observations suggest that at 12 weeks of gestation, methylmalonic acid is undetectable in the amniotic fluid surrounding a normal fetus and, probably, a fetus who is heterozygous for the (recessive) gene (ref 24). Goodman, et al. (ref 25) have successfully diagnosed argininosuccinic aciduria in a 16 week fetus. This involved detection of argininosuccinic acid in the amniotic fluid plus enzymatic studies on cultured amniotic fluid cells. Normally this compound is not detected in amniotic fluid at 16 weeks of gestation. There are reports of other conditions being diagnosed in the fetus by direct assay of amniotic fluid (ref 26) as well.

The accuracy of diagnostic procedures probing amniotic fluid for soluble constituents has been questioned because of the possibility of contamination with maternal blood and because of lack of information on the normal state at various times of gestation. If the fluid component could be used for pre-natal diagnostic purposes, the phenotype of the fetus could be detected relatively rapidly as compared to the time required to culture sufficient amniotic cells. Hereditary diseases which are potentially amenable to diagnosis by analysis of the soluble constituents of amniotic fluid are those in which the accumulating metabolite is not cleared by the placenta but is expected to appear in fetal urine. Defects in epithelial transport, e.g., cystinuria and Hartnup disease, are examples of such conditions. However, it is clear that this class of metabolic errors is not the only one which might be detectable by direct assay of amniotic fluid. The examples provided above suggest that fetuses with overflow type metabolic errors may also be detected.

The development of diagnostic and screening techniques suitable for various inborn errors of metabolism will require a suitable computer based methodology for screening a large selected sample of subjects with the subsequent resolution of data into classifications describing normal states and ranges as well as specific correlations of GC/MS analysis abnormalities with disease states. With a modest augmentation of existing instrumentation facilities, we can accomplish these analytical tasks on increasing numbers of patients.

B. SPECIFIC AIMS

a) We plan to use GC/MS to screen urine and plasma from normal individuals of various ages, including premature and newborn infants, in order to establish adequate control data and to understand variations encountered.

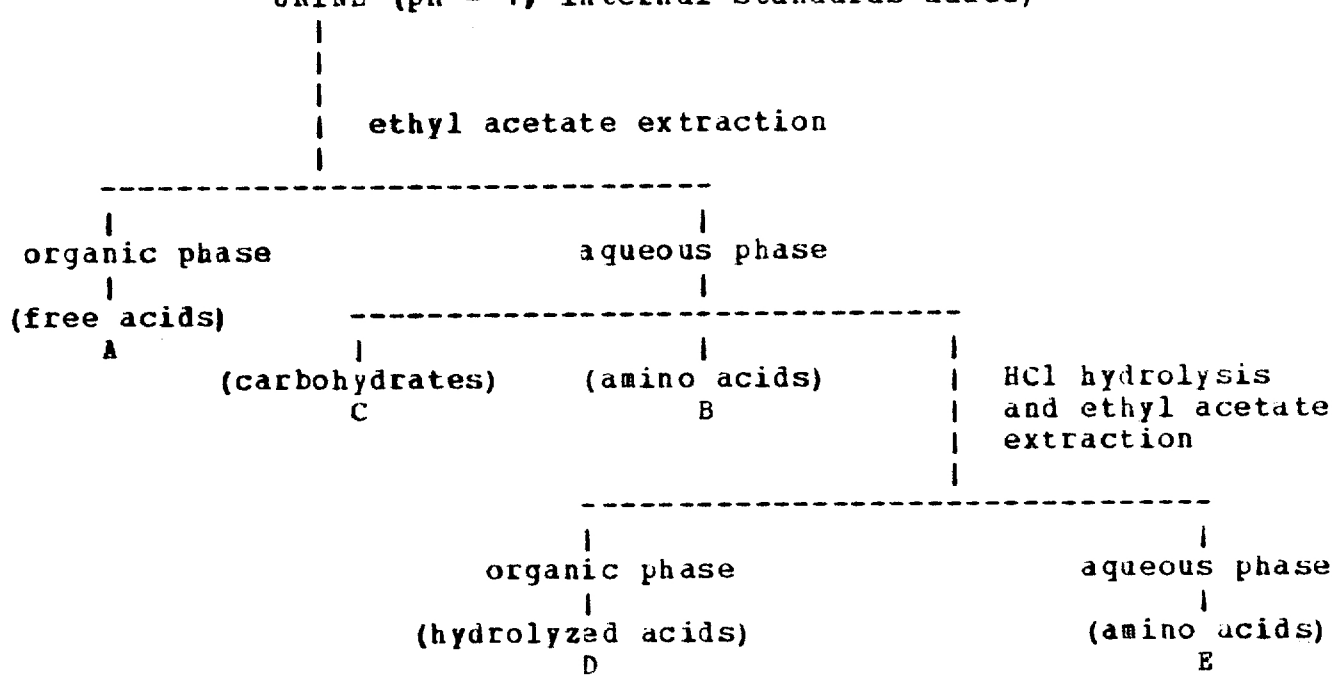
b) We plan to use GC/MS in the diagnosis of inherited metabolic abnormalities and in the detection and study of previously unrecognized metabolic disorders.

c) We plan to use GC/MS to study normal variation of clinically significant metabolites in amniotic fluid and then to apply these techniques to pre-natal diagnosis of hereditary inborn errors of metabolism.

d) In support of the above goals, we plan to augment our existing GC/MS system and to more fully automate it for screening larger numbers of patients through improved computer management. This would fit with current concepts of regionalization of diagnostic facilities for genetic disorders and would be appropriate for a Genetics Research Center.

C. METHODS OF PROCEDURE

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. Two internal standards, n-tetracosane and 2-amino octanoic acid are then added. Ethyl acetate extraction isolates the free acids (fraction A) which are then methylated and analyzed by GC/MS. An aliquot of the aqueous phase (0.5 ml.) is concentrated to dryness, reacted 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). Another aliquot (0.5 ml) of the aqueous phase can be derivatized (TMS) for the detection of carbohydrates (Fraction C).

Concentrated hydrochloric acid (0.15 ml) is added to the urine (1.5 ml) after ethyl acetate 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).

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. A different gas chromatography column and derivatization procedure would be required. Large molecular weight compounds (e.g., tripeptides and mucopolysaccharide fragments) cannot be analyzed by this system without their degradation to smaller unit molecules.

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 27, copy attached). Our most recent efforts have focussed on facilitating user interaction with the data system and on automating various aspects of data analysis (ref 3c). The research proposed in this application entails the analysis of increased numbers of body fluid samples, beyond the capacity of the present system. We therefore propose to augment the current system to handle the larger sample volume and at the same time to extend the capabilities of the system to reduce GC/MS data.

Our present system is built around the NIH-subsidized ACME time-shared computer facility (IBM 360/50) with the real time data interface being through an IBM 1800 computer. These machines will disappear at the termination of the ACME grant in July 1973 and will be replaced by a fee-for-service IBM 370/158 system with more limited real time support capabilities. With the transition implied by the machine changeover, we have reevaluated the approach to be taken in implementing our GC/MS support needs relative to the new requirements presently proposed, the projected costs of alternative approaches, and the evolving computer technology. Mini-computer capabilities have advanced significantly since the previous decision was made in 1965 to implement the existing system on the ACME facility.

After examining the options of implementing a data system on the new time-shared facility or on a stand-alone mini system, we feel that a mini-system is the more advantageous for our needs. Such a system is the cheaper of the two based on projected costs and is more responsive to real time needs in view of a smaller set of demands on the mini-machine. There are limitations inherent in mini-machines, however, in terms of memory size and software/language support. The large central system (370/158 or SUMEX if approved) will act as a backup for the mini-system in the relatively small number of instances where these considerations are important in low resolution GC/MS

applications.

In preparation for the transition this July, we are implementing a minimal data system on a PDP-11/20 computer. This machine is well suited for "front end" data collection and filing activities as well as simple analysis tasks. It cannot simultaneously support data acquisition and the more sophisticated analysis activities contemplated in this program, however, because of capacity limitations. In addition, it lacks the arithmetic speed desirable for these analysis procedures. Since the proposed research program will entail a heavy duty cycle of data collection throughout the day to analyze the increased number of body fluid samples, data analysis and program development would have to take place during off hours. We propose to solve this problem by acquiring a second machine to support the PDP-11/20. This machine, tentatively selected as a PDP-11/45, would provide for data analysis and program development support and would be well suited to anticipated data analysis functions because of its speed and extended arithmetic capabilities. We have also considered a PDP-11/40 machine but feel the PDP-11/45 to be a better choice because of the relatively small cost differential (approximately $10,000) in return for a factor of 2-3 in performance. The proposed system configuration is shown in Figure 1. Our choice of Digital Equipment Corporation (DEC) machines is based on our existing hardware and software expertise with this equipment. Our existing high resolution MS system utilizes a similar PDP-11/20 machine as the data acquisition computer as well.

The PDP-11/45 would have 24K words of memory, floating point hardware and a programmable clock. The memory size is based on projected needs for FORTRAN-based analysis programs. The two disk drives (an existing fixed head drive, 262K words and a proposed moving head drive, 1.2M words) provide needed space for system software, programs (source and object files), and for spectral data. It should be noted that a single GC/MS run contains some 700 spectra, each containing 500 12-bit spectral amplitude measurements. Each such file therefore requires 350,000 words (uncompressed) so that our proposed disk space would be quickly consumed by several such files in residence. We can increase the effective space available by compressing the data files to eliminate insignificant measurements. Note that this requires at least one data analysis pass over the full file, however. We propose to augment this disk capacity in the second year by adding an additional drive.

In general, data files will be stored on magnetic tape. Both DEC tape and industry compatible tape drives are provided. The former is required for system maintenance and is ideal for relatively small private program files, data files, etc. The industry compatible tape provides for large volume storage of raw data during data acquisition and for archival storage.

This augmented data system will allow increased sophistication in the analysis of GC/MS runs and in prescreening

GC profiles. In processing body fluid samples to establish norms and to investigate specific clinical abnormalities, full MS analyses of the GC effluent will be required. This involves extracting from the approximately 700 spectra collected during each run, the 100 or so representing the components of the body fluid sample for identification. The raw spectra are 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.

The results of this work can also lead to reliable prescreening analyses of GC traces alone by having available a detailed list of expected GC effluent positions and amplitudes for the particular body fluid fraction under consideration. 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. If so, a more thorough GC/MS analysis can be made. This type of prescreening is valuable in processing body fluid samples which may or may not be abnormal and saves by committing the expensive mass spectrometer instrumentation to analyzing only suspect samples. This, of course, assumes that norms have been previously established by processing body fluid samples in detail from a large population of normal subjects.

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 (NIH RR-00612). 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 most appropriate target material for this developmental effort is surely the metabolic output of NORMAL subjects under controlled conditions of diet and other intakes. The eventual application of this kind of analytical methodology to the diagnosis of disease obviously depends on the establishment of normal baselines, and much experience already tells us how important the influence of nutrient and medication intake can be in influencing the composition of urine, body fluids, and breath (ref 18). Among the most attractive subjects for such a baseline investigation are newborn infants already under close scrutiny in the Premature Research Center and the newborn nurseries of the Department of Pediatrics at this institution. Such patients are, for valid medical reasons, under a degree of dietary control difficult to match under any other circumstance. Many other features of their physiological condition are being carefully monitored for other purposes as well. The examination of their urine and other effluents is therefore accompanied by the most economical context of other information and requires the least disturbance of these subjects.

Two obvious factors which could profoundly influence the excretion of metabolites detected by GC/MS are maturity and diet. We have already initiated a program for serial screening of urinary metabolite excretion in premature infants of various gestational ages and a determination of changes in the pattern of excretion of various metabolites as a function of age following birth. A synopsis of this research is presented later in this section. These studies are being performed on infants admitted to the Center for Premature Infants and the Intensive Care Nursery at Stanford, a source of some 500 premature infants per year. In addition, in conjunction with an independent study on the effects of both quality and quantity of oral protein intake on the incidence and pathogenesis of late metabolic acidosis of prematurity, we plan to measure the urinary excretion patterns of various metabolites and thereby partially assess the effect of diet on this screening method.

We shall use the analyses on blood and urine specimens from normal individuals in the final development of rapid, automated identification of compounds described by mass spectrometry. By compiling records of the gas chromatographic profiles and associated computer-identified constituents of the body fluids of normal individuals, we will establish the statistical norms and expected variations for the component and levels present. Quantitation is achieved by introducing internal standards into

the body fluid fractions prior to analysis. We will seek to minimize or at least stabilize the variations in measurement results by assessing the effects of various aspects of diet and medication. Normal blood and urine specimens will also allow us to test the system's operational capacity for rapid and accurate metabolite identification.

Given our ability to identify various constituents of urine and plasma and to understand normal variation, we shall apply the GC/MS system to pathology, making use of patients with already identified metabolic defects for control purposes. The main application will, of course, be diagnostic and patients with suggestive clinical manifestations, such as psychomotor retardation and progressive neurologic disease, as well as suggestive pedigrees (e.g. affected offspring of consanguineous parents or multiplex sibships) will be investigated. Actually, we have been studying limited numbers of such patients already. These patients are seen relatively frequently at any university hospital, and their presence in the various in-patient and out-patient services of the Stanford Department of Pediatrics is well documented.

The GC/MS system will be invaluable in diagnosing various inborn errors of metabolism, especially those involving excretion of various fatty and other organic acids. Some of these are isovaleric acidemia (ref 15), methylmalonic acidemia (ref 28), the recently reported inherited disorder of isoleucine metabolism causing accumulation of alpha-methylacetoacetic acid and alpha-methyl-beta-hydroxybutyric acid (ref 29), lactic acidemia (ref 20), Refsum's disease (a defect in the oxidation of phytanic acid - ref 31), orotic aciduria (ref 32), and as illustrated above, ornithine transcarbamylase deficiency (ref 19). We recognize the potential of this methodology for defining previously undescribed ("new") inborn errors of metabolism (e.g., ref 15).

In considering the strategy for applying GC/MS techniques to problems of screening and characterizing inborn errors of metabolism, particular limitations in our present system in terms of detection capabilities or throughput capacity must be accounted for. For example, our present system is somewhat limited for screening and diagnosing those conditions whose phenotypes include the accumulation of amino acids in urine and/or blood. We can detect all of the naturally occurring alpha amino acids except homocystine, cystine, and tryptophan. In addition, the derivatization procedure being used, converts asparagine and glutamine respectively to aspartic acid and glutamic acid. Thus the presence and quantity of asparagine and glutamine cannot be separately measured from that of aspartic acid and glutamic acid by current procedures. This situation could be remedied by the use of a different GC column in order to detect homocystine, cystine, and tryptophan. By using an additional derivatization procedure, asparagine and glutamine could be measured. This approach, while rigorous in its ability to detect and quantitate amino acids, complicates the higher

volume screening throughput. In effect, it would be necessary to install and operate an additional gas chromatograph just for this purpose because the temperature programming conditions for the new column differ from those required for the column presently used for acid and carbohydrate fractions. We are aware that an amino acid analyzer, separate from the GC/MS system, offers a practical solution to larger scale screening for metabolic errors involving the accumulation of amino acids. This approach also has the advantage of providing increased assurance that the clinical analysis of amino acid accumulations can be performed at times when the GC/MS system may be tied-up, under repair, or undergoing engineering change. Such an analyzer, using ion exchange chromatography, does not require derivatization of the specimen and can detect all of the naturally occurring amino acids. We therefore propose to acquire an amino acid analyzer to assist in the screening of the increased number of samples expected from our work at Stanford and from collaborating researchers elsewhere. In selected cases where the amino acid analyzer alone cannot provide sufficient resolution and quantitative accuracy in characterizing amino acid accumulations, the more lengthy, augmented GC/MS approach will be available.

Another area in which our existing equipment would limit throughput is in the capacity of the single low resolution mass spectometer. The present GC/MS system can process 1-2 specimens per day (including all five derivatized fractions). This throughput has sufficed for small sample research applications but we anticipate that this load will at least be tripled when we are actively studying normal variations and are screening patients for metabolic errors. We could, of course, eliminate the bottleneck by buying more mass spectrometers. This would be quite expensive. An alternative approach appears feasible.

By passing the samples submitted for screening through a preliminary GC profile analysis (without MS), we feel it will be possible to identify those deviating from established normal limits and warranting a more rigorous GC/MS analysis. This will allow more efficient use to be made of the MS instrument time. For abnormal samples, the deviation, under MS analysis, may be readily identified by library search techniques or may require more lengthy analysis procedures. These could require different chemical derivatization techniques or additional information such as high resolution mass spectrometry. This additional analysis could consume days or weeks of effort. Thus it is important to make efficient use of these analysis capabilities. The number of samples eliminated in GC prescreening depends, of course, on the nature of the samples submitted. Prescreening will be less useful for the processing of samples which have been carefully selected as clinically suspect than on a general sampling of "normal" individuals. It is in this latter case that we see a benefit to developing the prescreening capability. For this purpose, we propose to add to our laboratory a 4 column gas chromatograph.

The Department of Pediatrics has affiliations with the Kaiser-Permanente Medical Center in Santa Clara, the Santa Clara

County Hospital, and the Children's Hospital at Stanford. These clinical units will funnel samples (urine, blood, amniotic fluid, etc.) from suspect patients into the laboratory of this program. We are already receiving samples from various physicians in California (e.g., Fresno - see below) and from Dr. Jose M. Garcia-Castro, Medical Geneticist of the University of Puerto Rico Medical School (see below also). Arrangements are in process to obtain samples from the newborn nurseries of the Los Angeles County Hospital. The proposed augmentation of our instrumentation with an amino acid analyzer and an additional gas chromatograph will assist in being able to handle this anticipated specimen load. With the experience afforded by our augmented screening and diagnostic facilities, we will have the capabilities to develop into a regional center for the screening and diagnosis of various metabolic errors.

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 (figure 2) of the amino acid fraction of his urine showed the presence of an abnormal peak (A). The mass spectral analysis of this chromatographic peak (Figure 3) identified this component as beta-amino ISObutyric acid (BAIB) from a comparison with a literature spectrum of authentic material (ref 32a). The literature on human urinary BAIB excretion is extensive and it has been reviewed in reference 33 . Awapara (ref 34) noted increased urinary BAIB excretion from patients receiving nitrogen mustard therapy while Rubini (ref 35) reported high levels of BAIB excretion in people exposed to excessive radiation levels. Wright and Fink (ref 36) found elevated urinary BAIB levels from mongoloid children and non-mongoloid mental defectives but these conclusions were subsequently disputed (ref 37). Harris in England (ref 38) and Calchi-Novata in Italy (ref 39) found that 6-10% of a sample population had elevated urine concentrations of BAIB while DeGrouchy and Sutton (ref 40) in a study of families of oriental extraction, and Yani, et al. (ref 41) 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 42), Apache Indians and Black Caribs of British Honduras (ref 35) was due to genetic difference at one locus. Later studies showed that thymine was probably a precursor of BAIB in man (ref 44).

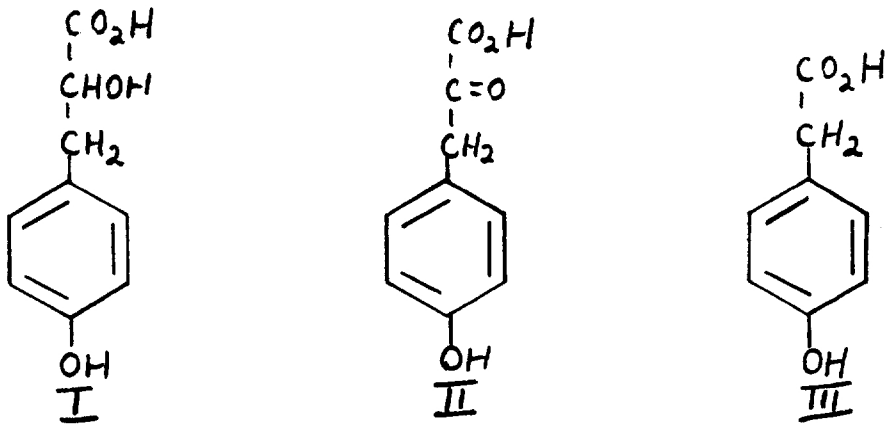
At least two authors (refs 33 and 41) have questioned the accuracy of the methods used for the detection and quantitation of BAIB in urine. The early work (refs 32 and 51), including the first isolation of BAIB from urine (ref 38), was completed using paper chromatography and reaction with ninhydrin to develop the appropriate spot for detection and quantitation. As suggested (refs 25 and 34) 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 47) and paper electrophoresis (ref 48). 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 mass spectrum. 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 3a and 3b), we can quantitate for BAIB under these conditions. In this technique, a characteristic fragment ion from the mass spectrum of BAIB (figure 3, m/e=153 or 182, for example) 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. This work is almost finished and a paper describing a convenient way to quantitate BAIB in urine will be prepared in the near future.

We have examined urine samples from 18 patients suffering from leukemia and have observed BAIB in a total of five individuals. The patient whose urine was used to record the chromatogram shown in figure 2 was excreting 1.2 grams of BAIB per day and following drug therapy this compound could no longer be detected nor was it detected in a urine sample collected 3 months later. Awapara (ref 34) claimed that all leukemic patients excrete varying amounts of BAIB which was not detected in normal urine. Killmann, et al. (ref 49) 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 50) 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 45, 49, and 50), suggest that during the disease cycle, leukemic patients may excrete large amounts of BAIB but this ceases following chemotherapy. Schreier (ref 51) 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.

As mentioned earlier in this section, we started pilot GC/MS studies of biological fluids by investigating the urinary metabolic output of premature children. So far we have studied over 80 urine specimens from a total of 11 premature or "small for gestational age" infants. Of this population, six infants were closely studied for periods of between six and eight weeks beginning at day 3 of life. Five of the six babies showed clinical symptoms of late metabolic acidosis (ref 52) and all five showed the same abnormal metabolic profile of their acidic fractions. All five children excreted excessive amounts of p-hydroxyphenyllactic acid (I), together with smaller quantities

of p-hydroxyphenylpyruvic acid (II), and p-hydroxyphenylacetic acid (III) (see figure 4A and the structures shown below). After reaching a maximum, the daily excretion of these abnormal metabolites diminished until they were almost completely absent when the child's pH and weight gain had returned to normal values (see figure 4-B). The sixth infant showed no clinical symptoms of acidosis and excreted only minute amounts of I, II, and III during the observation period. None of the six subjects showed any increased level of urinary tyrosine excretion. A blood sample, from a patient who excreted large urinary amounts of I, II, and III, showed no detectable quantity of these acids.



The heavy urinary content of I and II by premature infants ingesting a relatively high protein diet (cow's milk) was described in 1941 (ref 53). Classical chemical isolation procedures were used for the identification of I and II and quantitation was achieved by two colorimetric assays. The quantity of I was determined from the difference between the two colorimetric assays, assuming that no p-hydroxyphenylacetic acid (III) was present. Our finding of appreciable amounts of III in the urine of these premature infants suggests that the quantitation values given previously (ref 53) for the excretion of (I) are incorrect. Figure 4 illustrates the gas chromatographic trace from the analysis of the acid fraction (as their methyl esters) present in the urine of one premature infant while acidotic (A) and after recovery (B). The identification of the compounds on Figure 4 follow from their mass spectral signatures while quantitation was achieved from measuring the area under the gas chromatographic peaks using tetracosane as an internal standard.

During the past six months we have commenced a preliminary investigation of the use of GC/MS for analyzing body fluids from both normal patients and those whose clinical history suggested they had a high risk of suffering from a metabolic disorder. The first two cases reflect some of the results we have obtained while the third describes the preliminary work undertaken with amniotic fluid from normal pregnancies being terminated for medico-social reasons.

The first case involves a deceased child, whose parents were first cousins and who was suspected of having a branched chain amino aciduria as indicated by thin layer chromatography at another institution (ref. 54). Preliminary screening by GC/MS of the urine which was sent to us showed no abnormal levels of leucine, isoleucine or valine, nor of their respective alpha-keto acids. In order to verify this point we analyzed the amino acid fraction using our recently developed mass fragmentography technique (refs. 2, 3). A copy of the computer output for this analysis is reproduced as Figure 5. Clearly there are no elevated quantities of any of the branched chain amino acids, which we know we can detect, and the cause of this child's death remains unknown at this time. It is possible that this child showed intermittent excretion of branched chain amino acids (54). This urine was sent to us by Dr. Jose M. Garcia-Castro, Puerto Rico (see note on Collaborative Arrangements).

Recently a child died 33 hours after birth in Fresno, California, with the classical signs of hypophosphatasia (ref 55). This genetic defect is marked by high phosphoethanolamine (PEA) concentrations in urine of affected homozygotes and unaffected heterozygotes. After derivatization (in this instance the TMS ethers of the water soluble carbohydrate fraction were prepared) we were able to detect by GC/MS large concentrations of ethanolamine and phosphoric acid but not PEA itself. The derivatization procedure we used most likely hydrolyzed PEA (ref 56). We were able to quantitate for this compound in the infant's urine using an amino acid analyzer, and PEA excretion was extremely high (over 200 times normal values for infants) confirming the diagnosis. Next we examined urine samples from the child's parents, presumed heterozygotes, by GC/MS and by the amino acid analyzer. Again, no PEA was detected by the former method although the presence of ethanolamine and phosphoric acid was demonstrated. We determined the following excretion levels of PEA by amino acid analyzer:

Newborn infant:	94 micromoles per 100 ml.	(Normal 0.21-0.33)
Father:	269 micromoles per 24 hours	(normal 17-99)
Mother:	32 micromoles per 24 hours	(normal 17-99)

It is of interest that in this family the affected infant and his unaffected father both show subnormal serum alkaline phosphatase activity. The mother, who did not excrete increased amounts of PEA, was found to have normal activity of this enzyme in her serum. The following table summarizes the serum phosphatase activity measurements:

Newborn infant:	0.2 units*	(normal 2.8-6.7)
Father:	0.7 units	(normal 0.8-2.3)
Mother:	3.4 units	(normal 0.8-2.3)

(* - 1 unit is that phosphatase activity which will liberate 1 millimole of p-nitrophenol per hour per liter of serum)

These findings are quite consistent with hypophosphatasia. The heterozygous unaffected parents of children with hypophosphatasia may or may not show biochemical evidence of the carrier state.

The analysis of the metabolic content of amniotic fluid has been pursued by many investigators with the aim of identifying metabolic defects IN UTERO. Historically the first studies on amniotic fluid utilized paper chromatography (refs 57, 58, 59). Amino acid quantitation in amniotic fluid was attempted by a visual comparison of paper chromatographic spots with standard spots (refs 60, 61) and by spectrophotometrically measuring the intensity of spots eluted from paper chromatograms. (ref 62). Ion exchange chromatography was applied (refs 20, 21) for the analysis of the free amino acids in amniotic fluid while gas chromatography (refs 63, 64) and GC/MS (ref 65) were used for the determination of the free acid content of amniotic fluid. On the basis of their results, Levy and Montag suggested (ref 21) that amniotic fluid arises from at least 3 sources: maternal blood, fetal blood, and fetal urine.

Our own endeavors in the field of amniotic fluid analysis has involved a study to determine those constituents found in normal amniotic fluid. Ten specimens have been analyzed. All of these samples were obtained at Stanford, most at 14-16 weeks of gestation for chromosome studies of the fetus, usually because of increased maternal age. Following separation by centrifugation and removal of the cells for culture, the amniotic fluid was again centrifuged (2900 rpm, International Clinical Centrifuge, for 10 minutes) and the supernatant was stored at -20 degrees C until analyzed by GC/MS. To date we have identified the following acids in amniotic fluid: citric, myristic, palmitic, stearic, oleic and esters of phthalic acid (probably leached from the plastic containers used to store the specimens). The amniotic fluid from one patient contained salicylic acid (aspirin ingestion) and from another caffeine (tea or coffee ingestion). The following amino acids have been identified in these "normal" amniotic fluid samples: alanine, threonine, serine, glycine, valine, i-leucine, leucine, proline, methionine, aspartic acid, phenylalanine, tyrosine, glutamic acid and lysine. In view of the current interest in the pre-natal diagnosis of methylmalonicaciduria, we can state that methylmalonic acid was not detected in the acidic fraction of any of the "normal" amniotic fluid specimens. We are continuing these baseline studies of normal amniotic fluid in order to eventually identify abnormal accumulations of metabolites in the at-risk pregnancy. Variables which we will consider include gestational age, diagnosis and status of the fetus, maternal plasma concentration of the relevant substances and maternal plasma concentrations of hormones and medications and their metabolites. We are presently planning to investigate variation in amniotic fluid pregnanetriol, 17-ketosteroids and some of the specific androgens which are elevated in the adrenogenital syndrome (ref 66).

At this time we are not prepared to predict that GC/MS analysis of amniotic fluid alone will provide the accurate diagnostic information about the fetus required for a decision for selective abortion. Heterozygous fetuses and contamination of the amniotic fluid with maternal blood (the result of the amniocentesis procedure) raise possibilities for false positive diagnoses which may lead to abortion of unaffected fetuses if cultured amniotic fluid cells are not also studied. However, studies relating quantity of the pertinent metabolite in amniotic fluid to enzyme activity in the cultured cells should be helpful in providing information for discriminating between heterozygous and homozygous fetuses by GC/MS. In other words, we will monitor at-risk pregnancies with GC/MS and with biochemical studies of cultured cells until we are confident we can predict the homozygous state from GC/MS analysis alone. Maternal blood contamination will be monitored by gross and microscopic examination of amniotic fluid samples, and the behavior of contaminated fluid on GC/MS analysis will be anticipated by studying a series of amniotic fluid samples to which various amounts of blood from a heterozygous individual (The mother in the high risk pregnancy is usually a heterozygote) have been added.

D. SIGNIFICANCE

An accurate diagnosis is especially important for genetic counseling purposes. The diagnosis allows reference to published data on the mode of inheritance and, thus, expresses the recurrence risk. Furthermore, accurate diagnosis of the accumulated metabolite provides insight into the biochemical pathogenesis and into therapeutic approaches to the control of hereditary inborn errors of metabolism. The GC/MS system, with its potential for automated and rapid identification of many metabolites, provides the diagnostic accuracy necessary for a clinical program. This system also provides the methodology for detecting previously unrecognized inherited metabolic errors.

The methodology developed by this project will decrease the time required for antenatal diagnosis of certain metabolic disorders. The elapsed time until diagnosis is important because legal, psychological and, perhaps, obstetrical considerations have set a deadline of 20 weeks of gestation for selective abortion. This deadline is sometimes not met when amniotic cells obtained for diagnostic purposes fail to divide sufficiently rapidly in culture to provide adequate material for biochemical testing.

The study of system designs for automated GC/MS systems in the clinical environment will pave the way for a prototype system which will make more routinely available these powerful analytical tools. Such tools will be important in the inevitable regionalization of facilities for the screening, diagnosis and study of hereditary inborn errors of metabolism.

E. FACILITIES AVAILABLE

We will derive much of the clinically significant material for analysis from patients in the Premature Research Center and the Clinical Research Center of the Department of Pediatrics. Analyses will be performed in part on existing GC and MS equipment in the Department of Genetics. We now have a two column Varian Aerograph 2100 gas chromatograph used for specimen prescreening and an older, less effective Varian Aerograph 1200 gas chromatograph connected to a Finnigan 1015 quadrupole mass spectrometer. We have access to a Varian-MAT 711 high resolution GC/MS system to assist in the identification of compounds not readily identified by low resolution spectrometry alone. Also available to the project are the electronics facilities and software experience of the Instrumentation Research Laboratory of the Department of Genetics. Assuming the approval of the SUMEX resource proposal, we will have access to large scale computing support for the later applications of artificial intelligence to this research from the SUMEX PDP-10 machine.

F. COLLABORATIVE ARRANGEMENTS

This project involves an interdisciplinary collaboration between Drs. J. Lederberg (Principal Investigator) and A. Duffield (Associate Investigator) of the Department of Genetics, Drs. N. Kretchmer and H. Cann (Associate Investigators) of the Department of Pediatrics, and the Instrumentation Research Laboratory (including Dr. E. C. Levinthal and Mr. T. C. Rindfleisch). Dr. Jose M. Garcia-Castro, University of Puerto Rico School of Medicine has agreed to send us samples (urine and blood) from selected patients. We will also receive samples from collaborators at the Kaiser-Permanente Medical Center in Santa Clara, the Santa Clara Valley Medical Center, the Children's Hospital at Stanford, and the Los Angeles County Hospital.

This arrangement is a prototype of efforts to organize a systematic network of support to physicians at outlying centers for the mutual benefit of better care for their patients, and providing pre-screened, high-yield material for scientific study. Before this is formalized, we wish to build up practical experience with collaborations where personal understanding allows good communication about respective needs and flexibility in meeting urgent requirements.

REFERENCES

1) Harvey, D. J., and Horning, M.G., "Characterization of the Trimethyl-Silyl Derivatives of Sugar Phosphates and Related Compounds by Gas Chromatography-Mass Spectrometry," J. Chromat., 76, Page 51 (1973).

2) Biemann, K., Althaus, J.R., Biller, J, Donaghue, P.F., Evans, D.A., Forster, H.-J., Hertz, H.S., Hignite, C.E., Murphy, R.C., Preti, G., and Reinhold, V., Experientia, 26, Page 714, (1970).

3a) Pereira, W. E., Hoyano, Y., Reynolds, W. E., Summons, R. E., and Duffield, A. M., "The Simultaneous Quantitation of Ten Amino Acids in Soil by Mass Fragmentography," Anal. Biochem., in press (preprint attached).

3b) Summons, R. E., Pereira, W. E., Reynolds, W. E., Rindfleisch, T. C., and Duffield, A. M., "Analysis of Twelve Amino Acids in Biological Fluids by Mass Fragmentography Using a Quadrupole Mass Spectrometer-Computer System," Anal. Chem. in preparation.

3c) "Cytochemical Studies of Planetary Microorganisms - Explorations in Exobiology," Annual reports from 1962 to the present, NASA Grant NsG 81-60.

4) Smith, D.H., Buchanan, B.G., Englemore, R.S., Duffield, A.M., Yeo, A., Feigenbaum, E.A., Lederberg, J., and Djerassi, C., "Applications of Artificial Intelligence for Chemical Inference VII. An Approach to the Computer Interpretation of the High Resolution Mass Spectra of Complex Molecules: Structure Elucidation of Estrogenic Steroids," J. Amer. Chem. Soc., 94, p. 5962, (1972).

5) Schwartz, M.K., "Biochemical Analysis," Anal. Chem., 44, p. 9R, (1972).

6) Heathcote, J.G., Davies, D.M., and Haworth, C., "The Effect of Desalting on the Determination of Amino Acids in Urine by Thin Layer Chromatography," Clin. Chim. Acta, 32, p. 457, (1971).

7) Davidow, B., Petri, N.L., and Quame, B., "A Thin Layer Chromatographic Screening Procedure for Detecting Drug Abuse," Amer. J. Clin. Pathol., 50, p 714, (1968).

8) Efron, K. and Wolf, P.L., "Accelerated Single-column Procedure for Automated Measurement of Amino Acids in Physiological Fluids," Clin. Chem., 18, p 621, (1972).

9) Burtis, C.A., "The Separation of the Ultraviolet-absorbing Constituents of Urine by High Pressure Liquid Chromatography," J. Chromatog., 52, p 97, (1970).

10) Wilson-Pitt, W., Scott, C.D., Johnson, W.F., and Jones, G., "A Bench-top, Automated, High-resolution Analyzer for Ultraviolet Absorbing Constituents of Body Fluids," Clin. Chem., 16, p. 657, (1970).

11) Dalgliesh, C.E., Horning, E.C., Horning, M.G., Knose, K.L., and Yarger, K., "A Gas-liquid Chromatographic Procedure for Separating a Wide Range of Metabolites Occurring in Urine or Tissue Extracts," Biochem. J., 101, p. 792, (1966).

12) Teranishi, R., Mon, T.R., Robinson, A.B., Cary, P., and Pauling, L., "Gas Chromatography of Volatiles from Breath and Urine," Anal. Chem., 44, p. 18, (1972).

13) Pauling, L., Robinson, A.B., Teranishi, R., and Cary, P., "Quantitative Analysis of Urine Vapor and Breath by Gas-liquid Partition Chromatography," Proc. Nat. Acad. Sci. USA, 68, p. 2374, (1971).

14) Zlatkis, A. and Liebich, H.M., "Profile of Volatile Metabolites in Human Urine," Clin. Chem., 17, p. 592, (1971).

15) Ando, T., Nyhan, W. L., Bachmann, C., Rasmussen, K., Scott, R., and Smith, E. K., "Isovaleric Acidemia: Identification of Isovalerate, Isovalerylglycine and 3-Hydroxyisovalerate in Urine of a Patient Previously Reported as Having Butyric and Hexanoic Acidemia," J. Pediatrics, 82, 243 (1973).

16) Mrochek, J.E., Butts, W.C., Rainey, W.T., and Burtis, C.A., "Separation and Identification of Urinary Constituents by Use of Multiple-analytical Techniques," Clin. Chem., 17, p. 72, (1971).

17a) Horning, E.C. and Horning, M.G., "Human Metabolic Profiles Obtained by GC and GC/MS," J. Chromatog. Sci., 9, p. 129, (1971).

17b) Jenden, D.J. and Cho, A.K., "Applications of Integrated Gas Chromatography/Mass Spectrometry in Pharmacology and Toxicology," Ann. Rev. of Pharmacology, 13, Page 371, (1973).

18) Jellum, E., Stokke, O., and Eldjarn, L., "Combined Use of Gas Chromatography, Mass Spectrometry, and Computer in Diagnosis and Studies of Metabolic Disorders," Clin. Chem., 18, p. 800, (1972).

19) Campbell, A.G.M., Rosenberg, L.E., Snodgrass, P.J., and Nuzum, C.T., "Ornithine Transcarbamylase Deficiency: Neonatal Hyperammonemia in Males," New England J. of Med., 288, p. 1, (1973).

20) Daum, R. S. Seriver, C. R., Mamer, O. A., Delvin, E., Lamm, P., and Goldman, H., "An Inherited Disorder of Isoleucine Catabolism Causing Accumulation of Apha-methylacetoacetate

21) Levy, H. L. and Montag, P. P., "Free Amino Acids in Human Amniotic Fluid. A Quantitative Study by Ion Exchange Chromatography," Pediat. Res., 3, page 113 (1969).

22) Nadler, H. L., "In-Vitro Detection of Familial Metabolic Disorders." Pediatrics,49, 329 (1972).

23) Mahoney, M. J., Rosenberg, L. E., Waldenstrom, J., Lindblad, B., and Zetterstrom, R., Prenatal Diagnosis of Methylmalonicaciduria" (abstract), Pediat. Res., 7, 342 (1973).

24) Rosenberg, L. E., Disorders of Propionate, Methylmalonate and Vitamin B12 Metabolism", In the Matabolic Basis of Inherited Disease, Ed. by Stambury, J. B., Wyngarden, J. B., and Fredrickson, D. S., 3rd Edition, p. 440.

25) Goodman, S. I., Mace, J. W., Turner, B., and Garrett, W. J., "Antenatal Diagnosis of Arginosuccinic aciduria" (abstract), Clin. Res., 21, 295 (1973).

26a) Nichols, J., "Antenatal Diagnosis and Treatment of the Adenogenital Syndrome" Lancet, 1, 83 (1970).

26b) Matalon, R., Dorfman, A., Nadler, H. L., and Jacobson, C. B., "A Chemical Method for the Antenatal Diagnosis of Mucopolysaccharidoses." Lancet, 1, 83 (1970).

26c) Nadler, H. L., and Messina, A. M., In-Utero Detection of Type II Glycogenosis (Pompe's Disease)" Lancet, 2, 1277 (1969).

27) Reynolds, W.E., Bacon, V.A., Bridges, J.C., Coburn, T.C., Halpern, B., Lederberg, J., Levinthal, E.C., Steed, E., and Tucker, R.B., "A Computer Operated Mass Spectrometer System," Anal. Chem., 42, Page 1122, (1970).

28) Morrow, G., Schwartz, R.H., Hallock, J.A., and Barness, L.A., "Prenatal Detection of Methylmalonic Acidemia," J. Pediatrics, 77, p. 120, (1970).

29) Daum, R. S., Seriver, C. R., Mamer, O. A., Delvin, E., Lamm, P., and Goldman, H., "An Inherited Disorder of Isoleucine Catabolism Causing Accumulation of Alpha-methylacetoacetate and Alpha-methyl-beta-hydroxybutyrate and Intermittant Metabolic Acidosis, Pediat. Res., 7, 149 (1973).

30) Haworth, J.C., Ford, J.D., and Younoszai, M.K., "Familial Chronic Acidosis due to an Error in Lactate and Pyruvate Metabolism," Canad. Med. Ass. J., 97, p. 773, (1967).

31) Herndon, J.H., Steinberg, D., and Uhlendorf, B.W., "Refsum's Disease: Defective Oxidation of Phytanic Acid in Tissue Cultures Derived from Homozygotes and Heterozygotes," New England J. of Med., 281, p. 1034, (1969).

32) Fallon, J.H., Smith, L.H., Graham, J.B., and Burnett, C.H., "A Genetic Study of Hereditary Orotic Aciduria," New England J. of Med., 270, p. 878, (1964).

32a) Lawless, J.G. and Chadha, M.S., "Mass Spectral Analysis of C(3) and C(4) Aliphatic Amino Acid Derivatives," Anal. Biochem., 44, p. 473, (1971).

33) Sutton, H. E., "BAIBuria in Inherited Biochemical Disease," New York, McGraw-Hill, 1960, pp.792-806.

34) Awapara, J., "The Leukemias," International Symposium (Detroit) New York, Academic Press, 1956, p. 353.

35) Rubini, J. R., Cronkite, E. P., Bond, V., and Fleednir, T. M., "Urinary Excretion of BAIB in Irradiated Human Beings." Proc. Soc. Exper. Biol. and Med., 100, Page 130 (1959).

36) Wright, S. W. and Fink, K., "The Excretion of BAIB in Normal, Mongoloid and Non-Mongoloid Mentally Defective Children," Am. J. Ment. Deficiency, 61, Page 530 (1957).

37) Berry, H. K., "Individual Metabolism Patterns: II. Excretion of BAIB." Metabolism, 9, Page 373 (1960).

38) Harris, H., "Family Studies on the Urinary Excretion of BAIB," Ann. Eugenics, 18, Page 43 (1953).

39) Calchi-Novati, C., Cepellini, R., Biancho, I., Silvestroni, E. and Harris, H., "BAIB Excretion in Urine. A Family Study in an Italian Population," Ann. Eugenics, 18, Page 335 (1954).

40) DeGrouchy, J. and Sutton, H. E., "A Genetic Study of BAIB Excretion," Am. J. Human Genetics, 9, Page 76 (1957).

41) Yanai, J., Kakimoto, Y., Tsujio, T., and Sano, I., Am. J. Human Genetics, 21, Page 115 (1969).

42) Gartler, S. M., "A Family Study of BAIB Excretion," Am. J. Human Genetics 8, Page 120 (1956).

43) Gartler, S. M., Firschein, I. L., and Kraus, B. S., "An Investigation into the Genetics and Racial Variation of BAIB Excretion," Am. J. Human Genetics, 9, Page 200 (1957).

44) Gartler, S. M., "A Metabolic Investigation of Urinary BAIB Excretion in Man, Arch. Biochem. 80, 400 (1959).

45) Fink, K., Henderson, R. B., and Fink, R. M., "BAIB: A Possible Factor in Pyrimidine Metabolism," Proc. Soc. Exptl. Biol. Med., 78, Page 135 (1951).

46) Crumpler, H.R., Dent, C.E., Harris, H., and Westall, R.G., "BAIB, A New Amino Acid Obtained from Human Urine," Nature, 167, Page 307, (1951).

47) Goedde, H.W. and Brunschede, H., "BAIB: A TLC Method for the Quantitative Estimation in Human Urine," Clin. Chim Acta, 11, page 485, (1965).

48) Fessas, P.H., Koniavitis, A., and Zeis, P.M., J. Clin. Path., 22, Page 154, (1969).

49) Killmann, S.A., Rubini, J.R., Cronkite, E.P., and Bond, V.P., "Urinary Excretion of Beta-Amino Isobutyric Acid (BAIBA) in Leukemia and Polycythemia Vera," Acta Haemat., 25, Page 81, (1961).

50) Lee, M.B., Neill, D.W., and Bridges, J.M., "Urinary Amino Acid Excretion in Subjects with Leukemia," J. Clin. Path., 20, Page 70, (1967).

51) Schreier, "The Behavior of Amino Acids in Body Fluids During Development and Growth: Physiology and Pathology in Amino Acid Pools," J.T. Holden, Editor, Elsevier, New York, Page 276, (1962).

52) Ranlov, P. and Siggaard-Andersen, O., "Late Metabolic Acidosis in Premature Infants; Prevalence and Significance," Acta Ped. Scand., 54, Page 531, (1965).

53) Levine, S.Z., Marples, E., and Gordon, H.H., "A Defect in the Metabolism of Tyrosine and Phenylalanine in Premature Infants, I: Identification and Assay of Intermediary Products," J. Clin. Invest., 20, Page 199, (1941).

54) Dancis, J. and Levitz, M, "Abnormalities of Branched-Chain Amino Acid Metabolism: The Metabolic Basis of Inherited Disease," Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., Editors, McGraw-Hill, New York, 3rd Edition, Page 426.

55) Bartter, F.C., "Hypophosphatasia: The Metabolic Basis of Inherited Disease," Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., Editors, McGraw-Hill, New York, 3rd Edition, Page 1295.

56) Rasmussen, K., "Phosphorylethanolamine and Hypophosphatasia, Danish Med. Bull., 15 (Suppl. II), 1 (1968).

57) Bevis, D.C.A., "Composition of Liquor Amnii in Haemolytic Disease of Newborn," Lancet, ii, Page 443, (1950).

58) DeMarco, C., "Aminoacidie Chetoacidi Presenti nel Liquido Amniotico," G. Biochem., 3, Page 296, (1954).

59) Thomas, J.A. and Thiery, J.P., "The Use of Deproteinized and Purified Amniotic Fluid. Culture and Prolonged Conservation of the Tissues; Microbiological Cultures," Bull. Soc. Chim. Biol., 35, Page 841, (1953).

60) Orlandi, C., Torsello, R.V., and Bottiglioni, F., "Analisi Qualitativa e Dosaggio Semiquantitativo degli Aminoacidi Contenuti nel Liquido Amniotico," Attual. Ostet. Ginec., 4, Page 871, (1958).

61) Wirtschafter, Z.L., "Free Amino Acids in Human Amniotic Fluid, Fetal and Maternal Serum," Amer. J. Obstet. Gynec., 76, Page 1219, (1958).

62) Sassi, D., "Sulla Presenza degli Aminoacidi nel Liquido Amniotico," Monogr. Ostet. Ginec., 33, Page 683, (1962).

63) Warren, C., Allen, J.T., and Holton, J.B., "Assessment of Foetal Lung Maturity by Amniotic Fluid Fatty Acid Analysis," Clin. Chim. Acta, 44, Page 457, (1973).

64) Reiner, E. and Hicks, J.J., "Pyrolysis-GLC Studies on Amniotic Fluid: A Prospective Method for Confirming Inborn Errors of Fetuses," Chromatographia, 5, Page 529, (1972).

65) Hagenfeldt, L. and Kagenfeldt, K., "Gas Chromatographic-Mass Spectrometric Analysis of Organic Acids in Amniotic Fluid," Clin. Chim. Acta, 42, Page 219, (1972).

66) New, M. I., "Antenatal Diagnosis of the Adrenogenital Syndrome," Lancet, 1, 83 (1970).

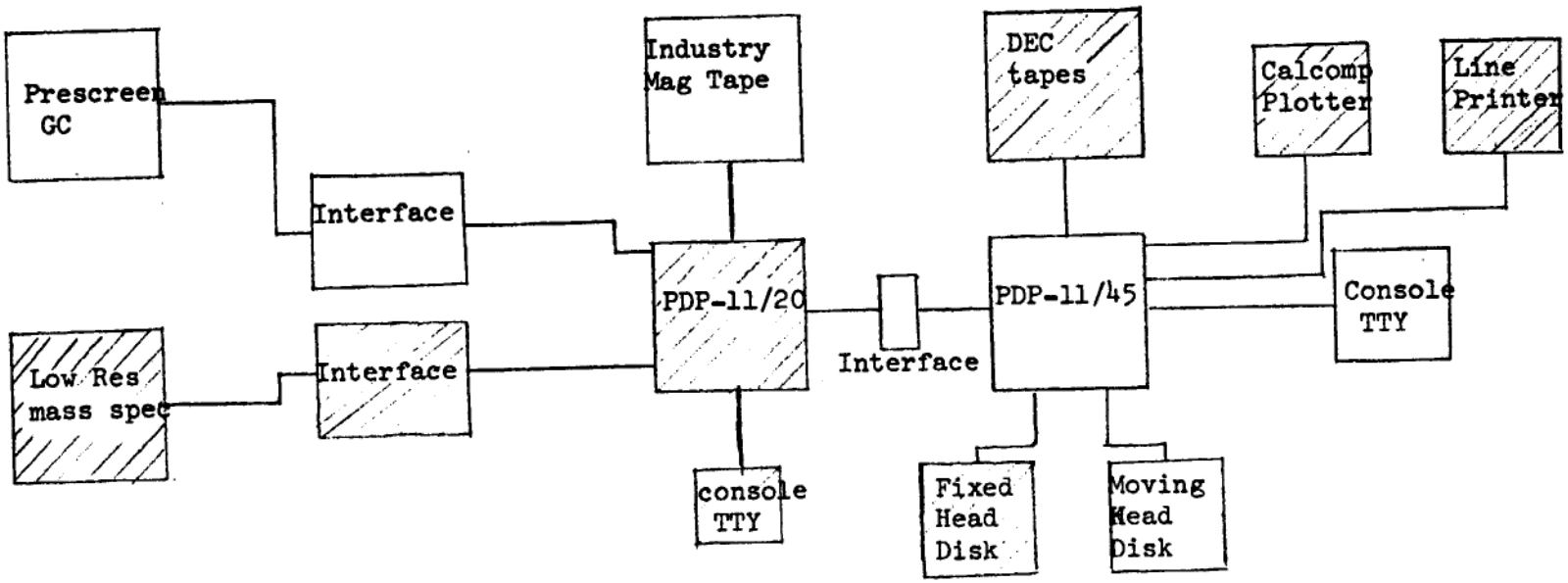


FIGURE 1. PROPOSED DATA SYSTEM HARDWARE CONFIGURATION

Prof. J. Lederberg
Department of Gene

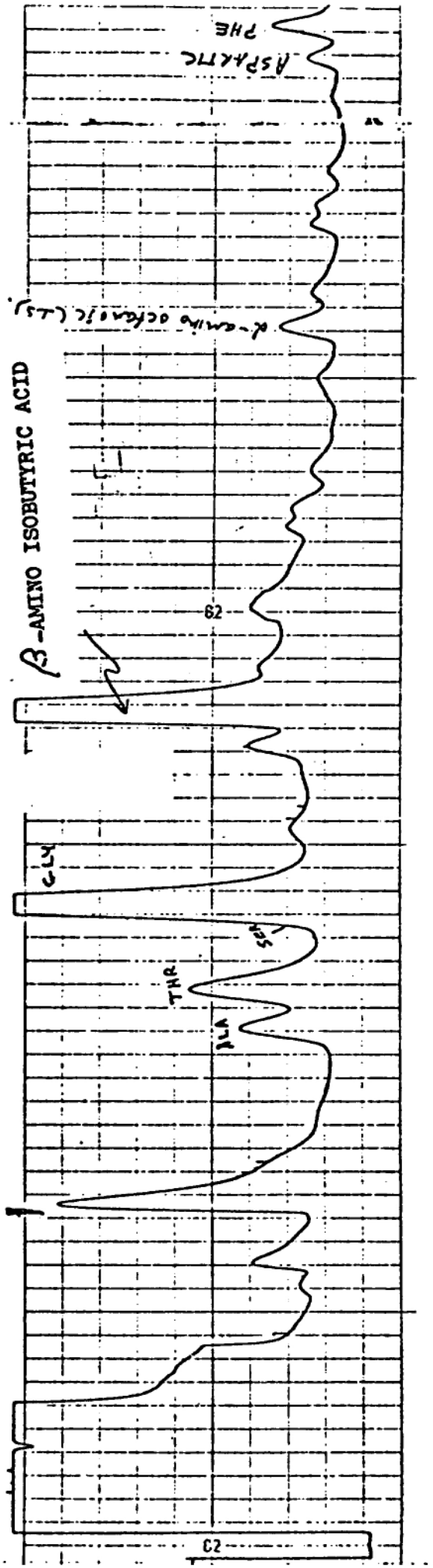


FIGURE 2

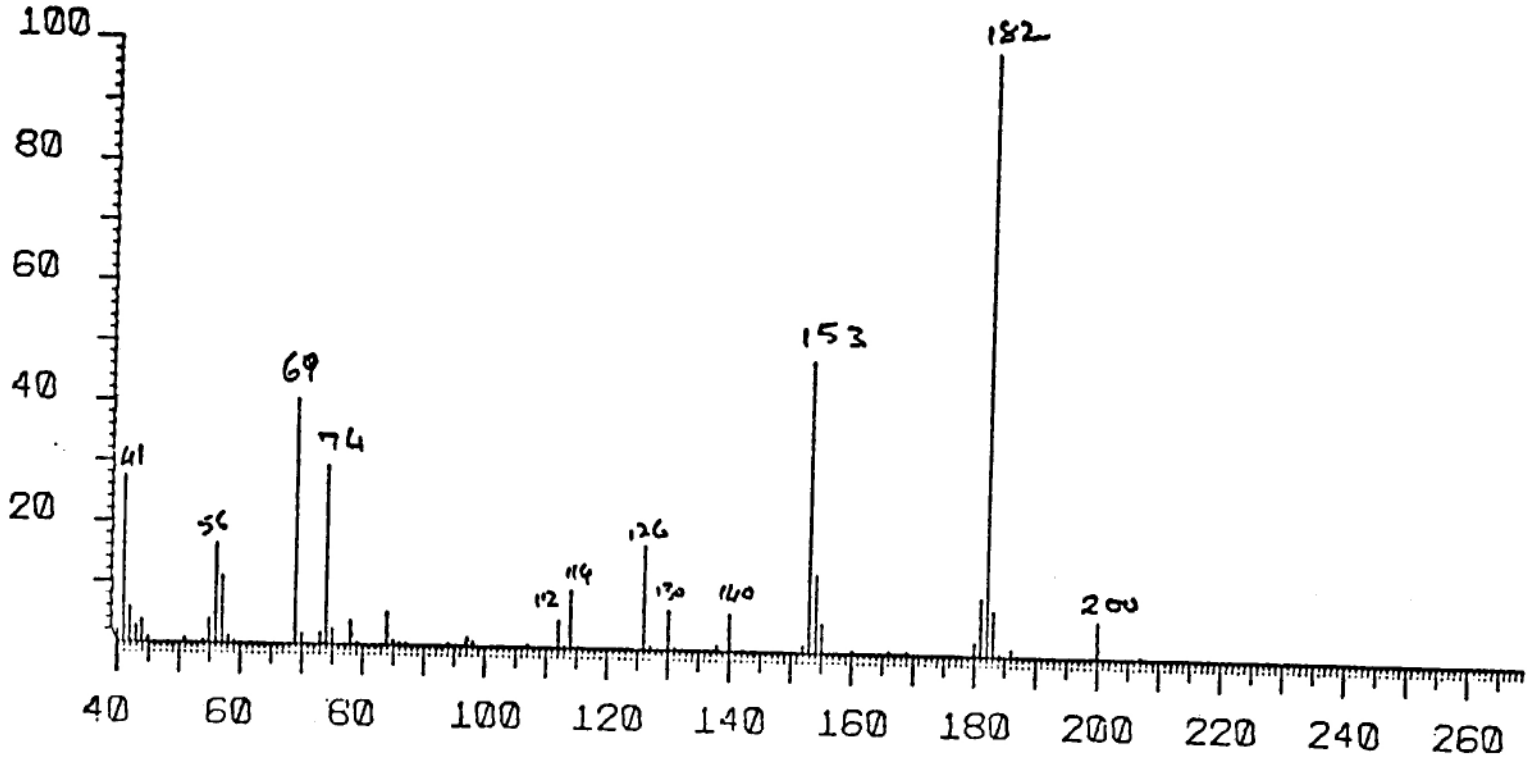


FIGURE 3

Mass spectrum of β-amino isobutytic acid