radioantibody Methods			Sensitive, selective with multiagent capability; low background interferences
H. Nucleic Acid Methods			Involves ultimate agent composition; simple radio tracer methods available
I. Virus Detection Utilizing Synthetic Particles			Flexible methods allowing multiagent detection
J. Agglutination			Rapid, simple technique with easy readout
K. Physical			
1. Bioelectrochemical detection		Comparatively unpromising	
2. EPR		Too insensitive to detect free-radical intermediates of bacterial enzymes	
3. Spectrophotometry, Fluorimetry			Provides indispensable comparisons of bacteria with interfering background
L. Rapid Detection of Group A Arboviruses (Utah)			General properties of Group A arboviruses indicated unique problems

Table 3-3 (Continued)
DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
M. Virus Purification (UCLA)		Objectives achieved	
II. COLLECTION, SEPARATION & CONCENTRATION			
A. Electrophoresis			
1. Magnetically Stabilized Electrophoresis	Provides highly sensitive separations; selected for breadboarding		Too sensitive method for field utilization
2. Thin-Film Electrophoresis			
3. Zone Electrophoresis			Not demonstrated
4. Electromagnetophoresis	Separations based on particle conductivities, may be quite selective		
5. pH-Density Gradient Electrophoresis			Not sufficiently sensitive, selective or rapid
B. Electric Field Gradient	Offers several separation principles based on conductivity and polarity; continuous mechanization possible		
C. Porous Electrode Electrostatic Precipitators			Recovers micron-sized particles; efficiently; qualified by operating experience

Table 3-3 (Continued)

DISPOSITION OF TASKS ON BW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
D. Electrostatic Spray Collectors			Promising method which avoids water distribu- tion aspects of impinge- ment on washed electrodes
E. Inertial Methods			
1. Double-Plate Impeller		Simpler devices give equally sharp separations, less holdup	
2. Density-Gradient		Continuous operations pose engineering problems	
F. Primary Separators			
1. Cyclone Classifiers		Useful, but not as simple as foam	Proven usefulness; simple, sharp separations; inexpensive
2. Open Cell Foam Classifier			
3. May Pre-Impinger		Has possibilities but not as simple as foam	
G. Combined Force Effects			
1. Liquid Partition			Highly selective in separating bacteria and viruses from background; rapid, reliable method
2. Concentration in Foams		Simple, novel method appeared selective in first trials, but with engineering problems	

Table 3-3 (Continued)
DISPOSITION OF TASKS ON HW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
H. Concentration by Filtration			
1. Sonication Washer-Concentrator			Promising, reliable, useful in washing
2. Vertical Filter-Concentrator			Probably effective, little data
III. BACKGROUND CHARACTERIZATION			
		Supplied useful data; further investigations associated with individual detectors	
IV. BREADBOARD EVALUATION			
1. Chemiluminescence Detector			Based on catalase-luminol reaction, rapid, sensitive, non-selective, simple; rugged instrumentation
2. Bacteriophage Detector			Based on bacteriophage research; prototype utilization of tracer techniques, selective
3. Collection-separation Subsystems			
a. Porous Electrode Electrostatic Precipitator (PEEP) Liquid			Based on two former research techniques; promises small, reliable methods for collection and separation of background from biological materials; no sampling rate limitations

Table 3-3 (Continued)

DISPOSITION OF TASKS ON EW RESEARCH PROGRAM

<u>Research Concept</u>	<u>Originally Proposed</u>	<u>Terminated</u>	<u>Added</u>
b. PEEP-Thin Film Electrophoresis			Most promising combination for utilization of electro- phoresis in practical de- vices; for low sampling rate detectors

3.6

PERSONNEL

Table 3-4 indicates the approximate level of effort which was expended in each technical area from the inception of the program.

CONTRACT DA 18-064-AMC-157(A), NEW AND NOVEL CONCEPTS IN
BW DETECTION, MANPOWER EXPENDITURES ON TECHNICAL TASKS

APPROXIMATE MANPOWER (MANMONTHS)																	
Spec-Gen Comptation		Biochem Laboratories		Metabolic Associates		Hydrol Laboratories		UCLA		Univ. of Utah		Bio-Science Laboratories		I-4 Chem. & Nuclear		TOTALS	
P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*
I. DETECTION RESEARCH																	
A. HOST PARASITE RELATIONS																	
1. Immunological																	
a. Immunofluorescence		24.8	4.9					20.1	6.9							44.9	23.2
b. Hypersensitivity		26.5	3.9					0.9	0.5							27.4	4.4
c. Viremia		33.7	4.6					1.0		8.4	10.0	10.3	9.0		0.5	53.9	26.0
2. Bacteriology		19.8	7.1					0.5								20.3	7.1
B. METABOLISM AND STRUCTURE																	
1. Biochemical Methods																	
a. Chromatography		11.6	6.6													11.6	6.6
b. Enzymes		23.9	16.1													23.9	16.1
c. pH Changes		1.0	-	8.7	4.0											9.7	4.0
d. Others		6.1	1.9	5.4**	5.4**								0.3			11.8	7.3
C. PHYSICAL METHODS		1.0	0.3													1.1	0.3
D. NEW APPROACHES		5.1	0.4													5.1	0.4
II. COLLECTION, SEPARATION & CONCENTRATION RESEARCH																	
A. APPLIED ELECTRIC FIELD METHODS																	
1. Electrophoretic		25.5	3.4	31.9	12.8											57.4	16.2
2. Electrokinetic		2.0	0.3			34.9	6.4									36.9	6.7
3. Electrostatic Field Gradients		1.9	1.4													1.9	1.4
B. INERTIAL METHODS		1.5	0.3	2.0	0.5	5.2	1.0									8.7	1.8
C. COMBINED FORCE EFFECTS																	
1. Liquid Particles		18.1	4.2													18.1	4.2
2. Films		0.5				1.5										2.0	-
3. Microbubbles		0.4	0.4													0.4	0.4
IV. BACKGROUND CHARACTERIZATION		15.4	2.1	5.3	2.6	13.1	2.0									33.8	6.7
IV. BACKGROUND EVALUATION																	
A. CHEMILUMINESCENCE		10.9	13.1													10.9	13.1
B. BACTERIOLOGY & MACROPHAGES		5.5	3.7													5.5	3.7
C. SEPARATION SUBSYSTEMS		15.9	2.0													15.9	2.0
V. ADMINISTRATION AND PROGRAM SUPPORT		19.8	17.8													19.8	17.8
TOTALS		270.9	107.9	53.3	25.3	54.7	9.4	22.5	7.4	8.4	10.0	10.3	9.0	0.1	-	421.0	169.4

* P = Professional Man Months
S = Support Man Months

** Major Component

Management's active interest in the EW research program is evidenced by the more than 131 meetings in which key management personnel participated. Numerous contributions have been provided by F. W. Lehan, President, Dr. J. E. Froehlich, Executive Vice President, and C. E. Roth, Jr., Vice President and General Manager, on a day-by-day basis in direct personal contacts and technical discussions with project personnel. In addition, Dr. J. C. Fletcher, former Chairman of the Board of Directors, now President of the University of Utah, evidenced his interest in this program throughout its term.

To provide a continuing review of the status of the program and a means of rapidly implementing decisions at the project level, a schedule of bi-weekly and bi-monthly program meetings have been conducted by the Program Manager, which included Space-General personnel as well as appropriate subcontractor representatives. The purpose of these meetings has been to review and guide the week-to-week progress of the technical efforts. Meetings of cognizant Space-General personnel with each subcontractor were nominally scheduled at bi-monthly intervals for purposes of technical direction, and have been held more or less frequently as circumstances required.

Initial program plans recognized the importance of meeting with Fort Detrick program management for purposes of review and possible reorientation. Major meetings with cognizant Fort Detrick personnel for comprehensive program review were held at El Monte and at Fort Detrick on a schedule approximating every second month, involving management and technical personnel from Fort Detrick, Space-General Corporation, and the participating subcontractors. A number of additional meetings have been held, both at Fort Detrick and El Monte, to maintain mutual up-to-date understanding of both progress and problem areas.

A summary of meetings and conferences pertinent to the research program on EW detection is presented in Table 3-5. In addition to the meetings described above, the list contains many special meetings held for the solution of specific problems, and reflects the widespread participation in the program of many agencies and individuals.

Table 3-5
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

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<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
May 13-14 1963	Fort Detrick Frederick, Md.	Contract Negotiations	F. W. Lehan Pres. Space-General Corp. R. B. McKinley Vice Pres., SGC E. Mishuck, Program Manager (5/63-9/64) R. W. Tew, Research Group Leader, Sensing Techniques R. D. Davis, Manager, Contracts Q. T. Parsely, Controller J. R. Rowe, Mgr. - SGC Wash. Office
May 27	Douglas Aircraft Co. Santa Monica, Calif.	Review Partichrome Analyzer	F. W. Lehan E. Mishuck R. W. Tew R. N. Ghose L. T. Carleton, Research Group Leader, Physical Sensing Techniques T. B. Weber, Program Manager, Beckman Instruments M. A. Robinson, Project Engi- neer, Beckman Instruments
May 28	Space-General El Monte, Calif.	Program Review	H. H. Aldinger, Douglas Program Manager W. E. Zisch, Pres. Aerojet- General Corp. F. W. Lehan A. L. Antonio, V.P., Formerly Gen. Mgr. Space-General Corp. (Currently Group Vice Pres., Aerojet-General Corp.) J. C. Fletcher, Formerly Chairman of the Board of Directors, SGC. (Currently President, University of Utah.)

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
May 28	General Mills Minneapolis, Minn.	Review Electrostatic Collector	J. N. Brown, Dir. Marketing SGC E. Mishuck K. H. Sweeny, Research Group Leader, Collection and Concentration P. L. Magill, Metronics Assoc.
June 7	Fort Detrick Frederick, Md.	Subcontract Negotia- tions	A. Smallberg, Contracting Of- ficer, Fort Detrick L. Sanders - Assistant Con- tracting Officer, Fort Detrick G. Bledsoe, Manager, Material Division, SGC
June 11-14	Dugway Proving Grounds BW-CW Indoctrination Utah		F. W. Lehan
June 21	Space-General El Monte, Calif.	Program Management Review	J. C. Fletcher F. W. Lehan A. L. Antonio E. Mishuck
June 24-25	Space-General El Monte, Calif.	Program Review for Ft. Detrick Personnel	Fort Detrick C. R. Phillips, Chief, Physical Defense Division B. Marshowsky, Chief, Physical Detection Branch Project Officer A. Pital, Chief, Bio- detection Branch

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

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<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
July 2	Space-General El Monte, Calif.	Program Review	A.R.T. Dernes, Chem. Corps, Consultant A. M. Cowan - Project Of- ficer, Douglas Program Space-General F. W. Lehan J. C. Fletcher A. L. Antonio E. Mishuck Program Personnel W. E. Zisch A. O. Beckman Pres. Beckman Instruments R. Erickson, Exec. Vice Pres. Beckman Instruments F. W. Lehan A. L. Antonio E. Mishuck E. R. Roberts, V.P. Aerojet-General W. Mullane, Group V.P. AGC J. N. Brown
July 8	Fort Detrick Frederick, Md.	Technical Coordination Meeting of Tri-Service Work on C-B Detection	E. Mishuck R.W. Tew W. Ware, Contract Adminis- trator, SGC J. R. Rowe

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
July 17	Space-General El Monte, Calif.	Program Analysis	J. C. Fletcher F. W. Lehan J. N. Brown E. Mishuck R. W. Tew
July 19	Space-General El Monte, Calif.	Program Review	Brig. Gen Delmore, Command- ing Officer, Fort Detrick W. E. Zisch J. C. Fletcher F. W. Lehan W. B. Mullane A. L. Antonio E. Mishuck
July 19	Space-General El Monte, Calif.	Preparation for Brain- storming Session	J. C. Fletcher F. W. Lehan A. L. Antonio E. Mishuck
July 23	Fort Detrick Frederick, Md.	Management Discussions	Fort Detrick A. Smallberg C. R. Phillips B. Warshowsky A. Pital A.R.T. Denues M. Guggenheim Space-General F. W. Lehan J. R. Rowe J. Convy, Wash. Rep. AGC

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON EW DETECTION

Date	Location	Purpose	Participants
July 25	Space-General El Monte, Calif.	Review of FAST Breadboard	A. Cowan V. Mumma - Fort Detrick E. Mishuck C. E. Hendrix, Research Group Leader, FAST Breadboard
July 26	Space-General El Monte, Calif.	Technical Advisory Board	A. F. Langkykke, Director, R and D Laboratories, E. R. Squibb and Sons A. O. Beckman W. A. Perkins, President Metronics Associates L. Zernow, Director of Research, Ordnance Division, Aerojet-General T. B. Weber E. Mishuck EW Program Personnel
July 31	Space-General El Monte, Calif.	Program Analysis	W. E. Zisch W. B. Mullane F. W. Lehan R. D. Geckler, Vice Pres., Future Operations, Aerojet-General Corp. A. L. Antonio J. N. Brown J. Convy E. Mishuck

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Aug. 5	Space-General El Monte, Calif.	Preparation & Agenda for Brainstorming Meeting	J. C. Fletcher E. Mishnuck L. T. Carlton R. W. Tew
Aug. 15	Naval Biological Laboratories Oakland, Calif.	Evaluation of Facilities for Handling Pathogens	E. Mishnuck R. W. Tew K. H. Sweeney
Sept. 4-5	Miramar Hotel, Santa Monica, Calif.	Brainstorming Meeting on New Concepts in BW Detection	B. Warshowsky A. Pital A. Dennes D. Campbell, Program Consultant Cal. Tech. R. Donovick, Program Consultant, Squibb Institute for Medical Research H. Halvorsen, Program Consultant, Univ. of Wisconsin A. Kolin, Program Consultant, UCLA A. Marr, Program Consultant, Univ. of Calif. (Davis) J. C. Fletcher F. W. Lehan E. Mishnuck L. T. Carleton C. E. Hendrix C. W. Molander, Senior Research Biologist. K. H. Sweeney R. W. Tew S. Witz, Scientist
Sept. 18	Space-General El Monte, Calif.	Program Review & Analysis of Minutes from Brain- storming Session	J. C. Fletcher F. W. Lehan J. E. Froehlich, Exec. V.P., SGC.

Table 3-5 (Continued)

SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAMS ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Sept. 18 Cont'd.	Space-General El Monte, Calif.	Program Review & Analysis	A. L. Antonio E. Mishuck
Sept. 26	Space-General El Monte, Calif.	Program Review	J. C. Fletcher F. W. Lehan A. L. Antonio J. N. Brown E. Mishuck
Sept 26-27	Monterey, Calif.	West Coast Technical Meeting of the Air Pollution Control Associates	K. H. Sweeny
Sept. 30	Fort Detrick, Frederick, Md.	Program Discussions	B. Warshowsky A. Pital R. Boyle, Assistant Project Officer, Ft. Detrick C. W. Molander C. E. Hendrix M. A. Robinson, Project Engineer, Beckman Instruments
Oct. 3	Berkeley, Calif.	First Aerobiology Meeting Sponsored by Naval Biological Laboratories	L. T. Carleton B. W. Grunbaum - Consultant, Univ. of Calif. (Berkeley)
Oct 24	Fort Detrick, Frederick, Md.	Program Discussion	A. Smallberg A. L. Antonio
Oct. 30-31	Space-General El Monte, Calif.	Program Review	B. Warshowsky R. Boyle T. Kerr, Deputy Director of Medical Research, Fort Detrick L. Spero, Chief, Chemistry Branch, Fort Detrick

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Oct. 30-31 cont'd.	Space-General El Monte, Calif.	Program Review	F. W. Lehan A. L. Antonio E. Mishuck Program Personnel, SGC R. Gafford, Program Manager, Beckman Instruments W. Henderson, Optical Engineer, Beckman Instruments A. Strickler, Senior Scientist, Beckman Instruments W. Perkins P. Magill, Senior Scientist, Metronics Associates T. Asher, Program Manager, Hyland Laboratories
Nov. 6	Space-General El Monte, Calif.	Review of System Parameters and Spec- ifications for FAST Breadboard	J. C. Fletcher W. A. Perkins E. Mishuck C. E. Hendrix K. H. Sweeny M. A. Robinson
Nov. 12	Litton Industries Minneapolis, Minn.	Review Development of Electrostatic Collection Unit	D. Lundgren, Project Scientist, Litton Industries E. Mishuck
Nov 18	Aerojet Delft TRG Long Island	Image Intensification Optics; Lasers as Light Sources	J. J. Van Der Sande, President, Aerojet Delft L. Goldmuntz, President, IFC E. Mishuck C. E. Hendrix
Nov 19-22	Dugway Proving Grounds, Utah	CBR Weapons Orientation	J. E. Froehlich A. L. Antonio

Table 3-5(continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Nov 20	Fort Detrick, Frederick, Md.	Review Immunofluorescent Tagging Techniques	B. Warshowsky A. Pital R. Boyle R. W. Tew T. Asher
Dec 5	Residence of Dr. A. Kolin	Review Research Effort on Microelectrophoresis	A. Kolin B. W. Grunbaum E. Mishnuck L. T. Carleton R. Gafford A. Strickler A. Kaplan
Dec 11	Douglas Aircraft Santa Monica	Discuss Characteristics of Background Atmosphere	H. Aldinger, Program Manager, Douglas Aircraft E. Mishnuck K. H. Sweeny C. W. Molander
Dec 23	Communicable Disease Center Atlanta, Ga.	Discuss Fluorescent Antibody Staining Techniques	W. B. Cherry, CDC J. Z. Biegeleisen, CDC M. D. Moody, CDC R. W. Tew C. E. Hendrix

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Jan 6-7 1964	California State Dept. of Public Health Berkeley, Calif.	Sixth Conference on Identification of Airborne Particulates and Air Sampling	K. H. Sweeny
Jan 15	Fort Detrick Frederick, Md.	Task II Follow-on Negotiations	C. R. Phillips B. Marshowsky R. Boyle L. Sanders, Contract Negotiator, USABL L. Gesell, Price Analyst, USABC E. Mishuck W. H. Ware L. G. Nieman, Subcontract Admini- strator, SGC R. M. Spalding, Program Control, SGC J. W. Rowe
Jan 20	Space-General El Monte, Calif.	Beckman Subcontract Negotiations	R. Gafford W. Hartman, Cost Control, Beckman Instruments N. Tonjes, Contract Administrator, Beckman Instruments E. Mishuck L. T. Carleton K. H. Sweeny S. Witz L. G. Nieman R. M. Spalding
Jan 22	Space-General El Monte, Calif.	Ryland Laboratories Subcontract Negotiations	T. Asher G. Key, Manager, Biological Products Hyland Laboratories

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Jan 22 (Cont'd)	Space-General El Monte, Calif.	Hyland Laboratories Subcontract Negotiations	W. Olson, Project Staff, Hyland Laboratories E. Mishuck R. W. Tew C. W. Molander
Jan 23	Space-General El Monte, Calif.	Review Management Aspects of Program	J. C. Fletcher A. L. Antonio E. Mishuck
Jan 24	Space-General El Monte, Calif.	U.C.L.A. Subcontract Negotiations	M. J. Pickett, Principal Investigator, UCLA C. R. Manclark, Scientist, Long Beach State College E. Mishuck R. W. Tew C. W. Molander
Jan 28	Space-General El Monte, Calif.	Evaluate Technical Status of BW Detection Program	J. C. Fletcher A. L. Antonio J. B. Cowen, Vice-President, Marketing, SGC J. N. Brown E. Mishuck
Feb 6	Space-General El Monte, Calif.	Review Status of BW Detection Program	J. C. Fletcher F. W. Lehan A. L. Antonio J. B. Cowen J. N. Brown E. Mishuck

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Feb 10	Space-General El Monte, Calif.	Presentation on BW Detection and Tour of SGC Facilities	V. L. Ruwet, Deputy Commanding Officer, USABC R. D. Housewright, Scientific Director, USABC B. Warshowsky A. M. Cowan J. C. Fletcher A. L. Antonio J. N. Brown E. Mishuck C. E. Hendrix J. K. Kuhn R. W. Tew K. H. Sweeny R. A. Gafford W. A. Perkins
Feb 26-28	Chicago, Ill.	8th Annual Biophysics Conference	R. E. Williams, Scientist, SGC
Feb 28	San Diego, Calif.	Discuss Evaluation Criteria on Task II Which Were Mailed to Fort Detrick on 2 March	J. C. Fletcher E. Mishuck R. W. Tew J. K. Kuhn L. T. Carleton
April 13-16	Dugway Proving Grounds, Utah	CBR Weapons Orientation	E. Mishuck

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

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<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
April 13-16	Dugway Proving Grounds Utah	CBR Weapons Orientation Course	E. Mishuck, Program Manager
May 3-7	Washington, D.C.	Meeting of American Society for Microbiology	R. W. Tew C. W. Molander
May 25	Space-General El Monte, Calif.	Review of Promising Detection Techniques	J. C. Fletcher F. W. Lehan E. Mishuck
June 1-4	Dugway Proving Grounds Utah	CBR Weapons Orientation	J. K. Kuhn, Systems Engineer
June 15	Airborne Instrument Laboratories Long Island, New York	Discuss Optical Instru- mentation Related to Detection Devices	H. P. Mansberg, Program Manager, A.I.L. E. Mishuck
June 16	Space-General El Monte, Calif.	Program Review	Fort Detrick B. Warshovsky L. Graf, Chief, Physical Detection Branch A. Cowan V. Munna J. E. Froehlich C. E. Roth, Jr., Vice-President and General Manager E. Mishuck Senior Program Personnel
June 19	Gelman Instrument Co. Chelsea	Discuss Preparation & Properties of Filter Tapes	K. H. Sweeny
June 22	Millipore Filter Corp. Bedford, Mass.	Review SGC's Requirements for Special Filter Tapes	K. H. Sweeny

Table 3-5 (Continued)

SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

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<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
June 23-25	San Diego, Calif.	Symposium for Biomedical Engineering	C. E. Hendrix
June 25 & 26	Fort Detrick Frederick, Md.	Discuss Enzyme Applications Relative to BW Detection	B. Warshowsky L. Graf R. Boyle H. A. Neufeld, Section Leader, Biochemistry, Fort Detrick L. Spero, Chief, Chemistry Branch, Fort Detrick S. Witz
June 30 & July 2	Fort Detrick Frederick, Md.	Review Progress & Problem Areas in Aerosol Sampling Research	C. R. Phillips B. Warshowsky L. Graf V. Munna K. H. Sweeny
July 6	Space-General El Monte, Calif.	Discuss A.I.L.'s Participation on the BW Detection Program	H. P. Mansberg E. Mishuck D. J. Sibbett, Scientist, Prospective Program Manager R. W. Tew
July 8	University of California	Discuss Microanalytical Techniques Related to BW Detection	B. Grunbaum, Consultant, University of California P. Kirk, Biochemist, Stanford University E. Mishuck R. W. Tew L. T. Carleton

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON HW DETECTION

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<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
July 9	Space-General El Monte, Calif.	Review Virus Detection Research	John Holland, Virologist, Univ. of Washington E. Mishuck R. W. Tew C. W. Molander
July 16	Stanford Medical School Palo Alto, Calif.	Discuss Nucleic Acid Reactions Relative to Detection of Microorganisms	P. Berg, Stanford Univ. D. J. Sibbett A. F. Wells, Research Biochemist
July 16	Space-General El Monte, Calif.	Review Aerosol Sampling Techniques	H. Aldinger, Douglas Aircraft, Santa Monica J. Finklestein, Douglas J. Cannady, Douglas W. Hartung, Douglas E. Mishuck K. H. Sweeny R. W. Tew P. H. Wright, Program Manager, FAST Development Program
July 21-25	Management Conference Rancho, Santa Fe, Calif.	Review of HW Detection Highlights	C. E. Roth, Jr.
July 24	Fort Detrick Frederick, Md.	Discuss Advanced Detection Requirements	B. Warshowsky J. K. Kuhn
July 28	Hyland Laboratories Los Angeles, Calif.	Review Hyland Subcontract Effort	F. Marquardt, Pres. Hyland Laboratories R. B. McKinley, Vice President, Finance & Administration, SGC E. Mishuck

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Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Aug 11	Space-General El Monte, Calif.	Discussion of Program Accomplishments & Problem Areas	F. W. Lehan E. Mishuck
Aug 14	Space-General El Monte, Calif.	Discuss Dr. Fletcher's Continued Participation on the BW Detection Program	J. C. Fletcher E. Mishuck
Aug 14	Space-General El Monte, Calif.	Review Proposal for Continuation of Research on BW Detection	C. E. Roth, Jr. R. B. McKinley R. N. MacCracken, Director of Contracts R. L. Henry, Manager of Contracts W. H. Ware, Manager, Contracts Administration E. Mishuck D. J. Sibbett
Aug 27	Space-General El Monte, Calif.	Review of BW Detection Programs	W. L. Jacobs, Chief, Program Coordinations Office, USABC C. E. Roth, Jr. E. Mishuck F. H. Wright Senior Program Personnel
Sept 1	Space-General El Monte, Calif.	Discussion of Program Accomplishments & Problem Areas	F. W. Lehan E. Mishuck

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Sept 1	Space-General El Monte, Calif.	Discussion of Program Accomplishments & Problem Areas	F. W. Lehan E. Mishuck
Sept 2	Space-General El Monte, Calif.	Review of FAST Development Program and Research on New Concepts and Principles	Fort Detrick A. Cowan I. Abelow, Chief, BW Detection Systems Office L. Graf V. Munna S. Janowitz SGC P. H. Wright E. Mishuck Senior Program Personnel
Sept 21	Fort Detrick Frederick, Md.	Follow-on Negotiations	Fort Detrick B. Warshowsky L. Graf C. Huston R. Boyle L. Sanders L. Gesell SGC E. Mishuck D. J. Sibbett R. W. Tew W. H. Ware L. G. Nieman

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Sept 25	Space-General El Monte, Calif.	Review Requirements for Pathogen Facility	R. Erlich, IIT Research Institute E. Mishuck R. W. Tew J. P. Kispersky, Safety Coordinator M. K. Madel, Biological Supervisor, FAST Development
Oct. 2, 1964	Space-General El Monte, Calif.	Review of Plans for Operation of Pathogenic Facilities	C. E. Roth, Jr. R. B. McKinley E. Mishuck Chemical & Biological Operations G. F. Binnings, Manager, Plant Services
Oct. 5	Space-General El Monte, Calif.	Revision of Pathogenic Facilities	C. E. Roth, Jr. E. Mishuck
Oct. 5 & 6	Space-General El Monte, Calif.	Discussion of Research on Virus Detection and Aerosol Collection	C. E. Roth, Jr. G. Middlebrook, Consultant E. Mishuck D. J. Sibbett, Program Manager L. T. Carleton R. W. Tew K. H. Sweeney C. W. Molander S. Witz

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Oct 12	Space-General El Monte, Calif.	Analysis of Beckman Instruments Plan of Performance	D. J. Sibbett L. G. Nieman, Subcontracts Control H. K. Bladow, Procurement R. Gafford W. Wimer, Contracts Division Beckman Instruments C. Neisser, Customer Relations, Beckman Instruments
Oct 13	University of California at Los Angeles	Review of Electrophoretic Separation Methods	A. Kolin, Consultant R. B. McKinley E. Mishuck
Oct 19	Space-General El Monte, Calif.	Analysis of Bacteriophage	A. Trakula, Physical Sciences Division, USABC E. Mishuck D. J. Sibbett R. W. Tew C. W. Molander
Oct 22	Space-General El Monte, Calif.	Review of Plans and Progress on New and Novel Concepts in BW Detection Contract	B. Warshowsky A. Pital L. Graf I. Abelow V. Muma C. Harp, Defense Systems Office, Commodity Management Division Edgewood Arsenal, Maryland E. Mishuck D. J. Sibbett BW Detection Research Program Staff

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Oct 28	University of Utah Salt Lake City, Utah	Discussion of Research Contract on Pathogenic Virus Detection	J. Fletcher, President, University of Utah L. P. Gebhardt, Chairman, Department of Microbiology Henry Eyring, Dean of the Graduate Faculties D. W. Hill, Asst. Professor, Department of Microbiology E. Mishuck R. W. Tew K. H. Sweeney
Oct 30	Space-General El Monte, Calif.	Review of Hyland Laboratories Planned Activities	F. Marquart T. Asher E. Mishuck D. J. Sibbett R. W. Tew
Nov 5	Space-General El Monte, Calif.	Review of Progress of Beckman Research on Thin-Film Electrophoresis	A. Strickler, Group Leader, Beckman Instruments D. J. Sibbett K. H. Sweeney L. T. Carleton
Nov 10	Space-General El Monte, Calif.	Analysis of Detection System Design	S. Golomb, Consultant, U. So. Calif. E. Mishuck D. J. Sibbett R. W. Tew L. T. Carleton

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT AND SPECIAL MEETINGS

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Nov 19	California Institute of Technology Pasadena, Calif.	Review of Research on Virus Detection and Bacteriophage Reactions	R. S. Edgar, Professor of Microbiology, Cal Tech D. J. Sibbett C. W. Molander
Dec 1	Space-General El Monte, Calif.	Discussion of Progress in BW Detection	V. Pratt, Test Engineering and Technical Division, Deseret Test Center, Salt Lake City E. Mishuck D. J. Sibbett R. W. Tew K. H. Sweeney L. T. Carleton
Dec 16	Metronics Associates Palo Alto, Calif.	Discussion of Aerosol and Collection Research	W. A. Perkins K. H. Sweeney
Dec 17	Space-General El Monte, Calif.	Biological Detection Research	E. Merrick, Life Detection Systems Branch, Exobiology Division, NASA's Ames Research Center E. Mishuck D. J. Sibbett R. W. Tew S. Witz
Jan 6 1965	Space-General El Monte, Calif.	Status Review, BW Detection Research	F. Lehan E. Mishuck D. J. Sibbett R. W. Tew L. T. Carleton K. H. Sweeney C. W. Molander S. Witz

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT AND SPECIAL MEETINGS

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Jan 7	Beckman Instruments Fullerton, Calif.	Progress in Thin-Film Electrophoresis	A. Strickler D. J. Sibbett L. T. Carleton K. H. Sweeny
Jan 11	Cal Tech Pasadena, Calif.	Progress in Hypersensitivity and Immunofluorescence	Dan Campbell, Consultant, Cal Tech Justine Garvey, Consultant, Cal Tech D. J. Sibbett R. W. Tew C. W. Molander
Jan 16	University of Utah Salt Lake City, Utah	Discussion of New Concepts in Virus Detection	J. C. Fletcher E. Mishuck L. Gebhardt
Jan 20	Stanford University Palo Alto, Calif.	Review of Detection Techniques for Group A Arboviruses	C. E. Schwerdt, Consultant, Stanford Univ. R. W. Tew
Jan 21	Space-General El Monte, Calif.	Progress in Virus Detection Methods Review	A. G. Marr E. Mishuck D. J. Sibbett R. W. Tew L. T. Carleton C. W. Molander S. Witz

Table 3-5 (Continued)

SUMMARY OF MANAGEMENT AND SPECIAL MEETINGS

<u>Date</u>	<u>Location</u>	<u>Purpose</u>	<u>Participants</u>
Jan 28	Space-General El Monte, Calif.	Review of Highlights of New Concept Program with Demonstrations	C. R. Phillips B. Warshowsky I. Abelow S. Yaverbaum, Bio Detection Branch, Physical Defense Division USABC V. Muma J. Selman, Directorate of Quality Assurance, MUCOM, Picatinney Arsenal A. G. Marr C. E. Roth, Jr. E. Mishuck D. J. Sibbett F. H. Wright L. T. Carleton R. W. Tew C. W. Molander S. Witz K. H. Sweeny Staff of BW Research Program
Jan 29	Space-General El Monte, Calif.	Brainstorming Session on Detection Research	B. Warshowsky I. Abelow S. Yaverbaum H. Eyring R. S. Edgar J. Garvey A. G. Marr (continued on next page)

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON EW DETECTION

Date	Location	Purpose	Participants
Feb 2	Space-General El Monte, Calif.	Review and Demonstration of New Developments	A. F. Rasmussen, Professor and Chairman, Department of Medical Microbiology and Immunology, UCIA, Consultant E. Mishuck D. J. Sibbett F. H. Wright R. W. Tew L. T. Carleton S. Witz K. H. Sweeney F. Delmore, Commanding General, Edgewood Arsenal V. Ruwet, Commanding Officer, USABC R. Housewright, Scientific Director, USABC B. Warshowsky I. Abelow A. L. Antonio C. E. Roth, Jr. E. Mishuck D. J. Sibbett Staff of EW Research Program
Feb 3 and 4	Space-General El Monte, Calif.	Review and Demonstration of Progress in EW Detection Research	B. Warshowsky I. Abelow A. Cowan, Technical Assistant to Scientific Directorate, USABC M. Guggenheim, Program Coordination Office, USABC G. Fleming, Chief, Defensive Systems Commodity Management, Edgewood Arsenal R. F. Franz, Jr., Chief, Defense Branch, Chem. and Biol. Office, Directorate of Special Weapons, OCRD, Dept. of Defense

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON HW DETECTION

Date	Location	Purpose	Participants
Feb 10	Space-General El Monte, Calif.	Briefing in HW Detection	V. E. Bowman, Physical Science Administrator, AMC A. Selman, Research and Engineering Directorate, MUCOM, Picatinney Arsenal S. Love, Deputy Director, Defensive Systems Division, Chemical Research and Development Laboratories, Edge- wood Arsenal, Maryland E. Mishuck D. J. Sibbett S. Witz R. W. Tew
Feb 15	USABC, Fort Detrick, Md.	Progress of Chemi- luminescence Research and Instrumentation	B. Warshowsky H. Neufeld L. Spero R. Boyle S. Witz
March 9	Space-General El Monte, Calif.	Status Review of Progress of Program	E. Mishuck D. J. Sibbett L. T. Carleton R. W. Tew K. H. Sweeny C. W. Molander S. Witz R. Pressman
March 10	Space-General El Monte, Calif.	Briefing on Progress in HW Detection Research	F. H. Lehan D. J. Sibbett

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

Date	Location	Purpose	Participants
March 10	Space-General El Monte, Calif.	Summary Review on Progress in Electrophoresis Research	D. J. Sibbett L. T. Carleton R. W. Tew K. H. Sweeney M. E. Concannon, Subcontracts Administration R. Gafford A. Strickler W. Wimer
March 18	Space-General El Monte, Calif.	Analysis of Operation of Chemiluminescence Detector	A. G. Marr E. Mishuck D. J. Sibbett L. T. Carleton S. Witz
March 26	U.C.L.A.	Discussion of Virus Detection	A. F. Pasmussen D. J. Sibbett
March 31	USABC, Fort Detrick, Md.	Summary of Progress and Plans in BW Detection Research Program	B. Warshowsky L. Graf R. Boyle C. Huston L. Sanders D. J. Sibbett R. W. Tew
April 30 May 1	Space-General El Monte, Calif.	Technical Discussion of Rapid Quantitation Problems in Virology	B. Warshowsky L. H. Graf C. E. Officer J. C. Fletcher, Chairman of Discussion M. A. Baluda, Department of Biology, City of Hope Medical Center D. H. Campbell, Department of Immunochemistry, CalTech

Table 3-5 (Continued)

SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

Date	Location	Purpose	Participants
			D. P. Durand, Department of Microbiology, University of Missouri
			R. S. Edgar, Department of Biology, CalTech
			J. S. Garvey, Department of Chemistry, CalTech
			D. W. Hill, Department of Microbiology University of Utah
			M. M. Jensen, Department of Medical Microbiology and Immunology, UCLA
			A. G. Marr, Department of Bacteriology, University of California (Davis)
			A. F. Rasmussen, Department of Medical Microbiology and Immunology, UCLA
			F. L. Schaffer, School of Public Health, University of California (Berkeley)
			J. Stevens, Department of Medical Microbiology and Immunology, UCLA
			J. Vinograd, Departments of Chemistry and Biology, CalTech
			J. E. Froehlich
			C. E. Roth, Jr.
			E. Mishuck
			D. J. Sibbett
			F. H. Wright
			L. T. Carleton
			C. W. Molander
			M. K. Nadel
			R. Pressman
			K. H. Sweeny
			R. W. Tev
			A. F. Wells
			S. Witz

Table 3-5 (Continued)

SUMMARY OF MANAGEMENT MEETINGS

RESEARCH PROGRAM ON HN DETECTION

Date	Location	Purpose	Participants
June 8	Space-General El Monte, Calif.	Review of Program Management	R. B. McKinley, Vice President, Finance and Administration A. P. Albrecht, Vice President, Asst. General Manager D. J. Sibbett
June 21, 22	Space-General El Monte, Calif.	Review of Virus Detection	G. Goldstein, Department of Micro- biology, University of Virginia E. Mishuck D. J. Sibbett R. W. Tew C. W. Molander R. Pressman L. T. Carleton S. Witz
June 26	Space-General El Monte, Calif.	Review of Virus Detection by Sialoresponsin Formation	S. Bogoch, Institute for Research on the Nervous System, Boston University D. J. Sibbett S. Witz M. K. Madel A. F. Wells Miss K. Benker
July 1, 2	Space-General El Monte, Calif.	Review of Progress in Chemiluminescence Detection	A. G. Marr D. J. Sibbett S. Witz L. T. Carleton
July 20-23	Space-General El Monte, Calif.	Review of Program and Demonstrations of Breadboards	B. Warsbowski L. B. Graf H. Neufeld M. Gordon C. A. Dixon

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON IW DETECTION

Date	Location	Purpose	Participants
Aug 2	Space-General El Monte, Calif.	Review of Instrumentation	E. Mishuck D. J. Sibbett L. T. Carleton R. W. Tew S. Witz C. W. Molander F. H. Lehan C. E. Roth, Jr. I. Stokes, Vice-President, Space-General D. J. Sibbett
Sept 7	Space-General El Monte, Calif.	Progress Review	L. F. Ayres, Adm. Assistant to W. E. Zisch F. H. Lehan C. E. Roth, Jr. J. E. Froehlich E. Mishuck F. H. Wright D. J. Sibbett F. H. Seubold
Sept 27, 28, 30	Space-General El Monte, Calif.	Review of Separation Methods	M. Bier, Research Prof. of Agricultural Biochemistry, University of Arizona D. J. Sibbett K. H. Sweeny L. T. Carleton C. W. Molander
Oct 1	Space-General El Monte, Calif.	Review of Applications of Ultrasonics to Biophysics	W. L. Nyborg, Prof. of Physics, University of Vermont D. J. Sibbett K. H. Sweeny L. T. Carleton S. Witz

Table 3-5 (Continued)
SUMMARY OF MANAGEMENT MEETINGS
RESEARCH PROGRAM ON BW DETECTION

Date	Location	Purpose	Participants
Oct 5	Space-General El Monte, Calif.	Review of Metronics Progress	W. A. Perkins E. Mishuck D. J. Sibbett L. T. Carleton K. H. Sweeney
Oct 20	Space-General El Monte, Calif.	Review of Program Progress	A. R. T. Denues E. Mishuck D. J. Sibbett L. T. Carleton

Section 4

FINANCIAL

The overall costs incurred under the Research Program on BW Detection have been maintained within the forecasted budget throughout the program, which extended from May 1963 through December 1965. Financial Chart No. 1 shows these expenditures. Financial Chart No. 2 summarizes the manpower expenditures in terms of professional and support personnel for all associated organizations during the same period.

CONTRACT DA 18-064-AMC-137(A)
RESEARCH ON NEW CONCEPTS AND PRINCIPLES IN BW DETECTION
ACTUAL EXPENDITURES
MAY 1963 THROUGH DECEMBER 1965
(Estimated Expenditures through December 1965 in Parenthesis)

Financial Chart No. 1

SPACE-GENERAL CORPORATION	TOTAL COSTS FOR PERIOD 5/15/63-9/30/63	10/1/63- 3/31/64	4/1/64- 9/30/64	10/1/64- 3/31/65	4/1/65- 9/30/65	10/1/65- 11/30/65	EST. COSTS 11/30/65- 12/31/65	TOTAL EST. COSTS Through 12/31/65
Direct Labor	38,041	58,100	66,781	62,426	70,755	19,284	(3,144)	320,531
Overhead**	45,884	74,478	90,633	85,449	99,022	26,612	(6,790)	428,890
Material	8,711	20,483	3,163	19,971	17,937	1,686	-	71,971
Travel	1,015	4,067	2,806	1,467	882	72	-	10,309
Subcontractors								
BECKMAN	83,358	75,680	46,633	37,925*	-	-	-	243,613
METRONICS	28,244	38,097	26,711	7,742	19,035	5,824	(7,323*)	132,698
HYLAND	7,609	29,483	18,894	13,001	7,999*	-	-	74,886
UCLA	-	3,565	8,734	-	-	-	-	12,299
UNIV. OF UTAH	-	-	-	1,000	10,861*	-	-	11,861
VARIAN	400	-	-	-	-	-	-	400
BIO-SCIENCE	1,000	-	-	-	-	-	-	1,000
INT. CHEM. & NUCLEAR	-	4,335	-	-	-	-	-	4,335
Consultants	4,765	2,961	2,666	2,116	7,301	937	-	20,746
Subtotal	219,024	311,249	267,053	231,117	233,412	64,118	(19,289)	1,335,229
G and A**	21,467	33,708	31,672	28,728	30,344	7,038	(2,561)	155,518
Total Cost	240,491	344,957	298,725	259,845	263,756	61,150	(21,820)	1,490,744
Cumulative	240,491	585,448	884,173	1,144,018	1,407,774	1,468,924	1,490,744	

* Estimated, prior to final audit.

** Overhead and G&A adjusted for actual rates experienced in FYs 1963 and 1964.

CONTRACT TOTALS

Task 1 \$ 205,856
Est. Task 2 \$1,490,744
Est. Total \$1,696,600

CONTRACT DA 18-064-AMC-137(A)
RESEARCH ON NEW CONCEPTS AND PRINCIPLES IN BW DETECTION
MANHOUR EXPENDITURES
MAY 1963 THROUGH DECEMBER 1965

TOTAL MANHOURS EXPENDED FOR PERIOD

	5/15/63 - 9/30/63		10/1/63 - 3/31/64		4/1/64 - 9/30/64		10/1/64 - 3/31/65		4/1/65 - 9/30/65	
	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*
SPACE-GENERAL CORPORATION	4,630	1,679	8,092	2,833	9,460	2,524	8,008	3,617	10,021	5,357
Cumulative	4,630	1,679	12,722	4,512	22,182	7,036	30,190	10,653	40,211	16,010
BECKMAN INSTRUMENTS	2,070	589	2,509	1,046	1,753	677	1,339	878	-	-
Cumulative	2,070	589	4,579	1,633	6,332	2,312	7,671	3,190	7,671	3,190
METRONICS ASSOCIATES	2,051	873	2,638	40	2,028	345	661	21	857	57
Cumulative	2,051	873	4,689	915	6,717	1,260	7,378	1,281	8,235	1,338
HYLAND LABORATORIES	565	310	1,901	542	1,129	330	- **	-	-	-
Cumulative	565	310	2,466	852	3,595	1,182	3,595	1,182	3,595	1,182
UNIV. OF CALIF. (at Los Angeles)	-	-	424	320	918	1,280	-	-	-	-
Cumulative	-	-	424	320	1,342	1,600	1,342	1,600	1,342	1,600
UNIV. OF UTAH	-	-	-	-	-	-	208	181	1,448	1,259
Cumulative	-	-	-	-	-	-	208	181	1,656	1,440
VARIAN ASSOCIATES	16	-	-	-	-	-	-	-	-	-
Cumulative	16	-	16	-	16	-	16	-	16	-
BIO-SCIENCE LABORATORIES	-	-	-	-	48	-	-	-	-	-
Cumulative	-	-	-	-	48	-	48	-	48	-
INT. CHEM. & NUCLEAR	-	-	86	66	-	-	-	-	-	-
Cumulative	-	-	86	66	86	66	86	66	86	66
MAGNA CORP. (Beckman Subcontract)	728	691	141	178	-	-	-	-	-	-
Cumulative	728	691	869	869	869	869	869	869	869	869
TOTALS	10,060	4,144	13,791	5,025	13,336	5,156	10,216	4,697	12,326	6,673
CUMULATIVE TOTALS	10,060	4,144	25,851	9,169	41,187	14,323	51,403	19,022	63,729	25,695

* Professional and Support hours,
** Hyland participation established in terms of
purchase of products at fixed price.
*** Estimated.

CONTRACT DA 18-064-AMC-137(A)
RESEARCH ON NEW CONCEPTS AND PRINCIPLES IN BW DETECTION
MANHOUR EXPENDITURES
MAY 1963 THROUGH DECEMBER 1965

Financial Chart No. 2

TOTAL MANHOURS EXPENDED FOR PERIOD

15/63 - 9/30/63		10/1/63 - 3/31/64		4/1/64 - 9/30/64		10/1/64 - 3/31/65		4/1/65 - 9/30/65		10/1/65 - 11/30/65		Estimated Expenditures 12/1/65 - 12/31/65		Totals	
P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*	P*	S*
4,630	1,679	8,092	2,833	9,460	2,524	8,008	3,617	10,021	5,357	2,547	1,005	593	254		
4,630	1,679	12,722	4,512	22,182	7,036	30,190	10,653	40,211	16,010	42,758	17,015	43,351	17,269	43,351	17,269
2,070	589	2,309	1,046	1,753	677	1,339	874	-	-	-	-	-	-		
2,070	589	4,579	1,635	6,332	2,312	7,671	3,190	7,671	3,190	7,671	3,190	7,671	3,190	7,671	3,190
2,051	875	2,638	40	2,028	345	661	21	857	57	215 ***	67	301	93		
2,051	875	4,689	915	6,717	1,260	7,378	1,281	8,235	1,338	8,450	1,405	8,751	1,498	8,751	1,498
565	310	1,901	542	1,129	330	- **	-	-	-	-	-	-	-		
565	310	2,466	852	3,595	1,182	3,595	1,182	3,595	1,182	3,595	1,182	3,595	1,182	3,595	1,182
-	-	424	320	918	1,280	-	-	-	-	-	-	-	-		
-	-	424	320	1,342	1,600	1,342	1,600	1,342	1,600	1,342	1,600	1,342	1,600	1,342	1,600
-	-	-	-	-	-	208	181	1,448	1,259	-	-	-	-		
-	-	-	-	-	-	208	181	1,656	1,440	1,656	1,440	1,656	1,440	1,656	1,440
16	-	-	-	-	-	-	-	-	-	-	-	-	-		
16	-	16	-	16	-	16	-	16	-	16	-	16	-	16	-
-	-	-	-	48	-	-	-	-	-	-	-	-	-		
-	-	-	-	48	-	48	-	48	-	48	-	48	-	48	-
-	-	86	66	-	-	-	-	-	-	-	-	-	-		
-	-	86	66	86	66	86	66	86	66	86	66	86	66	86	66
728	691	141	178	-	-	-	-	-	-	-	-	-	-		
728	691	869	869	869	869	869	869	869	869	869	869	869	869	869	869
10,060	4,144	15,791	5,025	15,336	5,156	10,216	4,697	12,326	6,673	2,762	1,072	894	347		
10,060	4,144	25,851	9,169	41,187	14,325	51,403	19,022	63,729	25,695	66,491	26,767	67,385	27,114	67,385	27,114

* Professional and Support hours,
** Myland participation established in terms of
purchase of products at fixed price.
*** Estimated.

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