RFP TITLE:

BIOCHEMICAL MARKERS OR ENZYME CHANGES THAT MAY PRESAGE THE PRESENCE OF CANCER

RFP No.

NCI-CB-74-29 (PROJECT NO. CB-43902-S)

SUBMITTED BY:

THE BOARD OF TRUSTEES OF THE LELAND STANFORD, JR. UNIVERSITY

PROPOSAL SUMMARY AND DATA SHEET	RFP NO. NCI-CB-74-29

NAME OF OFFEROR

The Board of Trustees of the Leland Stanford Jr. University

PLACE OF PERFORMANCE *(City, County, and State)*

Stanford, Santa Clara County, California

TOTAL ESTIMATED TIME REQUIRED TO COMPLETE THE PROJECT	TOTAL ESTIMATED COSTS	1ST YEAR *(Detailed budget required)*	4TH YEAR
3 years		$70,808	
		2ND YEAR 76,130	5TH YEAR
		3RD YEAR 81,855	

TYPE OF CONTRACT PROPOSED

☒ COST-REIMBURSEMENT ☐ COST-SHARING ☐ COST-PLUS-FIXED-FEE ☐ FIXED-PRICE
☐ OTHER

NAME OF INDIVIDUAL(S) AUTHORIZED TO NEGOTIATE	TITLE	TELEPHONE NUMBER
Jon A. Erickson	Sponsored Projects Officer	415 321-2300 X2883
Robert D. Simmons	Contracts and Grants Manager	415 321-2300 X2883
Jason C. Mavis	Manager, Sponsored Projects Office	415 321-2300 X2883
NAME OF INDIVIDUAL(S) AUTHORIZED TO EXECUTE AND SIGN CONTRACTS	**TITLE**	**TELEPHONE NUMBER**
Jason C. Mavis	Manager, Sponsored Projects Office	415 321-2300 X2883
Robert D. Simmons	Contracts and Grants Manager	415 321-2300 X2883

NUMBER OF EMPLOYEES CURRENTLY EMPLOYED	DOLLAR VOLUME OF BUSINESS PER ANNUM
7,000	$60 Million

Acceptance Period - The offeror agrees to allow 120 days from the date of this proposal for acceptance thereof by the Government *(120 days, if not otherwise specified).*

NAME AND ADDRESS OF COGNIZANT GOVERNMENT AUDIT AGENCY

Defense Contract Audit Agency
400 Cambridge Ave., Suite 404
Palo Alto, Calif. 94306

NAME OF PRINCIPAL INVESTIGATOR	TELEPHONE NUMBER	ESTIMATED HOURS WEEKLY	SOCIAL SECURITY NUMBER
Prof. Joshua Lederberg	415-321-1200 X5801	two	[redacted]
NAME OF CO-INVESTIGATORS AND SENIOR SCIENTISTS	**TELEPHONE NUMBER**	**ESTIMATED HOURS WEEKLY**	**SOCIAL SECURITY NUMBER**
Dr. Alan Duffield	415-321-1200 X5239	twenty	[redacted]
Dr. Wilfred Pereira	415-321-1200 X5239	Twenty	[redacted]

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II. INTRODUCTION

The objectives of this work are to develop further the uses of gas-liquid chromatography (GC) and mass spectrometry (MS) instrumentation, under computer management, for the study of derangements of human biochemical metabolism due to neoplastic change. The efficacy of these analytical tools has been demonstrated when applied to the determination of the metabolic content of limited populations of urine samples in the research laboratory environment. We propose to enlarge the clinical investigative applications of GC/MS technology and to demonstrate its utility for the screening of urine samples from clinically diagnosed cancer patients with a view to identifying new metabolites, or altered levels of known metabolites, which might be diagnostic for cancer.

Specific goals include the applications of GC/MS analysis capabilities to large and more diversified populations to establish better defined norms, deviations, and control parameters necessary to relate GC/MS analysis results to identifiable disease states. Control urine samples will be obtained from healthy volunteers and from patients hospitalized for disease other than cancer. These will be matched by age, sex, smoking history, drug and alcohol habits and clinical judgments of general state of nutrition length of illness (disturbance of work, exercise, and weight), and exposure to radiation and to surgery. It will not be possible to control dietary and medicinal intake, but these factors will be monitored for their expected influence on urinary output. In addition samples will be taken from healthy relatives of patients to serve as controls and as possible sources of evidence for genetic-metabolic predispositions to specific forms of cancer.

As promising biochemical markers for cancer are recognized we will collaborate with clinicians in applying this methodology for the practical early detection of the types of cancer studied.

III. TECHNICAL

A. Objectives and Scope.

(1) Gas Chromatography/Mass Spectrometry Screening of Urine from Cancer Patients for the Identification of Metabolites Related to this Disease.

We plan to use gas chromatography/mass spectrometry (GC/MS)to screen urine from control subjects and individuals suffering from the following forms of cancer:

(a) prostatic cancer
(b) bladder cancer
(c) Hodgkin's Disease
(d) Various lymphomas

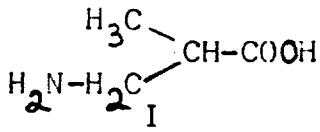
These samples will be obtained from the Clinical Research Center and the Urology Clinic of the Stanford University Medical

School through the cooperation of Dr. W. Fair (Associate Professor of Surgery, see attached letter of intent, Appendix A.)

As detailed in Section IIIC of this proposal these samples will be scrutinized to identify the metabolites present in the "acidic", "amino acid" and "carbohydrate" fractions using an adaptation of the procedure described by Jellum and his group (ref. 1). Comparison of the metabolic content of the urines from control and diseased subjects will then be made to detect any metabolites diagnostic for the various cancers studied.

(2) The Quantitation of Urinary Beta-Aminoiso-butyric Acid (BAIB) Levels from Cancer Patients.

During a pilot survey of the urinary metabolites excreted by children with diagnosed leukemias we observed several who excreted massive amounts (excess of 1 gram/24 hours) of BAIB (I). Others in our studied population excreted far smaller amounts of BAIB (milligrams/24 hours) and frequently other compounds were seen to co-elute which made it impossible to accurately quantitate for daily BAIB excretion. This experience led us to develop a method using mass fragmentography (see section IIIC (2)) for the quantitation of BAIB at the nanogram level (ref. 2; copy attached). Subsequently other investigators, using an amino acid analyzer reported on the significance of urinary BAIB excretion in cancer patients (ref. 3).



I

We will assay for BAIB those clinical samples from diagnosed cancers listed in section IIIA (1) together with samples received from Drs. T. Long and J. R. Wilbur (Children's Hospital, Stanford University; See letter of intent; Appendix B). In this latter instance we will monitor urinary BAIB levels of children as they receive treatment (chemotherapy and radiation) with a view of following BAIB excretion during the course of their disease. This approach might provide an insight into the efficacy of dose levels of various chemotherapeutic agents and radiation levels used to check leukemia. In addition a relationship will be sought between urinary BAIB levels and the activity of the disease state with special emphasis on predicting the first indications of relapse during clinical therapy. This objective would continue the work initiated in our pilot study (ref. 2).

(3) The Quantitation of Urinary Protein Amino Acid Levels in Cancer Patients

The literature contains reports of abnormal patterns of urinary excretion of protein amino acids by cancer patients. Thus ref. 4 describes marked differences (using paper chromatography) in the excretion of methionine, threonine, valine, leucine, tyrosine, histidine and aspartic acid between normal and leukemic patients. Another publication (ref 5) describes elevated urinary

methionine (using paper chromatography) in leukemic patients.

Quantitation by GC of the urinary amino acid levels is difficult because of the number and complexity (overlapping peaks) in the chromatogram. In addition gas chromatography is a non-specific method of detection (i.e. it detects a peak but it cannot identify what compound(s) are present under the peak envelope). Applying this laboratory's recently developed technique of quadrupole mass fragmentography (ref 6; copy attached to the quantitation of urinary protein amino acids will enable us to measure these compounds at the nanogram level (refs 6,7). We intend to measure amino acid levels in a statistically significant number of urines from normal and cancer patients. We will then search for subtle changes in the quantitative levels of amino acids between the normals and the various types (section IIIA (1)) of cancer investigated. Experimentally our computer programs will have to be slightly modified so that a single analysis will suffice for the quantitation of BAIB (section IIIA (2)) and the amino acids.

IIIB. BACKGROUND

The Instrumentation Research Laboratory was established in the Genetics Department under NASA auspices in 1961. Its task was to define and improve microanalytical methods for the detection of living processes that might be useful for the biological exploration of the planets. Many of the concepts that we explored have been embodied in NASA's planetary mission plans. However, we have not undertaken to design and build hardware for such missions. Instead, we have served as experienced advisors to the experiment teams responsible for scientific studies on the Mariner and Viking Mars programs. Our work on GC/MS is one of several lines of instrumentation effort.

Our original mandate from NASA included generous encouragement to seek health-related applications as a spin-off of the development work they were supporting. However, they have not been able to support the full fledged extension of space-related technology to disease research per se. The present application also comes at a time when overall funding for basic research by NASA is declining rapidly and may disappear within the next year. We have reduced our GC/MS laboratory staff in response to these cutbacks. It is therefore appropriate that we seek other support to help maintain this existing laboratory to apply its capabilities to problems of characaterizing metabolites and their levels associated with carcinomatous disease.

Our focus on mass spectrometry (ref 8) originally stemmed from the exquisite sensitivity, speed, and specificity of this technique for the identification of organic molecules. We have had some experience with instruments (like the Bendix Time-of-Flight Mass Spectrometer) which can generate a complete low resolution mass spectrum in 100 microseconds and whose sensitivity is limited by the statistics of the number of ionized fragments, and by the data handling problems of averaging

repetitive spectra emerging at a rate of 10,000 frames per second). We have also been led to look at the computational challenges of MS from another standpoint - namely the mechanization of the scientific thinking that is entailed by the interpretation of a mass spectrum. This task has been the focus of the research in "artificial intelligence" of the DENDRAL project. (See for instance ref. 9, 10.)

The application of these instruments to human metabolic research requires another dimension, namely the separation of complex mixtures, e.g., from body fluids, into individual components. These are then available for identification by the mass spectrometer. Gas chromatography has proven to be a useful companion to the mass spectrometer - the output gas stream can be fed directly to the inlet of the spectrometer and much of the carrier gas (helium) selectively deviated by a semi-permeable membrane. (Automated, continuous flow into a mass spectrometer of other chromatographic streams, e.g., high pressure liquid chromatography, is a speculation that may eventually materialize to great advantage but is not yet available for applications like ours).

For some time then we have been developing the means to integrate GC with MS under computer management. The present project represents the systematic application of these skills to the recognition and identification of metabolic variations, viewed as clinical problems of cancer in man. The sample populations will comprise healthy (control) and patients with clinically diagnosed forms of cancer who are under treatment in either of the (1) Clinical Research Center; (2) Urology Clinic and (3) Stanford Children's Hospital

The Techniques of MS and GC

The technique of mass spectrometry gives information about the structure of a molecular species by measuring the characteristic mass abundance pattern of fragments resulting from ionizing the parent molecule. Ionization is usually accomplished by electron bombardment. The compound under analysis must have a measurable vapor pressure at about 200 degrees C. (This temperature and a pressure of 0.01 microbars are the normal operating conditions of a GC-coupled mass spectrometer ion source). The ionizing electron beam (70 eV energy) removes one electron from some of the molecules of the sample vapor to yield excited positive molecular ions:

$$M + e \longrightarrow M(+) + 2e$$

The molecular ion, M(+), is generally unstable (especially if at a high energy of excitation) and may decompose within a few microseconds to yield a series of positively charged fragment ions. Each fragment ion can in turn decompose to ions of lesser mass. These ions are accelerated from the ion source of the mass spectrometer into the analyzer region where they are separated according to their mass-to-charge ratio. In sector instruments

this is done by a magnetic field, in quadrupole instruments by an electric field, and in time-of-flight instruments by an adjustable ion detection time delay. The mass spectrum of an organic compound thus consists of a table of positive ions of different masses and abundances. Molecular structure (for instance number and location of heteroatoms) determines the frequency with which bonds will rupture subsequent to ionization, thereby producing, for the most part, a characteristic mass spectrum for each compound. Geometrical isomers may show subtle differences within their respective mass spectra owing to the influence of the geometry of neighboring groups. Optical enantiomers yield identical spectra.

Although the technique of mass spectrometry was extensively used by petroleum chemists from the 1940's, it was not widely utilized in organic chemistry until the late 1950's. The first extensive monograph on biochemical applications of mass spectrometry has just appeared (ref 11). Our colleagues and close collaborators in the Stanford Chemistry Department, led by Professor Carl Djerassi, have been among the pioneers in the development of MS for natural product chemistry, especially as applied to steroids (4 books and in excess of 200 papers on various aspects of the theory and application of MS have been published by Prof. Djerassi's group since 1961). During the 1960's, mass spectrometry was applied to many different types of organic compounds. The accumulation of these reference mass spectra was necessary to establish fragmentation rules for the interpretation of unknown mass spectra. The experienced mass spectroscopist becomes adept at recognizing the mass spectral signatures of those types of compounds with which he works but he cannot encompass within his memory all the relevant information contained in the literature. In addition, many reference spectra determined by mass spectrometry laboratories have not been published. To overcome this problem, libraries of mass spectra are being compiled for computer storage and retrieval so that they will eventually be available for matching by computer against the mass spectra of unknown compounds. Progress is now being made toward compiling libraries of mass spectra relevant to general metabolic studies. These will match the accumulation already available for special classes of organic molecules and for some drugs whose spectra are important for emergency toxicological analyses (ref 12).

Instrumentation advances in mass spectrometry during the past decade like improved sensitivity, direct coupling with GC and the use of computers for the routine recording and presentation of mass spectra, all facilitate the large scale application of mass spectrometry to biomedical problems.

Body fluids and other materials encountered in biomedical research are complex mixtures. For example, urine is known to contain several hundred organic compounds at levels exceeding on the order of 1 nanogram per milliliter. The gas chromatograph is indispensable for the separation of such mixtures into discrete compontents. With medium resolution instruments, the mass

spectrometer can be scanned repeatedly once every 2-4 seconds. The gas chromatographic separation of a urine mixture may require 40-50 minutes: the result is the accumulation of over 600 mass spectra per analysis. The simplest way to identify these mass spectra is to search a library of known compounds. Even if the mass spectrum of a test compound does not reside in the library, the best match found may be a related compound. This can facilitate the manual interpretation based on the chemist's knowledge of and guesses about the rules of fragmentation. The problem of computerizing the identification of compounds whose mass spectra are not in a library is addressed by the DENDRAL project (refs 9, 10). Computer programs have been developed to interpret mass spectra of unknown compounds from first principles (i.e., to emulate the reasoning processes of organic chemists).

Frequently compounds of biochemical interest occur in small amounts in biological fluids. (By definition, many frontier problems concern compounds at the limit of easy detection by existing techniques). Thus, the effectiveness of GC/MS as a detector of biological materials is directly related to its sensitivity. Current systems routinely operate with sensitivities such that mixture components with as little as 50-300 nanograms of material can be measured. This limitation is imposed by the following instrument-related factors. In order to record an interpretable mass spectrum, a low resolution mass spectrometer must have input to its source on the order of 5 nanograms of material per second. Since a gas chromatographic peak lasts for approximately 5 to 30 seconds, in GC/MS operation some 25-150 nanograms per GC peak are required for mass spectral analysis. Inherent in the gas chromatographic column and in the semi-permeable membrane separator (used to preferentially remove the helium carrier gas from the effluent stream) are losses of up to 50% which increase the input sample requirements in a practical sense to about 50-300 nanograms of material per GC peak analysis.

Another limiting factor in the application of the GC/MS technique to biological extracts concerns the volatility of the material to be assayed. Before the system can detect many non-volatile components (e.g., carbohydrates, amino acids, etc.), they must be converted into volatile derivatives which will pass through the gas chromatograph at a maximum oven temperature of 300 degrees C. Above this temperature, column bleed from the gas chromatographic phase will tend to enter the ion source and complicate the recorded spectra. Thus the GC/MS technique is restricted to those organic compounds which can be converted to volatile derivatives and is not, in general, applicable to inorganic compounds. A recent report (ref 13) describes the analysis by GC/MS of ketose diphosphates (as their trimethylsilyl (TMS) derivatives) while aldose diphosphates (also as their TMS derivatives) proved to be too unstable to analyze. It is safe to assert, however, based on our own experience and the literature, that a broad spectrum of organic compounds of biological significance will be amenable to analysis by GC/MS methods.

The routine screening of normal and abnormal body metabolites, including drugs, in human body fluids (ref 14) is currently the object of several research programs. Various non-specific methods, including thin layer (ref 15,16), ion exchange (ref 17,18), liquid (ref 19), and gas chromatography (ref 20-23 and 24), are used primarily with the goal of separating a large number of unnamed constituent materials. Using these techniques, compound "identification" is made by a comparison of the migration, under identical conditions, of the unknown spot with reference compounds. This approach can lead to erroneous identifications, however. This point is illustrated by a recent article (ref 25) which describes the use of mass spectrometry in the identification of a case of isovaleric acidemia. Previously, the same patient was diagnosed as having butyric and hexanoic acidemia on the basis of chromatographic evidence alone. This type of error is especially important when analyzing a "new" (previously undescribed) metabolite where rigorous identification in various body fluids and tissues is essential.

For positive identification using mass spectrometry, the separated components must be transferred from the chromatographic medium to the mass spectrometer. The unknown spot can be leached from paper or thin layer chromatograms, or in the case of liquid chromatography, the solvent removed, and a mass spectrum recorded on the residual material. Trapping the various effluent components from a gas chromatograph, with subsequent introduction into the mass spectrometer, has been used. This approach requires considerable time and is inefficient when applied to complex separations. It has been superseded by the direct coupling of the gas chromatograph and mass spectrometer.

Gas chromatography is unquestionably the most convenient separation technique to couple to the mass spectrometer because the carrier gas can be removed efficiently and easily as the analysis proceeds. For recent examples of the use of the GC/MS technique for the analysis of body fluids see refs 1,25-27. Based on the work cited, as well as our own on-going programs, the ability of the GC/MS technique for the analysis of body fluids is well established. We have drawn upon the published literature in helping to design our experimental protocols (ref 1).

III. B (2) MASS FRAGMENTOGRAPHY

There is another mode of operation of a GC/MS system which enables greater sensitivities to be attained for the quantitation of KNOWN metabolites. If the mass spectrum and the gas chromatographic retention time of the compound to be quantitated are known, the mass spectrometer can be used as a specific detecting system for this compound. This technique is called mass fragmentography. Under these experimental conditions, the mass spectrometer is not scanned over the ENTIRE mass range but is directed to measure one or two SPECIFIC masses known to be characteristic of the compound(s) being quantitated. Consequently, there is an appreciable increase in sensitivity

since the mass spectrometer samples only the significant data points and can integrate the signal longer.

In this mode, existing GC/MS instrumentation matches the new fluorescent reagents for amines (reported to detect approximately 10 picomoles). It also embodies the specificity of the mass spectrum at individual mass numbers. At greater cost and cumbersomeness, the MS can be extended to a quantum-counting range of sensitivity. These methods are therefore likely to be complementary to the special purpose methods, like fluorescimetry, which are often cheaper and more efficient for well defined classes of compounds. On the other hand, the history of pesticide analysis shows how the GC can also be made ultrasensitive at the cost of some loss of specificity.

Using deuterated analogs as standards for the test compound, quantitation can also be achieved at sub-nanogram levels. We have recently exploited certain characteristics of the quadrupole mass spectrometer and its data system to develop a method for the quantitation of ten amino acids in soil extracts (ref 7, copy attached) and subsequently for the amino acid content of biological fluids (ref 6). This represents an advance in the technique of mass fragmentography since the sector mass spectrometers used up to this time, have been severely limited in the number of ions and the mass range they could monitor for any one experiment. Our technique of quadrupole mass fragmentography was used for the quantitation of the amino acids in the urine of a patient with suspected branch chain amino aciduria (see ref. 6).

As an example of the application of these methods to biomedical problems, we can use some recent studies we have undertaken on the urine of a patient suffering from acute lymphoblastic leukemia. The gas chromatographic profile of the amino acid fraction of his urine showed the presence of an abnormal peak . The mass spectral analysis of this chromatographic peak identified this component as beta-amino isobutyric acid (BAIB) from a comparison with a literature spectrum of authentic m material (ref 28). The literature on human urinary BAIB excretion is extensive and it has been reviewed in reference 29. Awapara (ref 30) noted increased urinary BAIB excretion from patients receiving nitrogen mustard therapy while Rubini (ref 31) reported high levels of BAIB excretion in people exposed to excessive radiation levels. Wright and Fink (ref 32) found elevated urinary BAIB levels from mongoloid children and non-mongoloid mental defectives but these conclusions were subsequently disputed (ref 33). Harris in England (ref 34) and Calchi-Novata in Italy (ref 35) found that 6-10% of a sample population had elevated urine concentrations of BAIB while DeGrouchy and Sutton (ref 36) in a study of families of oriental extraction, and Yani, et al. (ref 37) with Japanese families, found about 40% of their subjects excreted elevated levels of BAIB. Gartler concluded that the variation underlying BAIB excretion by New York families (ref 38), Apache Indians and Black Caribs of British Honduras (ref 39) was due to genetic

difference at one locus. Later studies showed that thymine was probably a precursor of BAIB in man (ref 40).

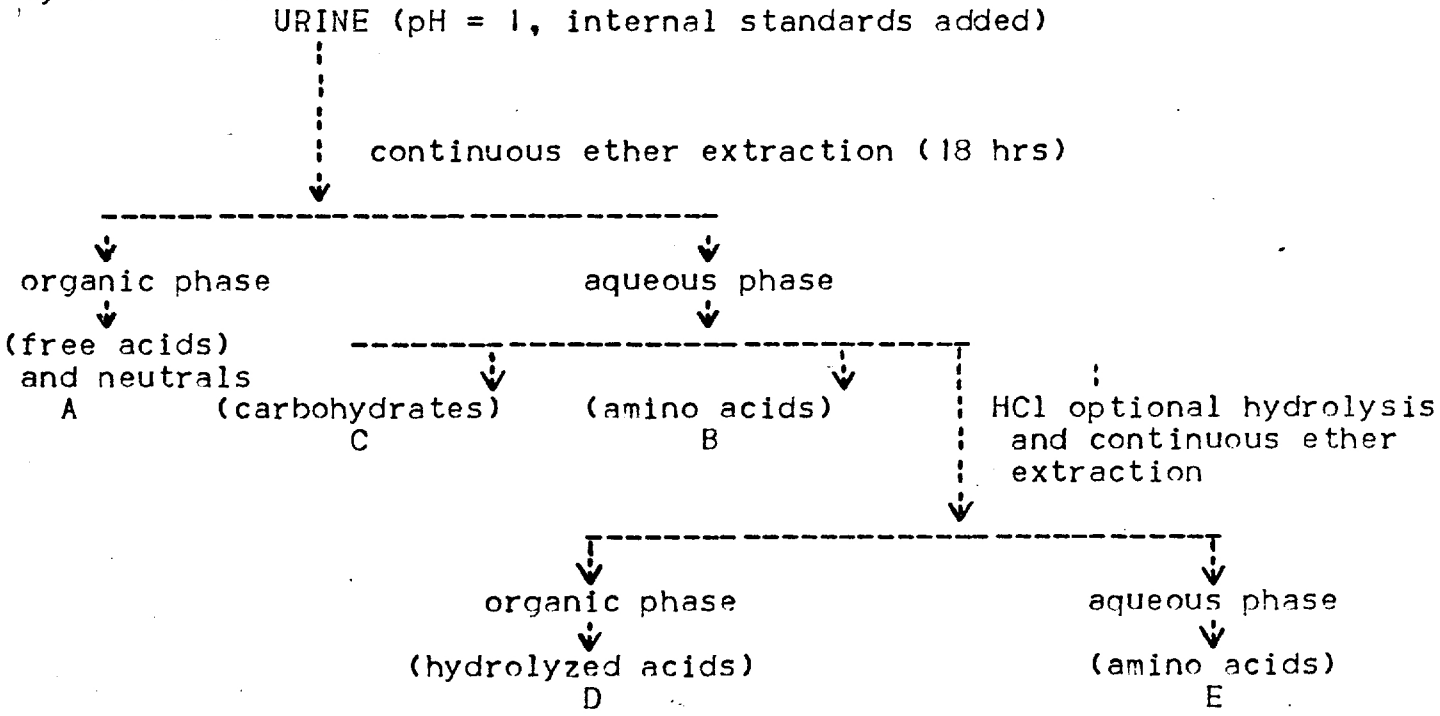
At least two authors (refs 29 amd '37) have questioned the accuracy of the methods used for the detection and quantitation of BAIB in urine. The early work (refs 36 and 41), including the first isolation of BAIB from urine (ref 34a), was completed using paper chromatography and reaction with ninhydrin to develop the appropriate spot for detection and quantitation. As suggested (refs 29 and 37) other compounds in urine migrate with characteristics similar to BAIB thereby introducing an element of imprecision into the published quantitation results. In those instances where BAIB is present in only a small amount, or for that matter in large amounts, it is always possible that another metabolite could be present within the confines of the "BAIB spot". Subsequent methods employed for BAIB quantitation include thin layer chromatography (ref 42) and paper electrophoresis (ref 43). These methods must also be considered as non-specific for the estimation of BAIB in urine. Our own procedure, must be considered far superior to these other techniques for both the detection and quantitation of BAIB in urine because it combines the quantitative aspects of gas chromatography with the positive identification of a specific ion detector. We have observed several instances in which the gas chromatographic peak associated with BAIB was shown by mass spectrometry to contain other components. Using the technique of mass fragmentography (refs 6 and 7) (copies attached), we can quantitate for BAIB under these conditions. In this technique, a characteristic fragment ion from the mass spectrum of BAIB is summed over the total ion chromatogram to give an area corresponding to the amount of BAIB present. Using an internal standard and a characteristic ion from its mass spectrum and knowing the ionization efficiency of both BAIB and the internal standard, quantitation can be achieved. For a description of this work see ref 2 (copy attached).

In a pilot program we have examined urine samples from over 30 patients suffering from leukemia and have observed various levels of BAIB in over 50% of these individuals. One patient was excreting 1.2 grams of BAIB per day and immediately following drug therapy this compound could no longer be detected nor was it detected in a urine sample collected 3 months later. Awapara (ref 30) claimed that all leukemic patients excrete varying amounts of BAIB which was not detected in normal urine. Killmann, et al. (ref 44) noted that two out of three patients with chronic granulocytic leukemia produced high levels of BAIB but following drug therapy, the level returned to normal. Lee, et al. (ref 45) examined the urine of 33 patients with leukemia receiving specific drug therapy and failed to observe BAIB excretion in any of these subjects. Our own observations, in agreement with the literature (refs 44-46), suggest that during the disease cycle, leukemic patients may excrete large amounts of BAIB but this ceases following chemotherapy. Schreier (ref 41) studied the urinary excretion of BAIB in leukemic subjects and noted extremely variable results with "massive amounts" of BAIB present

in the terminal stages of the disease.

III. C. EXPERIMENTAL

We use the following procedures to fractionate urine, blood, and amniotic fluid in preparation for their analysis by GC/MS. In the case of plasma, the protein is first precipitated (by the addition of ethanol) and the supernatant liquid dried IN VACUO and processed as for urine. Amniotic fluid is treated in the same way as urine.



The experimental procedure used for working with a fluid sample is as follows. To an aliquot (2.5 ml.) of fluid is added 6N hydrochloric acid until the pH is 1. Internal standards, n-tetracosane, 2-amino octanoic acid and trehalose are then added. Continuous ether extraction isolates the free acids (fraction A) which are then methylated and analyzed by GC/MS. An aliquot of the aqueous phase (1.0 ml) is passed through an ion exchange resin (Dowex 50 H+) and the column washed with water (15 ml) to elute the carbohydrate fraction (C). This column is then washed with 3N aqueous ammonia to elute the amino acids and any basic compounds present (fraction B). Fractions B and C are then concentrated to dryness and B derivatized by reaction with n-butanol/hydrochloric acid followed by methylene chloride containing trifluoroacetic anhydride. This procedure derivatizes any amino acids (or water soluble amines) which are then subjected to GC/MS analysis (fraction B). The carbohydrate fraction (C) is then derivatized by reaction at room temperature with Tri-Sil-Z.

As an additional option, one can hydrolyse portions of the urine to free those metabolites present in conjugated form, and

this is accomplished as follows: Concentrated hydrochloric acid (0.15 ml) is added to the urine (1.5 ml) after ether extraction and the mixture hydrolyzed for 4 hours under reflux. Ethyl acetate extraction then separates the hydrolyzed acid fraction (D) which is methylated and analyzed by GC/MS. A portion of the aqueous phase (0.5 ml), from hydrolysis of the urine, is concentrated to dryness and derivatized and analyzed for amino acids (Fraction E). We do not propose to hydrolyze all the urine samples screened but rather to limit this step to those samples having an indication of the presence of unusual metabolites. In these instances it will be of interest to hydrolyze te urine to determine whether the metabolite of interest is also present in conjugated form.

The above scheme represents a general method for preparing body fluids for analysis. The conditions required for gas chromatographic separation vary with different classes of compounds. For instance, using our method for chromatographing derivatized free acids, amino acids, and carbohydrates, steroids will not be detected . Large molecular weight compounds (e.g., tripeptides and mucopolysaccharide fragments) cannot be analyzed by this system without their degradation to smaller unit molecules. Also small neutral molecules (the so called "urinary volatiles") will be mostly lost during our isolation techniques.

A suitable computer data system can significantly ease the tedious burden of analyzing the large amounts of data emanating from GC/MS instrumentation. The computer assists in instrument set-up and calibration, data collection and filing, and data reduction and analysis. We have developed considerable experience with such data systems, both for low and high resolution instruments, since our first design was described in 1966 (ref 47, copy attached). Our most recent efforts have focussed on facilitating user interaction with the data system on automating various aspects of data analysis (ref 8) and on establishing a library search system for the automatic identification of metabolites from their recorded mass spectra.

At the end of July 1973 the NIH financial support ceased for the computer facility (ACME system of the Stanford University Medical School) to which our mass spectrometer was interfaced. At this time we commenced using a PDP-11/20 based mini-computer system (16K of core) and containing the following peripherals: disk storage (256K words), DEC tape drive for the recording of mass spectra, a line printer and a floating head disk (1.2 M words) for the support of a library of mass spectra and the associated search routines.

This system has the capacity to record on DEC tape those mass spectra recorded during the lifetime (approximately 40 minutes in our procedure) of a GC/MS analysis of one fraction from a urine sample. This system also operates the mass spectrometer in the mass fragmentography analytical procedure. The automatic data reduction phase of our previous system (ref. 6; copy attached) is currently being coded to operate on the PDP

11/20 system. It is anticipated that the recoding of these programs will be completed by April 1, 1974. During the lifetime of this proposal, effort will be applied to improving the routines previously used. To this effect we have budgeted 20% time for an experienced programmer to accomplish this task and to maintain existing software.

Our object in screening urine samples is to identify as broad a suite as possible of those metabolites present. This results in a relatively low daily throughput of samples but this is balanced by the detailed scrutiny each fraction receives. For instance our procedure fractionates urine into the following general metabolic classes:

(i) free acids and neutrals (isolated by solvent extraction)

(ii) amino acids and bases

(iii) sugars.

A GC/MS analysis after appropriate derivatization of one of these fractions occupies about 40 minutes but with the time required for preparation of the analytical conditions this relates to a sample through put of one per hour. In devoting 50% time to the screening of urine an organic chemist would have a throughput of one urine sample per day (time is required to plot back data stored in the data system and for the interpretation of the results). Some additional efficiency would be generated by limiting the GC/MS analysis to those urinary fractions displaying anomalous peaks after a pre-screening analysis through an existing dual column gas chromtograph. The incresed efficiency might allow between 5 and 7 urine samples to be analyzed per week with each analysis consisting of the three metabolic classes shown at the bottom of the previous paragraph. This method for the screening of urine allows a broad range of significnt compounds to be analyzed but only quantitative estimates of their concentration (use of internal standards) to be presented. More precise quantitation will be obtained from GC using the relative FID sensitivities of the unknown and internal standard (FID = flame ionization detector). This should be contrasted to the situation pertaining to mass fragmentography where a more limited range of metabolites are measured very precisely.

This data system will allow the necessary sophistication for the processing of body fluid samples to establish norms and to investigate specific urinary metabolic content for various forms of clinically diagnosed cancer. This involves extracting from the approximately 600 spectra collected during each run, the 100 or so representing the components of the body fluid sample for identification. The raw spectra may be contaminated with background "column bleed" and some are composited with adjacent constituent spectra unresolved by the GC.

We have begun to develop a solution to the problem of effectively increasing the resolution of the GC by computer analysis of the data. These programs will allow us to automaticaly locate the body fluid constituent spectra and remove the distortions caused by background and poor resolution. These "cleaned-up" spectra can then be analyzed by library search techniques or first principles as necessary. By using a disk-oriented matrix transposition algorithm developed for image processing applications, we can rotate the entire array of 700 spectra by 500 mass samples for each run. In this way we can gain convenient access to the "mass chromatogram" form of the data. This form of the data, displayed at a few selected masses, is used in mass fragmentography described elsewhere in this proposal. Mass chromatograms have the important property of displaying much higher resolution in localizing GC effluent constituents. The automatic analysis techniques currently being developed for mass fragmentograms can be extended to this more general case. Thus by transposing the raw data to the mass chromatogram domain, we can systematically analyze these data for baselines, peak positions, and correct amplitudes thereby deriving idealized mass spectra for the constituent materials. These spectra are free from background contamination and influences of adjacent unresolved GC peaks. This aspect of our work is funded by NASA (see Section VI. Cytochemical Studies of Microorganisms.), but on implementation it will be available to this proposal

The problem of spectrum identification is addressed by using rapid library search techniques for the identification of previously encountered compounds. Those not in the library will be identified by the manual interpretation of the spectrum using other information as available. These results will be incorporated into the library to extend its domain of usefulness. This progressive compilation of a library of biomedically relevant compounds will speed the throughput of the system. This library will be freely shared with other investigators. Eventually the extension of the library domain will be assisted by adapting the computer programs under development in the DENDRAL project (see for instance refs. 9 and 10). These seek to emulate the reasoning processes organic chemists employ in interpreting spectra using fragmentation rules and other knowledge to infer the correct molecular structure from among those possible. The compilation of mass spectra is currently well underway and should be operational by June 1974. We are in contact with Dr. S. Markey, University of Colorado Medical Center, Denver, who is compiling a library of the mass spectra of biologically significant compounds. This laboratory has contributed mass spectra to this compilation and we will receive a copy of the final library.

IIIC (2) and (3)

Quantitation of Urinary Beta-Aminoiso-butyric Acid and Protein Amino Acids using Quadrupole Mass Fragmentography.

In the preceding section we briefly touched upon the capability our laboratory has generated for the quantitation of BAIB and amino acids at the nanogram level using quadrupole mass fragmentography. In the case of the amino acids a deuterated amino acid mixture serves as an internal standard. By exploiting characteristics of the quadrupole mass spectrometer-computer system we can quantitate in a single analysis many more components than previously was possible using mass fragmentography.

The quantitation of urinary BAIB and amino acid levels are conveniently discussed together since experimentally one analysis will suffice to quantitate both. The fractionation procedure for urine described in section IIIC (1) provides a derivatized fraction (B) consisting of a carboxybutyl, N-trifluoroacetate derivative which our procedures use for the quantitation of BAIB and protein aminoacids. This quantitation will as we have already demonstrated be at the nanogram level of detection (refs. 2, 6, 7, 48, 49, copies attached). The PDP-11 computer system will calculate the concentrations of the following amino acids: BAIB, alanine, valine, glycine, isoleucine, leucine, proline, threonine, serine, phenylalanine, aspartic acid, glutamic acid, tyrosine and lysine present in the urine of control and cancer patients. Statistical correlations will then be sought between the levels of these metabolites and the respective disease states of the patients.

Our publications (refs. 6,7) describing the mass fragmentographic quantitation of amino acids did not measure tyrosine because we lacked deuterated tyrosine to serve as an internal standard. We now have this deuterated amino acid and routinely analyze for tyrosine.

One analysis using mass fragmentography will be completed every hour. The data acquisition phase of the experiment occupies about 30 minutes, the computation and presentation of the results should occupy another 15 minutes and 15 minutes are allowed for resetting various experimental parameters (sample preparation, resetting GC oven conditions, etc.). We believe that three samples per day will be analyzed (50% time of one organic chemist for a weekly output of 15 samples 750 on an annual basis).

GENERAL COMMENTS: An important aspect of our experimental protocols will involve the clinical study of the efficacy of metabolites or their concentration levels we may identify for the early detection of cancer. This aspect of our program will be addressed by screening a large number of urines from healthy subjects and clinically following those who register positive in our tests. Our clinical associates at Stanford see a large number of patients during their professional treatment of cancer and we expect to have ample control subjects and patients for the testing of any clinical applications resulting from this research project. At this time we have not formalized the protocols which might be used in such a clinical study and prefer to wait until the research develops results applicable to a detailed clinical

study.

IV. KEY PERSONNEL

J. Lederberg	Prof. of Genetics & Principal Investigator	5%
A Duffield	Research Associate	50%
W. Pereira	Research Associate	50%
R. Tucker	Computer Programmer	20%
E. Steed	Research Associate	10%
N. Veizades	Research Engineer	10%
M. Wyche	Research Assistant	30%
M. Allan	Secretary	10%

J. Lederberg, Principal Investigator

Professor Lederberg is the Joseph D. Grant Professor of Genetics and assumes overall responsibility for the research program outlined in this proposal. His original interest in the technique of GC/MS involved its use in exobiology for the detection of extra-terrestrial life. His present interest in GC/MS resides in its application to the analysis of the metabolic content of body fluids. He is the principal investigator of a comprehensive grant to start a Genetics Research Center, one project of which is the use of GC/MS to analyze the metabolic content of body fluids for the recognition of genetic disease.

Dr. Alan Duffield will assume responsibility for the integrity of the urine analyses. His experience is in organic chemistry and the applications of mass spectrometry to problems in organic chemistry and biochemistry. Since 1970 he has been active in the applications of GC/MS to biochemical and biomedical problems. This has involved research in the analysis of various body fluids for metabolic content primarily with a view to recognizing genetic disease. In the present proposal he will conduct those experiments designed to screen urine from cancer patients for specific metabolites which may be idiosyncratic with the disease.

Dr. Wilfred Pereira received his Ph.D. in pharmaceutical chemistry and has had experience in the analysis of body fluids by GC/MS. He developed the chemistry which led to the development of quadrupole mass fragmentography as a sensitive, specific and quantitative single analysis for trace quantities of over 12 organic compounds. In the present proposal Dr. Pereira will use quadrupole mass fragmentography for the quantitation of beta-amino-isobutyric acid and protein amino acids in the urines of control subjects and cancer patients.

Mr. Robert Tucker implemented and maintains the software used for the PDP 11/20 mini-computer system. He is responsible for the continuing development of the software requied for automatic reduction of mass fragmentography data.

Mr. Ernest Steed is responsible for the maintenance of the GC/MS system. The transfer lines (GC column, separator) in this

system are all glass (the membrane separator is made by Mr. Steed, who is an experienced glass blower). He also assumes responsibility for the maintenance of the mass spectrometer vacuum and electrical systems.

Mr. Nicholas Veizades will be required to maintain the interface between the mass spectrometer and the PDP-11/20 computer system. He will also be responsible for some maintenance of the data system.

Mrs. Wyche will support the chemical portion of this program. She assists in routine laboratory functions, preparation of standard solutions and data presentation from the mini-computer system.

V. FACILITIES AND EQUIPMENT.

We will derive our clinical urine samples from Dr. W. R. Fair (Associate Professor of Surgery; see attached letter, Appendix A) and from Drs. J. R. Wilbur and T. Long, Stanford Children's Hospital (see attached letter, Appendix B). Analyses will be performed on existing GC and MS equipment in the Department of Genetics. We have a two-column Varian Aerograph 2100 gas chromtograph used for pre-screening and a Varian Aerograph 1200 gas chromatograph interfaced to a Finnigan 1015 Quadrupole mass spectrometer by an all glass membrane separator of our own design. The mass spectrometer is interfaced to a PDP-11/20 mini-computer system (16K core) consisting of fixed head disk storage (256K) movable head disk storage (1.2 million) line printer, and for data storage, a DEC tape drive. We have access to a Varian MAT 711 high resolution mass spectrometer (interfaced to a Hewlett-Packard 7610 gas chromatograph) in the Chemistry Department for the recording of HRMS data in those instances where unknown metabolites cannot be uniquely identified from GC/LRMS data alone. Also available to the project are the electronic facilities and the software experience of the Instrumentation Research Laboratory (Dr. E. Levinthal, Director) of the Department of Genetics.

VI. RELATED ACTIVITIES:

Cytochemical Studies of Planetary Microorganisms. Explorations in Exobiology. (J. Lederberg, Principal Investigator). NASA Grant NGR-05-020-004.

This grant sponsored the activities of the Instrumentation Research Laboratory of the Department of Genetics since 1962 and these are summarized in ref. 8. The initial focus of this laboratory was to develop methodologies for the detection of extraterrestrial life and this led to the development of a sophisticated GC/MS-computer system (ref. 47). During the lifetime of this grant NASA has generously fostered applications of these methodologies for health related research. With recent budgetary restrictions which necessitated that NASA reduce its sponsorship of basic research we approached other granting

agencies in the field of public health for the financial support necessary to continue to apply advanced chemical methodologies to health related research.

Analytical Methodology for Biochemical Monitoring. (J. Lederberg,Principal Investigator). NASA NGR-05-020-632.

Under this grant several pilot projects were commenced aimed at devising non-invasive techniques for the diagnosis of human disease. An important aspect of this grant was its willingness to sponsor the software development required for the PDP-11/20 mini-computer system to be interfaced to our mass spectrometer. It was under this umbrella that our investigation of the metabolites present in urine from patients with leukemia and Hodgkin's Disease were commenced. During the sponsorship of this grant we have applied mass fragmentography to the analysis of body fluids. Another investigation partly generated by this grant was an investigation of the urinary metabolic content of premature infants following controlled dietary intake. An early draft of a manuscript describing this research is attached to this application (see Appendix C).

Genetics Research Center. (J. Lederberg, Principal Investigator) NIGMS Approved and awaiting funding. (Grant #P01-GM-20832-01)

The focus of this grant is the detection of clinical indicators of genetic disease. One section of the proposal deals with GC/MS methodology in the analysis of body fluids for the identification of metabolites which might be indicative of genetic disease. It is intended that this will not be a broad screening program but rather that its emphasis resides in analyzing body fluids from those patients where there are clinical symtoms indicative of possible genetic abnormalities. Another application within this grant of GC/MS methodology is the analysis of amniotic fluid for prenatal diagnosis of hereditary inborn errors of metabolism.

VII. PROGRAM MANAGEMENT.

The proposed program, because of its limited size and sharply focussed arena of application does not require special management protocols to be drawn up.

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