Technical Report No. IRL 1158

# CYTOCHEMICAL STUDIES OF PLANETARY MICROORGANISMS EXPLORATIONS IN EXOBIOLOGY

Status Report Covering Period January 1, 1972 to December 31, 1972
For
National Aeronautics and Space Administration
Grant NGR-05-020-004


Instrumentation Research Laboratory, Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

Report to the National Aeronautics and Space Administration

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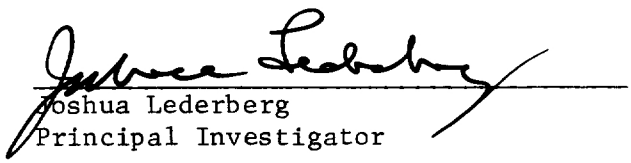
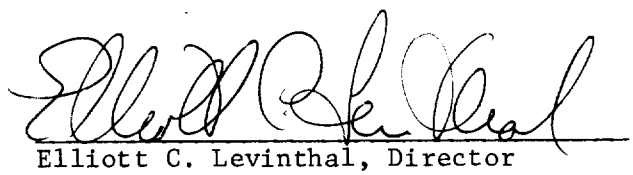


Joshua Lederberg
Principal Investigator



Elliott C. Levinthal, Director
Instrumentation Research Laboratory

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# A. INTRODUCTION

This report covers the activities of the Instrumentation Research Laboratory during the calendar year January 1, 1972 to December 31, 1972.

The main support of the IRL activities during this period continued to be the NASA grant NGR 05-020-004. Some funds have come from other grants, other agencies, and in some cases private institutions. This report includes all the activities of the laboratory which relate to or have benefited from NASA support regardless of whether or not they were primarily supported by this NASA grant.

The Dendral project receives direct support from NIH grant GM 00612 and indirectly from the ACME grant NIH RR 00311 and Professor Feigenbaum's ARPA grant SD-183.

Our work in the area of cell separation was supported in this period by NIH grant GM 17367.

The efforts on image processing for the 1971 Mars Mariner are a joint effort with the Artificial Intelligence Laboratory of the Computer Science Department under Professor John McCarthy. It receives support from NASA grant NGR-05-020-508 and JPL Contract 952489. Our work on Viking lander camera imagery receives support under Langley contract NAS 1-9682.

During the last six months of this report period we have had extensive discussions with the Manned Spacecraft Center at Houston on ways to exploit our experience in analytical methodology using gas chromatography and mass spectroscopy for the purpose of improved physiological monitoring of astronauts. In particular the work we have been doing on the analysis of the metabolic constituents of urine has led to a specific proposal. This proposal has been funded (NGR-05-020-632) for one year starting May 15, 1973.

We have continued to collaborate with Professor Marvin Chodorow of the Applied Physics Department on a preliminary evaluation of the application of microwave acoustic scattering to problems of cell discrimination and detection.

The general areas of the Program Resume, Part B of the report, are:

I. Chlorination of DNA Bases

II. Mass Fragmentography

III. Mass Spectrometry

IV. Urine Analysis

V. Analysis of Natural Products by Mass Spectrometry

VI. Computer Aided Research (DENDRAL)

VII. Cell Separation

VIII. Mariner Mars 1971 Orbiter Photography

IX. Viking Lander Imagery

## B. PROGRAM RESUME

### I. Chlorination of DNA Bases

The study of the action of aqueous hypochlorous acid under physiological conditions upon the bases present in DNA has been continued. Under these conditions thymine yields 5-chloro-6-hydroxy-5, 6-dihydrothymine while with one and two equivalents of reagent 6-methyluracil is converted into 5-chloro-6-methyluracil and 5,5-dichloro-6-hydroxy-5, 6-dihydro-6-methyluracil respectively. 1,3-Dimethyluracil reacts with both one and two equivalents of hypochlorous acid to form 5,5-dichloro-6-hydroxy-5,6-dihydro-1,3-dimethyluracil.

DNA bases containing the purine ring system, for instance guanine and adenine, react with hypochlorous acid to yield parabanic acid. Xanthine also yielded this product while the N-methylated purines caffeine and theophiline afforded N-methyl parabanic acid. Mechanistically these observations suggest that parabanic acid is derived from the six-membered ring of the purine system.

Our chlorination studies were extended to the nucleosides, cytidine and deoxycytidine and the nucleotide cytidine-5'-monophosphate. The products from all three compounds were the corresponding 4-N-chloro compound. This was determined from physical measurements including mass spectrometry and especially from their NMR spectra which

exhibited a characteristic 0.42-0.52 ppm diamagnetic shift for the chemical shift of the C-6 proton.

In addition the following papers on chlorination by hypochlorous acid have been either published or accepted for publication:

Chlorination Studies. I. The Reaction of Aqueous Hypochlorous Acid with Cytosine. By W. Patton, V. Bacon, A. M. Duffield, B. Halpern, Y. Hoyano, W. Pereira and J. Lederberg. Biochem. Biophys. Res. Commun., 48, 880 (1972).

Chlorination Studies. II. The Reaction of Aqueous Hypochlorous Acid with α-Amino Acids and Dipeptides. By. W. E. Pereira, Y. Hoyano, R. Summons, V. A. Bacon and A. M. Duffield. Biophys. Biochem. Acta (in press).

## II. Mass Fragmentography

Methods for the detection and quantitation of amino acids in varied environments by mass spectrometry is being pursued. As the approach used involves mass fragmentography we require the preparation of a suitable derivative which must enhance the mass spectral identification and in addition must possess suitable gas chromatographic properties. The derivative of choice for these purposes would appear to be the amino acid O-butyl ester, N-trifluoroacetate. Quantitation of the amino acid levels present in crude soil extracts has been achieved by the addition of an internal standard consisting of a known quantity of the deuterated amino acids to be analyzed. Using the quadrupole-mass spectrometer-computer system in the technique of mass fragmentography we have been able to simultaneously quantitate up to ten of the amino acids present in soil. The experimental details of this novel method await publication, see "The Simultaneous Quantitation of Ten Amino Acids in Soil Extracts by Mass Fragmentograph" by W. E. Pereira, Y. Hoyano, W. E. Reynolds, R. E. Summons and A. M. Duffield, Anal. Biochem., in press.

We have also extended this quantitative technique to the measurement of phenylalanine in plasma (i.e. phenylketonuria). See "The Determination of Phenylalanine in Serum by Mass Fragmentography" by W. E. Pereira, V. A. Bacon, Y. Hoyano, R. Summons and A. M. Duffield, Clinical Biochem., in press.

## III. Mass Spectrometry

Phenothiazines represent a class of drugs commonly prescribed in medicine as tranquilizers. Although the mass spectra of this group of compounds have been investigated in several laboratories no definitive study of their mass spectral decomposition processes using deuterium labeling has appeared in the literature. Suitable deuterated derivatives of promazine and promazine sulfoxide were prepared and from their mass spectra the types of rearrangements occurring in the mass spectral fragmentation processes of promazine and its sulphoxide were identified. See "A Study of the Electron Impact Fragmentation of Promazine Sulphoxide and Promazine using Specifically Dueterated Analogs," by M. D. Solomon, R. Summons, W. Pereira and A. M. Duffield, Austral. J. Chem., 26, 325 (1973).

## IV. Urine Analysis

Work is progressing on the organic chemical constituents of the urine of premature babies hospitalized in the Pediatrics Ward of the Stanford University Medical Center. Premature infants were selected for this study because they are on strict diets and their urinary metabolites should reflect body metabolites rather than food artifacts. Each urine specimen is processed for their free acid and amino acid constituents and these assays are repeated following hydrolysis of the urine. The complex series of compounds contained in each of the four fractions is separated by gas chromatography and the constituents of each chromatographic peak identified (where possible) by their mass spectra signatures. Currently our system can identify organic compounds present in derivatized extracts below the microgram level.

As an example of the application of GC-MS to biomedical problems we can cite preliminary studies on approximately 80 urine samples from a total of 11 premature or "small for gestational age" infants. This project was undertaken to investigate the phenomenon of late metabolic acidosis. This condition is characterized by low blood pH levels and poor weight gain and, as distinct from respiratory acidosis, occurs after the 2nd day of life. Its incidence is higher in infants whose birthweight is less than 1750 g (one study shows 92% incidence for these children) than in infants with birthweight greater than 1750 g (28%).

Of the 11 patients studied we were able to observe 6 closely and continuously for periods ranging from 6 to 8 weeks from day 3 of life. Three of these infants had birthweights below 1000 g and the other three were born weighing less than 1500 g. Of the 6, five showed symptoms corresponding to late metabolic acidosis and the other showed normal and even development. The five infants showing the acidosis all excreted very large amounts of p-hydroxyphenyllactic acid together with smaller amounts of p-hydroxyphenylpyruvic acid and p-hydroxyphenylacetic acid. After reaching a peak, the occurrence of these compounds in the urine gradually diminished and were almost completely absent at the time blood pH and weight gain had returned to normal. The infant who did not show symptoms of acidosis only excreted minute amounts of these compounds during the period of observation.

The occurrence of large amounts of these compounds in the urine indicates a temporary defect in phenylalanine - tyrosine metabolism and dietary factors such as protein and vitamin intake can be shown to affect the incidence and the severity of the condition. It is hoped that further studies will result in a clearer picture of relationships between the condition and diet and hence lead to a reduction in its occurrence.

V. Analysis of Natural Products by Mass Spectrometry

During the past year research has continued on the structural analysis by mass spectrometry of natural products isolated from plant, animal and marine sources. A list of publications resulting from this experimentation follows:


E. Ali, P. P. Gosh Dastidar, S. C. Pakrashi, L. J. Durham and A. M. Duffield. "Studies on Indian Medicine Plants - XXVII Sequiterpene Lactones of Enhydra Fluctuans Lour. Structures of Enhydrin, Fluctuanin and Fluctuadin." Tetrahedron 28, 2285 (1972).

A. M. Duffield and O. Buchardt. "Thermal Fragmentation of Quinoline and Isoquinoline N-Oxides in the Ion Source of a Mass Spectrometer." Acta Chem. Scand., 26, 2423 (1972).

A. N. H. Yeo and C. Djerassi. "Mass Spectrometry in Structural and Stereochemical Problems. CCX. Evidence for Transition States of Different Ring Sizes in the Loss of $C_4H_8$ from Phenyl n-Butyl Ether in the Mass Spectrometer." J. Am. Chem. Soc. 94, 482 (1972).

B. A. Brady, W. I. O'Sullivan and A. M. Duffield. "The Electron-Impact Promoted Fragmentation of Aurone Epoxides." Organic Mass Spectrometry 6, 199 (1972).

P. Sedmera, A. Klasek, A. M. Duffield and F. Santavy. "Pyrrolizidine Alkaloids. XIX. Structure of the Alkaloid Eruoifoline." Coll. Czech. Chem. Commun., 37, 4112 (1972).

P. Perros, J. P. Morizur, J. Kossanyi and A. M. Duffield. "Spectrometrie de Masse VIII. Elimination d'can Induite par Impact Electronique dans le Tetrahydro-1,2,3,4-Naphtal-ene-diol-1,2. Org. Mass Spectrom. 7, 357 (1973).

Y. M. Sheikh, R. J. Liedtke, A. M. Duffield and C. Djerassi. "Mass Spectrometry in Structural and Stereochemical Problems CCXVII. Electron Impact Promoted Fragmentation of O-Methyl Oximes of some $\alpha,\beta$-Unsaturated Ketones and Methyl Substituted Cyclohexanones." Canad. Jour. Chem. 50, 2776 (1972).

## VI. Dendral

### A. Computer Science

Recent Progress in the Dendral Artificial Intelligence Project is summarized in the following report.

## B. Computer Aided Research Instrumentation

### 1. Objectives and Background

The objectives of this work are to develop and demonstrate computer techniques for automating complex laboratory instrumentation and procedures. Within these objectives, three main areas of investigation are of interest including: 1) methodologies for computer modeling and control of instrument performance, 2) the integration of intelligent data analysis and interpretation programs into closed loop systems to optimize and economize problem solutions, and 3) the organization of computing and hardware resources needed to implement automated systems. We have chosen gas chromatography/mass spectrometry (GC/MS) as a specific environment in which to develop and test ideas. This choice is based on the importance of GC/MS to on-going research in medicine, biochemistry, and computer science described elsewhere in this report, as well as a genuine need for system automation because of severe data volume and analysis complexity problems.

### 2. Progress

Since the last report, we have made progress in developing aspects of computer-aided GC/MS instrumentation as summarized below.

a) MASS SPECTROMETER DATA SYSTEM AUTOMATION

Concentrating initially on the MAT-711 high resolution mass spectrometer, we have made progress toward a reliable, automated data acquisition and reduction system for scanned low and high resolution spectra. This system is largely failsafe and requires no operator support or intervention in the calculation procedures. Output and warnings to the operator are provided on a CRT adjacent to the mass spectrometer. The system contains many interactive features which permit the operator to examine selected features of the data at his leisure. The feedback currently provided to the operator to assist in instrument set-up and operation can just as well be routed to hardware control elements for these functions thereby allowing computer maintenance of optimum instrument performance.

Progress in this area is an integration of our efforts in hardware and software improvements:

HARDWARE - The basic system consists of the mass spectrometer interfaced to a PDP-11/20 computer for data acquisition, pre-filtering, and time buffering into the ACME time-shared 360/50. The more complex aspects of data reduction are done in the 360/50 since the PDP-11 has limited memory and arithmetic capabilities. New interfaces for mass spectrometer operation and control have been developed. The interfaces can handle (through an analog

multiplexer) several analog inputs and outputs which require that the PDP-11 computer be relatively near the mass spectrometer. We now have the capability for the following kinds of operation through the new interfaces.

i) Computer selection of digitization rate

ii) Computer selection of data path (interrupt mode or direct memory access (DMA)

iii) Direct memory access for faster operation in the data acquisition mode.

iv) Computer selection of analog input and output channels.

v) Sensing of several analog channels through a multiplexer (e.g., ion signal, total ion current).

vi) Magnet scan control. This control can be exercised manually or set by the computer. It controls both time of scan and flyback time. Coupled with selection of scan rate, any desired mass range can be scanned at any desired scan rate.

vii) The computer can monitor the mass spectrometer's mass marker output as additional information which will be used to effect calibration.

Another development has been a signal conditioner for

the ion signal which incorporates a box-type integrator to sum the ion signal between A/D converter readings. This modification makes successive intensity readings independent of each other because the integrator is reset after each reading. It also provides for low pass filtering the ion current signal with a bandwidth automatically adjusted correctly for different sampling rates and hence lessens intensity measurement uncertainties caused by external noises.

SOFTWARE - Automatic instrument calibration and data reduction programs have been developed to a high degree of sophistication. We can now accurately model the behavior of the MAT-711 mass spectrometer over a variety of scan rates and resolving powers. Our instrument diagnostic routines are depended upon by the spectrometer operator to indicate successful operation or to help point to instrument malfunctions or set-up errors. Some features of these programs are described below.

i) Data Acquisition. Programs have been written which permit acquisition of peak profile data at high data rates using the PDP-11 as an intermediate data filter and buffer store between the mass spectrometer and ACME. This allows data acquisition to proceed even under the time constraints of the time-sharing system. Storage of peak profiles rather than all data collected has greatly reduced the storage

requirements of the program and saves time as the background data (below threshold) are removed in real-time. An automatic thresholding program is in operation which statistically evaluates background noise and thresholds subsequent data accordingly. Amplifier drift can thus be compensated. We have developed some theoretical models of the data acquisition process which suggest that high data acquisition rates are not necessary to maintain the integrity of the data. Demonstration of this fact with actual data has helped relieve the burden of high data rates on the computer system, particularly as imposed by GC/MS operation, and permits more data reduction to be accomplished in real-time or alternatively reduces the required data acquisition computer capacity.

ii) Instrument Evaluation. A high resolution mass spectrometer operating in a dynamic scanning mode is a complex instrument and many things can go wrong which are difficult for the operator to detect in real-time. In order for the computer to assist in maintaining data quality, it must have a model of spectrometer operation on the basis of which data quality can be assessed and processing suitably adapted as well as instrument performance optimized. We have developed a program which monitors the state of the mass spectrometer. This preliminary program checks the following items:

1) Data acquisition parameters such as scan range and time constants, background threshold, a dynamic peak model to determine resolution and threshold acceptance levels for peak width and intensity, the number of peaks collected, and data storage utilization statistics.

2) Calibration of the mass/time relation to be used as a model for subsequent spectra, output of the mass range over which the scale is calibrated, calibration peaks missed, if any, and a graph of extrapolation error versus mass. Any irregularities in this output point to scan problems.

3) The dynamic resolution versus mass is determined and output as a graph. This allows the operator to adjust to more constant resolution over the mass range.

iii) Data Reduction. A program has been written which allows automatic reduction of high resolution data based on the results of the prior instrument evaluation data. Conversion of peak positions in time to the corresponding mass values is effected by mapping each spectrum into the calibration model developed previously. The interpolation algorithm between reference calibration points incorporates a quadratically varying exponential time constant to account for the second order character of a magnet discharging through a resistance and a capacitance. It also takes into account a mass offset at infinite time which affects

accuracy in determining low masses and which results from residual magnetization at the end of a scan.

Perfluorokerosene (PFK) peaks, introduced into high resolution mass spectra for internal mass calibration, are distinguished from unknown peaks by a pattern recognition algorithm which compares the relationships between sequences of reference peaks in the calibration run with the set of possible corresponding sequences in the sample run. The candidate sequence is selected which best approximates calibrated performance within constraints of internally consistent scan model variations. This approach minimizes the need for selection criteria such as greatest negative mass defect for reference peaks, the validity of which cannot be guaranteed. Excellent performance results from using sequences containing 10 reference peaks.

Unresolved peaks are separated by a new analytical algorithm, the operation of which is based on a calculated model peak derived from known singlet peaks rather than the assumption of a particular parametric shape (e.g., triangular, Gaussian, etc.) This algorithm provides an effective increase in system resolution by a factor of three thereby effectively increasing system sensitivity. By measuring and comparing successive moments of the sample and model peaks, a series of hypotheses are tested to establish the multiplicity of the peak, minimizing computing

requirements for the usually encountered simple peaks. Analytic expressions for the amplitudes and positions of component peaks have been derived in the doublet case in terms of the first four moments of the peak complex. This eliminates time consuming iteration procedures for this important multiplet case. Iteration is still required for more complex multiplets.

Elemental compositions are calculated from high resolution mass values with a new, efficient table look-up algorithm developed by Lederberg (ref. 1) and appended herewith.

Future work will extend these ideas to a system for the acquisition of selected metastable information as well as to include the quadrupole system used in the routine low resolution clinical work.

b) GAS CHROMATOGRAPHY/HIGH RESOLUTION MASS SPECTROMETRY

We have recently verified the feasibility of combined gas chromatography/ high resolution mass spectrometry (GC/HRMS). Using the programs described above we can acquire selected scans and reduce them automatically, although the procedures are slow compared to "real-time" due to the limitations of the time-shared ACME facility. We have recorded sufficient spectra of standard compounds to show that the system is performing well. A typical experiment

which illustrates some of the parameters involved was the following. A mixture (approximately 1 microgram/ component) of methyl palmitate and methyl stearate was analyzed by GC under conditions such that the GC peaks were well separated and of approximately 25 sec. duration. The mass spectrometer was scanned at a rate of 10.5 sec/decade, and a resolving power of 5000. The resulting mass spectra displayed peaks over a dynamic range of 100 to 1 and were automatically reduced to masses and elemental compositions without difficulty. Mass measurement accuracy appears to be 10 ppm over this dynamic range. A more definitive study of mass measurement accuracy will be carried out shortly to accurately determine the performance of the system.

We have begun to exercise the GC/HRMS system on urine fractions containing significant components whose structures have not been elucidated on the basis of low resolution spectra alone. Whereas more work is required to establish system performance capabilities, two things have become clear: 1) GC/HRMS will be a useful analytical adjunct to our low resolution GC/MS clinical studies to assist in the identification of significant components whose structures are not elucidated on the basis of low resolution spectra alone, and 2) the sensitivity of the present system limits analysis to relatively intense GC peaks. This sensitivity limitation is inherent in scanning instruments where one gives up a factor of 20-50 in sensitivity over photographic

image plane systems in return for on-line data read-out. This limitation may be relieved by using television read-out systems in conjunction with extended channeltron detector arrays as has been proposed by researchers at the Jet Propulsion Laboratory. The development of such a sensor system is beyond the current scope of our effort. We can nevertheless make progress in applying GC/HRMS techniques to accessible effluent peaks and can adapt the improved sensor capability when available.

Recent experiments in operation of the mass spectrometer in conjunction with the gas chromatograph have also shown that the present ACME computer facility cannot provide the rapid service required to acquire repetitive scans at either high or low resolving powers. We can, however, acquire scans on a periodic basis, meaning most GC peaks in a run can be scanned once at high resolving power. We are presently implementing a disk on the PDP-11 to act as a temporary data buffer between the mass spectrometer and ACME. This disk will allow acquisition of repetitive scans, while data reduction must be deferred to completion of the GC run.

c) AUTOMATED GC/MS DATA REDUCTION

The application of GC/MS techniques to clinical problems has made clear the need for automating the analysis of the results of a GC/MS experiment. Previous paragraphs

dealt with the problems of reducing raw data in preparation for analysis. At this point the data must be analyzed with a minimum of human interaction in terms of locating and identifying specific constituents of the GC effluent. The subsequent problem of identification is addressed by the library search and DENDRAL mass spectrum interpretation programs. The problem of locating effluent components in the GC/MS output involves extracting from the approximately 700 spectra collected during a GC run, the 50 or so representing components of the body fluid sample. The raw spectra are in part contaminated with background "column bleed" and in part composited with adjacent constituent spectra unresolved by the GC.

We have begun to develop a solution to this problem with very promising results. By using a unique disk oriented matrix transposition algorithm developed for image processing applications, we can rotate the entire array of 700 spectra by 500 mass samples per spectrum to gain convenient access to the "mass fragmentogram" form of the data. This form of the data, displayed at a few selected mass values, has been used at Stanford, MIT, and elsewhere for some time to evaluate the GC effluent profile as seen from these masses. Mass fragmentograms have the important property of displaying much higher resolution in localizing GC effluent constituents. Thus by transposing the raw data to the mass chromatogram domain we can systematically

analyze these data for baselines, peak positions, and amplitudes, and thus derive idealized mass spectra for the constituent materials free from background contamination and influences of adjacent GC peaks unresolved in the overall gas chromatogram. These spectra can then be analyzed by library search techniques or first principles as necessary.

The results of this work can also lead to reliable prescreening analysis of GC traces alone by having available a detailed list of GC effluent positions and expected amplitudes for say a urine fraction. By dynamically determining peak shape parameters for detected GC singlet peaks, interpretation of more complex peaks can be made to determine if unexpected constituents or abnormal amounts of expected constituents are present.

d) CLOSED-LOOP INSTRUMENT CONTROL

In the long term, it would be possible for the data interpretation software to direct the acquisition of data in order to remove ambiguities from interpretation procedures and to optimize system efficiency. The achievement of this goal is a long way off but we feel the above developments along with progress in the computer interpretation of mass spectra (DENDRAL) represent important preliminary steps toward closed-loop control.

The task of collecting different types of mass spectral

information (e.g., high resolution spectra, low ionizing voltage spectra and selected metastable information) under closed loop control during a GC/MS experiment is difficult and may not be realizable with current technology. We are studying this problem in a manner which will allow the system to be used for important research problems (e.g., routine analysis of urine fractions without fully closed loop control) while aspects of instrument control strategy are developed in an incremental fashion.

The essence of this approach is to develop a multi (two or three)-pass system which permits collection of one type of data (e.g., high resolution mass spectra) during the first GC/MS analysis. Processing of these data by DENDRAL will reveal what additional data are necessary on specific GC peaks during a subsequent GC/MS run to uniquely solve the structure or at least to reduce the number of candidate structures. This simulated closed-loop procedure will demonstrate the ability of DENDRAL type programs to examine data, determine solutions and propose additional strategies, but will not have the requirement of operating in real-time, although some parameters in the acquisition of metastable data will require change between consecutive GC peaks.

Studies such as these will identify in some detail the feasibility and necessity of closed-loop automation as well as the portions of the procedure which must be improved to

meet the time constraints imposed by limited sample quantities and GC/MS operation. We have already identified the problem of the rate at which resolution can be changed and have determined a potential solution. Additional problems under study are those of instrument sensitivity and strategies for metastable ion measurement.

## 3. Future Plans

Our future plans represent extensions of the on-going work described above under "PROGRESS" as well as the continued routine maintenance of the GC/MS systems. Specifics are briefly summarized below. A significant impact will occur with the termination of NIH support of the ACME computing facility in July 1973. We now perform most of our data reduction processing on the ACME 360/50. The follow-on facility to ACME will be an unsubsidized, fee-for-service facility mounted on a 370/158 computer along with other Stanford Hospital administrative computing functions.

We are in the process of implementing interim, stand-alone support for our GC/MS work on available PDP-11/20 machines. We have a number of proposals pending with NIH which would support longer term solutions, either through augmented stand-alone PDP-11 capabilities or through funds to operate on the fee-for-service 370/158.

### a) MASS SPECTROMETER DATA SYSTEM AUTOMATION

Future efforts will include the transition of the existing ACME-based system to a follow-on system, the character of which will depend on NIH funding approval for one of the alternatives. We will adapt previously developed concepts for use in the Finnigan low resolution GC/MS system being used for routine urine analysis. We will develop data system extensions for the MAT-711 system which allow semi-automated acquisition and reduction of metastable information to support fragmentation pathway studies, Heuristic DENDRAL program development, and closed-loop simulation. This metastable system will incorporate calibration procedures and automated peak detection and resolution procedures based on the high resolution system. The existing hardware interface will be used to control source or electrostatic analyzer voltages in conjunction with the magnet scan to measure specific parent-daughter ion relationships.

b) GAS CHROMATOGRAPHY/HIGH RESOLUTION MASS SPECTROMETRY

We will complete the intermediate disk buffer in conjunction with the follow-on computing system transition to allow routine collection and filing of sequential spectra. We will exercize the system on body fluid samples in support of our clinical applications and the development of interpretation programs. As developments occur which improve sensitivity, we will incorporate these to extend the

power of the system.

c) AUTOMATED GC/MS DATA REDUCTION

The approach described above is still in the formative stage. We will complete the development and implementation of these ideas, test them in the clinical application domain and produce an automated system suitable for routine use by the biochemist.

d) CLOSED-LOOP INSTRUMENT CONTROL

With the development of a more automated method for acquiring information on metastable peaks (under subtask (a) plans), we will develop and exercise the strategy planning aspects of the Heuristic DENDRAL programs in connection with managing a urine analysis GC/MS run. This will be a simulation of closed-loop operation intended to demonstrate the feasibility and need for an actual implementation of these ideas. In support of these closed-loop simulations we will investigate the feasibility of instrument mode switching and simple control function such as ion source and electrostatic analyzer potentials and magnet scan.

## REFERENCE

1) Lederberg, Joshua, "Rapid Calculation of Molecular Formulas from Mass Values," Journal of Chemical Education, Vol. 49, Page 613, September, 1972.

## VII. Cell Separation

While this work was initiated by the subject grant, most of the support is now coming from NIH Grant GM 17367. The work has obvious applications in the medical field as well as possible applications for exobiology.

### A. High Speed Fluorescent Cell Sorter

This unit is designed to measure the fluorescence of cells in a jet of liquid, break-up the jet into uniform drops and collect the drops in a series of containers, with all drops containing cells with similar fluorescent characteristics collected in the same container.

Our last annual report for the period ending December 31, 1971, described a multichannel cell separator which had just been completed and upon which testing was just commencing. The new instrument simultaneously measures fluorescence and scattering cross section of each cell. Both signals are used as sorting parameters. Much of our recent efforts have been devoted to evaluating, operating and improving this new instrument.

Installation of a second, more powerful laser (4 watts) has improved the sensitivity and made it possible to operate the new system completely independently of the old instrument which continues to be frequently used. Many modifications to the fluid and sample handling components

of the system have resulted in more reliable operation, accurate monitoring of sample flow rate, rapid flushing and sample changing, and fast recovery from nozzle blockages. The electronic signal processing logic has been replaced with improved circuitry that permits independent specification of scattering and fluorescence signal limits for each of two separated fractions. Detected cells that do not satisfy the criteria for sorting are positively isolated to the undeflected fraction, thus preventing contamination of the wanted cells. Purity of the separated fractions has also been improved by identifying pairs of cells too closely spaced to properly sort, and also isolating these cells to the undeflected fraction. These improvements also keep most empty droplets and much unwanted debris from either separated fraction. Cells are now processed at rates of several thousand per second with separated fraction purities routinely between 90% and 99%. Further refinement of the decision making circuitry is expected to increase both processing rate and fraction purity.

Programs for processing and plotting data from the two parameter 1024 channel pulse height analyzer have been written. A storage indicator and circuitry for displaying two-parameter "scatter plots" of cell parameters has been added to the system.

The minimum detectable number of molecules of rhodamine or fluorescein has been determined using radioassay techniques and conconavalin A (Con A), a protein from jack bean meal which binds to carbohydrate

residues on the cell surfaces. The Con A was labelled with both the fluor and with $I^{125}$. The $I^{125}$ count on cell suspensions was used to determine the number of Con A molecules, and thus of fluor molecules bound. The number of fluor molecules bound was reduced until it was just possible to detect a signal above background noise in the cell separator. This minimum number seemed to be less than 4000 for either fluor.

In most of our work fluors are conjugated with immunoglobulins. Considering that each immunoglobulin molecule will usually contain more than one fluor molecule, and that further amplification can be obtained using the immunofluorescence "sandwich" technique, it appeared that cells with on the order of a few hundred active sites on their surface should be separable under ideal conditions using this unit. This was confirmed by tests on thymocytes carrying a few hundred molecules of synthetic polypeptide, T, G, A-----L, labelled with $I^{125}$, as a marker for autoradiography, reacted with a fluorescent antibody to T, G, A-----L and separated. The practical separation limit is usually set by nonspecific adhesion of fluor-containing molecules to the cells.

A major effort has been devoted to achieving sufficiently asceptic operation that separated fractions can be cultured. This has involved basic redesign of the flow system and much more care in sample and machine treatment. The work has been successful and several cultures have been grown, indicating anew that cells are viable after passing

through the unit.

The scatter channel has proved more useful than anticipated. The signal it provided with various red blood cell samples was compared to that from a Coulter counter. The variation with volume appeared to be somewhat less than linear. However larger cells gave larger signals, and the resolution of neighboring signals was significantly better in the scatter channel than in the Coulter.

This equipment makes many desirable but previously impossible biological experiments not only possible but relatively easy. The following are among the many applications which have been made:

1. Rabbit lymphocytes bearing different membrane antibody light chain markers have been separated and shown to give rise preferentially to plasma cells bearing the same markers.

2. Spleen cells from mice immunized with two different antigens were incubated with one of the antigens after the latter had been made fluorescent. These fluorescent cells were then separated. The non-fluorescent portion retained all of its antibody titer to the second antigen but lost most of its titer to the first, indicating that the activity was on different cells.

3. Populations of different apparent sizes have been separated from both mouse spleen and mouse thymus lymphocyte suspensions. Preliminary indications are that the spleen lymphocytes giving

the smaller scatter signal are dead, but the smaller thymus fraction appears to be functionally different than the larger, or at least to react differently to hydroxycortisone treatment of the animal. Experiments in this area, and in those discussed below are continuing.

4. Electron micrographs of antigen reactive cells have been made showing the location of molecules of antigen on the cell surface.

5. Preliminary results on treatment of separated cells with mitogens like PHA (phytohemagglutinin) and PWM (Pokeweed mitogen) indicate these reagents can successfully stimulate division in T but not B cells. Interferon is also produced in T but not B cell cultures, in contradiction to earlier speculations that B-cells are also interferon producers.

6. A series of experiments was conducted to determine the minimum leakage of Rh+ fetal cells into an Rh- maternal circulation which could be detected. In these tests Rh+ antibody was added to suspensions of Rh- cells containing various proportions of Rh+ cells, and the suspension then treated with fluorescent goat antihuman gamma globulin. Results showed that Rh+ cells could be detected easily by the separator at dilutions of $10^{-5}$, and with care as low as $10^{-6}$.

7. Work has started on using the separator to fractionate fetal lymphocytes from maternal peripheral blood using HLA antigens as markers. Preliminary experiments indicate that a sandwich technique, in which the cells are treated with antiserum to the father's HLA antigens, then with fluoresceinated rabbit anti-human gamma globulin, and run through the separator should be able to provide considerable enrichment of the fetal cells. It may be possible eventually to use this technique to provide fetal cell cultures for use in prenatal diagnosis of chromosome abnormalities.

## VIII. Mariner Mars 1971 Orbiter Photography

The image processing work being carried out in cooperation with the Stanford Artificial Intelligence Project has resulted in about 500 image difference operations on MM'72 photos. Thirty-one images of the martian moons, Phobos and Deimos, were also processed to enhance their contrast and high frequency information. Late in the reporting period special emphasis was put on differencing images of the proposed landing sites for Viking 1975.

The image differencing operation which is described in detail in Technical Report IRL 1123 has represented our major interest. Candidate images for differencing are selected by ourselves and members of the MM-71 Image Team (primarily C. Sagan and J. Veverka). The necessary image data and navigation data is read from magnetic tapes supplied by Jet Propulsion Laboratory. The scientist requesting a particular set of image differences then has the opportunity to observe the work in progress and make decisions while the processing is being done. This interactive approach is possible through the availability of the Stanford AI time-sharing system operating on a PDP-10 computer and the associated image processing equipment.

Work has also been done in the area of image information management. An information retrieval capability has been implemented at the Stanford AI project which enables us to quickly review the planet coverage of the MM-71 TV Mission. It is primarily oriented toward revealing the extent

of repeated TV coverage of any area specified by latitude and longitude. It enables the user to quickly determine if an area has been photographed, and if so, how many times, on which orbits, by which camera, and by which pictures within an orbit. On a display screen is shown the disk of the planet, the footprint of the images, and vectors indicating view and sun angles (See Figure 1). Since more than seven thousand images exist, the need for such a system is obvious. This capability could prove quite useful in picture targeting and landing site refinement for the Viking mission.

It is important to note that this is an interactive system oriented towards the needs of the scientists. Its success depends on its ability to present data in a manner consistent in format and organization with the way the experimenters view the object under investigation.

The above mentioned capability actually represents the initial phase of the process for the projection and differencing operation. With the identifiers and footprints of all the images before the user the list can be pruned until just the footprints of interest are present. The user can then proceed directly to the projection and differencing steps.

The above capacity, when combined with a disc based storage system gives the scientist a significant degree of flexibility to review the image data and the processing carried out on it.

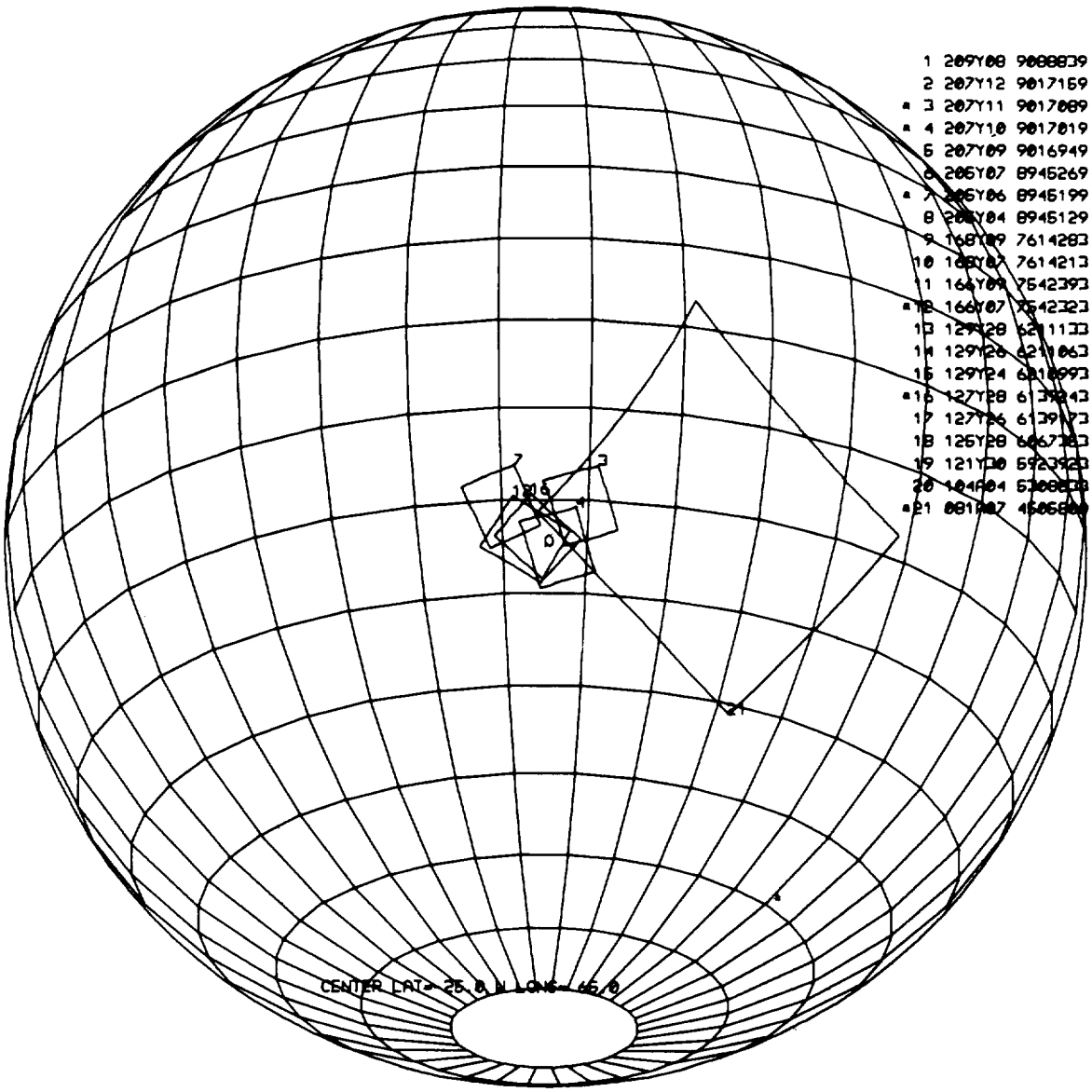
1 209Y08 9088839
2 207Y12 9017169
* 3 207Y11 9017089
* 4 207Y10 9017019
5 207Y09 9016949
6 206Y07 8945269
* 7 206Y06 8945199
8 206Y04 8945129
9 168Y09 7614283
10 168Y07 7614213
11 164Y09 7542393
*12 166Y07 7542323
13 129Y28 6211133
14 129Y26 6211063
15 129Y24 6210993
*16 127Y28 6139243
17 127Y26 6139173
18 125Y28 6067353
19 121Y30 5923923
20 104Y04 5100033
*21 081Y07 4506693
CENTER LAT= 25.0 N LONG= 65.0

Figure 1.

A total of forty-nine picture differences were made which covered the areas at or near nine of the ten candidate landing sites for the Viking 1975 mission. One such difference can be seen in Figure 2. This example shows the area around Viking Landing Site three. The upper left image was taken on orbit 168 and the upper right on orbit 209 forty days later. During this time significant clearing took place, the differences left minus right and right minus left are shown in the lower left and right respectively.

A portion of the Viking related work was presented at a Viking landing site selection meeting in November 1972.

Projects for the next reporting period include a comprehensive study of the albedo changes which occurred between the MM-6 and MM-7 period and the MM-9 mission, completing work on a catalog of enhanced Phobos and Deimos images and a limited number of additional MM-9 differencing.

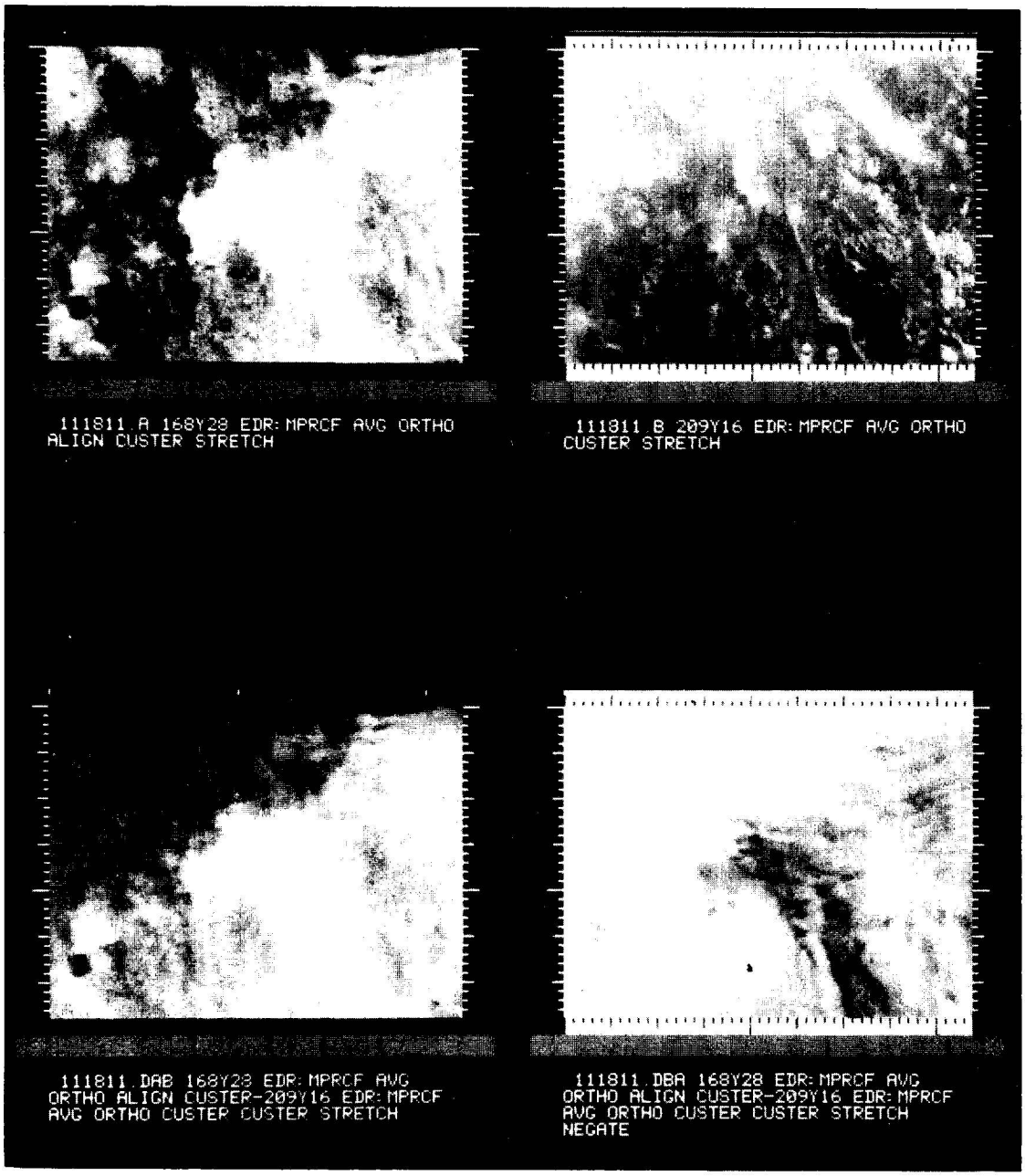
111811.A 168Y28 EDR: MPRCF AVG ORTHO ALIGN CUSTER STRETCH
111811.B 209Y16 EDR: MPRCF AVG ORTHO CUSTER STRETCH
111811.DAB 168Y28 EDR: MPRCF AVG ORTHO ALIGN CUSTER-209Y16 EDR: MPRCF AVG ORTHO CUSTER CUSTER STRETCH
111811.DBA 168Y28 EDR: MPRCF AVG ORTHO ALIGN CUSTER-209Y16 EDR: MPRCF AVG ORTHO CUSTER CUSTER STRETCH NEGATE

Figure 2

## IX. Viking Lander Imagery

During the last year, Drs. Levinthal and Liebes, as members of the Lander Camera Science Team have assumed team responsibility for the Science Operations Requirements Document (SORD), the Software Functional Descriptions (SFD's) derived from SORD and the more detailed Software Requirements Documents (SRD's) that, in turn, are derived from the SFD's. In addition they are part of the ad hoc Viking Image Processing System Steering Committee which is planning the hardware configuration to meet the requirements for the imaging science component of Mission Operations. All of the above activities, while separately supported by Langley, relate to, and benefit from previous efforts in connection with the Mariner Mission and some of the basic work supported by this grant.

## C. REPORTS AND PAPERS

This section lists reports and papers not referred to in preceding sections of the Program Resume.

### REPORTS


1. Annual Report for Period July 1, 1971 to June 30, 1972. JPL Contract 95289. Instrumentation Research Laboratory, Genetics Department, Stanford University. "Mariner Mars 1972". IRL Report 1143 (1972).


### PUBLICATIONS


1. A. M. Duffield, W. E. Reynolds, D. A. Anderson, R. A. Stillman, Jr., and C. E. Carroll. "Computer Recognition of Metastable Ions." Proc. Nineteenth Annual Conference on Mass Spectrometry and Allied Topics, D4, 63-67 (1971).

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3. W. A. Bonner, H. R. Hulett, R. G. Sweet and L. A. Herzenberg. "Fluorescence Activated Cell Sorting." Rev. Sci. Instr. 43, 404 (1972).

4. M. D. Solomon, W. E. Pereira, and A. M. Duffield. "The Determination of Cyclohexylamine in Aqueous Solutions of Sodium Cyclamate by Electron-Capture Gas Chromatography." Analytical Letters, 4(5), 301 (1971).

5. B. G. Buchanan, A. M. Duffield, A. V. Robertson. "An Application of Artificial Intelligence to the Interpretation of Mass Spectra." In "Mass Spectrometry: Techniques and Appliances" edited by George W. A. Milne, Published by John Wiley & Sons, Inc. 1971, pp. 121-178.

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10. A. Buchs, A. B. Delfino, C. Djerassi, A. M. Duffield, B. G. Buchanan, E. A. Feigenbaum, J. Lederberg, G. Schroll and G. L. Sutherland. "The Application of Artificial Intelligence in the Interpretation of Low Resolution Mass Spectra." Advances in Mass Spectrometry, Vol. 5 314 (1972).

11. E. Steed, W. E. Pereira, B. Halpern, M. D. Solomon and A. M. Duffield. "An Automated Gas Chromatographic Analysis of Phenylalanine in Serum." Clinical Biochemistry, in press.

12. V. Tortorella, G. Bettoni, B. Halpern, P. Crabbe. "Optical Properties of Dimedonyl Derivative of Aromatic Amines and Amino Acids." Tetrahedron 28, 2991 (1972).

13. E. C. Levinthal, W. B. Green, J. A. Cutts, E. D. Jahelka, R. A. Johansen, M. J. Sander, J. B. Seidman, A. T. Young and L. A. Soderblom. "Mariner 9 - Image Processing and Products." Icarus, in press.

14. T. A. Mutch, A. B. Binder, F. O. Huck, E. C. Levinthal, E. C. Morris, C. Sagan, and A. T. Young. "Imaging Experiment: The Viking Lander" Icarus, in press.

15. C. Sagan, J. Veverka, P. Fox, R. Dubisch, J. Lederberg, E. Levinthal, L. Quam, R. Tucker, J. B. Pollack, and B. Smith. "Variable Features on Mars: Preliminary Mariner 9 Television Results." Icarus, in press.