EEL

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

CONTRACT NUMBER NO1-CB-43902

(Renewal)

SUBMITTED BY:

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

Joshua Lederberg, Principal Investigator
Department of Genetics
Stanford University Medical School
Stanford, California 94305

PART I. TECHNICAL PROPOSAL

1. Summary Statement for Coming Year.

During the second year of this contract we intend to build upon the experience gained in the first year in the applications of gas chromatography-mass spectrometry-computer techniques (GC/MS) for the detection and quantitation of urinary metabolites. Our experimental procedures, and clinical arrangements, will remain essentially as described in the original and amended proposals. Our efforts in the second year will be augmented by a substantial increase in computer support with the utilization of a recently acquired PDP-11/45 computer system. The impact of this system on our analytical methodology will be discussed below.

By the beginning of the second year of this proposal we will have designed and operated an analytical method for the quantitation of urinary polyamine levels (putrescine, cadavarine, spermidine and spermine) in patients afflicted with prostatic cancer. Our technique is described under section 3 (Progress Report) and we believe it will introduce a degree of specificity and sensitivity currently lacking in polyamine analysis. This aspect of our research is aimed at investigating increases in urinary spermidine levels noted by Dr. Fair (Department of Urology) using a less specific analytical procedure. If tissue samples are available following surgery they will be examined for their polyamine content.

During the second year of this contract we shall press ahead with the evaluation of two metabolites, frequently observed in the urine of cancer patients, for the recognition of neoplastic disease. First it will be desirable to continue to quantitate levels of beta-aminoisobutyric acid (BAIB) in control and cancer urine in view of the occurrence of this compound in the urine of the majority of cancer patients examined to date. The second metabolite we have frequently observed in cancer patients' urine, but not in a limited number of controls, remains unidentified. It occurs in the amino acid fraction of urine and a copy of its low resolution mass spectrum is appended to this application as Figure 2. Identification of this material will require gas chromatography/high resolution mass spectrometry (GC/HRMS) analysis and this will be done in the Chemistry Department as soon as existing computer programs are fully operational. This experiment will be undertaken with the assistance of our colleagues in the Stanford University DENDRAL project.

We will continue to use GC/MS to search for new urinary metabolites which might be markers for various specific types of cancer. This aspect of our research program will benefit from the availability of a PDP-11/45 computer system. This system will be interfaced to our existing DEC PDP 11/20 which is used for the recording, processing and presentation of mass spectra. The addition of the PDP 11/45 system will allow us to store on magnetic tape the significant mass spectral data recorded for each patient. These data will then be available for computer search for those mass spectra common to patients with similar diagnosed carcinomas. This computer search will speed the task of comparing metabolic outputs between series of cancer patients.

Work is in progress (supported by another grant) for the use of the PDP-11/45 computer system for the removal of background mass spectra (derived from column or septum bleed) from the recorded mass spectral data. This work is being extended to the deconvolution of GC peaks containing more than one component as determined from the recorded mass spectra. The successful prosecution of this research will result in "cleaned-up" mass spectra being available for either human or machine (library search) matching with structures. To this end we have received a tape of a library of over 3,000 mass spectra of biologically relevant compounds compiled by Dr. S. Markey. These improvements will benefit the present contract by increasing the analytic power and sample throughput of our GC/MS computer system.

2. Updated Bibliography.

The following publications from this laboratory have appeared or are in press.

1. Applications of Artificial Intelligence for Chemical Inference. XII. Exhaustive Generation of Cyclic and Acyclic Isomers. By L. M. Masinter, N. S. Sridharan, J. Lederberg, and D. H. Smith, J. Amer. Chem. Soc., 96, 7702 (1974).

2. Applications of Artificial Intelligence for Chemical Inference. XIII. A General Method for Predicting Molecular Ions in Mass Spectra. By R. G. Dromey, B. G. Buchanan, D. H. Smith, J. Lederberg, and C. Djerassi. J. Org. Chem., in press (1975).

3. Progress Report for the Period July 1, 1974 to January 31, 1975.

A. Development of an Analytical Method for the Quantitation of Urinary Polyamine Levels.

We decided to use mass fragmentography for the quantitation of urinary polyamines in view of the high sensitivity and specificity available with this mode of analysis. The most appropriate internal standards for this analysis are deuterated polyamines which we have synthesized by the following procedures.

Putrescine-1,1,4,4-d_4 (1): This compound was obtained by condensation of 1,4-dibromobutane-1,1,4,4-d_4 with potassium phthalimide, and hydrolysis of the condensation product according to a published method. (J. Biol. Chem., 233, 907 (1958)).

Cadavarine-1,1,5,5-d_4 (2): This compound was obtained by the same general method using 1,5-dibromopentane-1,1,5,5-d_4.

Spermidine-d_2 (3) and Spermine-d_4 (4): Condensation of putrescine and acrylonitrile followed by reduction of the crude reaction product with lithium aluminum deuteride in tetrahydrofuran yielded a mixture of putrescine, and the desired products 3 and 4. The individual polyamine hydrochlorides were separated from this mixture by ion exchange chromatography.

$NH_2-CD_2-(CH_2)_n-CD_2-NH_2$

1. n = 2
2. n = 3

$NH_2-CD_2-(CH_2)_2-NH-(CH_2)_4-NH_2$

3.

$NH_2-CD_2-(CH_2)_2-NH-(CH_2)_4-NH-(CH_2)_2-CD_2-NH_2$

4.

Isolation of Polyamines from Urine.

The excretion levels of polyamines in urine are of the order of a few milligrams per day. In view of this dilution the isolation of a polyamine fraction from urine presents problems in chemical manipulation. We have investigated this problem using ^{14}C labeled polyamines as markers.

Ion Exchange Method.

Urine (50 ml) is hydrolyzed overnight with 6N hydrochloric acid and chromatographed on Dowex 50 (H^+) and the resin successively eluted with NaCl/$Na_3(PO_4)_2$ buffer, 1N HCl and finally 6N HCl. The polyamines are found in the 6N eluate in the following recoveries: putrescine (N.A.), spermidine (70-100%) and spermine (80-100%).

Using this procedure, and adding small amounts of polyamines to the urine, we have been able to detect putrescine and spermidine by mass fragmentography while spermine, although detectable is recovered in lower yield.

Butanol Extraction Method.

Urine is hydrolyzed overnight with 6N HCl, filtered and the filtrate concentrated to dryness. The dry sample is then made to pH 11 with sodium hydroxide, inorganic salts added and the solution extracted with butanol for 30 minutes. Using this method ^{14}C labeled spermidine was recovered in 60-80% yield.

GC/MS of Polyamines.

Derivatization of polyamines prior to GC analysis is accomplished by heating with trifluoroacetic anhydride in methylene chloride for 30 minutes in a sealed vial. The electron impact (EI) spectra of these N-TFA derivatives of the polyamines and their synthesized deuterated analogs have been recorded and the appropriate ions selected for mass fragmentographic detection.

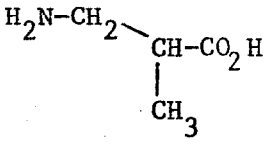
Using chemical ionization mass spectrometry (CI) (at the Applications Laboratory of Finnigan Instrument Corporation) we have recorded the CI spectra of the polyamine N-TFA derivatives. This technique appears to yield the optimum sensitivity for the detection of polyamines by mass spectrometry. Each polyamine TFA derivative yielded an intense quasi-molecular ion $(M+H)^+$. It is our intention, once we have demonstrated a mass fragmentography analysis using our existing equipment (EI), to collaborate with a laboratory equipped with a chemical ionization mass spectrometer in the development of a CI mass fragmentography analysis for polyamines in urine. We anticipate that this latter technique will result in a considerable enhancement of sensitivity for polyamine detection.

In the remaining period of the first year of the current contract we will develop and demonstrate a urinary polyamine analysis by mass fragmentography. In subsequent years this analysis will be available for the quantitation of urinary and tissue polyamines in cancer patients.

To date we have completed GC/MS profiles on the following distribution of cancer patients:

Bladder cancer	8
Non-Hodgkin Lymphomas	6
Cancer of the prostate	3
Leukemias	7

Each urine from these patients has been screened for acids + neutral compounds, amino acids and sugars. Examination of the mass spectra recorded for these fractions leads to two interesting observations. First, beta-amino-isobutyric acid (BAIB, 6) occurs in all 7 leukemic urines (in several in large amount), in 7 of 8 bladder cancer urines, 5 of 6 lymphoma urines and 1 in 3 prostatic cancer urines. Although it is known that approximately 10% of the Caucasian population are genetic excretors of BAIB (original proposal) it is striking that this compound occurs in 20 of 24 cancer urines examined. It is essential that we proceed with the quantitation of the urinary concentration of BAIB in these urines using the existing mass fragmentography method and the PDP-11/20 computer system.



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The second result of interest to evolve from the screening of urine from cancer patients is the frequent occurrence of an unidentified component in the amino acid fraction. This constituent elutes (Figure 1) under our gas chromatographic conditions (10% OV-17, N-TFA, O-n-Butyl derivative) between alpha-aminooctanoic acid and creatinine. The low resolution mass spectrum of this unidentified component is shown in Figure 2.

This compound occurs in the urine of 7 of 8 patients with bladder cancer, 5 of 6 with non-Hodgkin Lymphomas, 3 of 3 with prostatic cancer and 3 of 7 with leukemia. Of 40 children screened by our laboratory for genetic disease only one positively contained this unidentified compound while a second may have been positive but it was present at too low a level for a positive identification to be made. This compound has been identified in 2 of 3 control urines examined to date.

It is probable that this compound is of little significance as a marker for the detection of cancer but we shall continue to run controls in order to better understand its distribution in urine especially with relation to children and adults.

We believe the identification of the compound should precede the assessment of its significance for cancer prediction, and indeed of the delineation of controlled epidemiological studies.

We intend to record the mass spectra of several compounds in an attempt to positively identify this compound. If this fails we intend to use the GC/HRMS system of the DENDRAL project. This analysis will provide the empirical composition of the ions shown in Figure 2, and should facilitate identification of the unknown. At the present time the GC/HRMS system is undergoing final testing and it should then be available to this project for the analysis of this and any other analytical challenges of the future.

4.

	Personnel	Title	Soc.Sec.No.	% Time
(a)	LEDERBERG, Joshua	Professor of Genetics	[redacted]	5%
	Duffield, Alan M.	Research Associate	[redacted]	40%
	Everhart, Edwin T.	Research Assistant	[redacted]	100%

(b) To date the work under this contract is in a fact-building stage and has not yet resulted in the publication of any scientific research papers.

(c) No invention reports have resulted from work sponsored by this contract.