

**PROPOSAL FOR A
GENETICS RESEARCH CENTER**

**Submitted to the
National Institute of
General Medical Sciences**

June 1, 1973

**School of Medicine
Stanford University**

SECTION 1

Form Approved
Budget Bureau No. 68-R0249

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE GRANT APPLICATION	LEAVE BLANK		
	TYPE	PROGRAM	NUMBER
	REVIEW GROUP		FORMERLY
	COUNCIL (Month, Year)		DATE RECEIVED

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 15A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)

Genetics Research Center

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)

Lederberg, Joshua

2B. TITLE OF POSITION

Professor and Chairman

2C. MAILING ADDRESS (Street, City, State, Zip Code)

 Department of Genetics
 Stanford University School of Medicine
 Stanford, California 94305

2D. DEGREE

Ph.D.

2E. SOCIAL SECURITY NO.

[REDACTED]

2F. TELEPHONE DATA

 Area Code: 415
 TELEPHONE NUMBER AND EXTENSION: 321-1200 Ext. 5801

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)

Depts. of Genetics and Pediatrics

2H. MAJOR SUBDIVISION (See Instructions)

N/A

7. Research Involving Human Subjects (See Instructions)

 A. NO B. YES Approved:
 C. YES - Pending Review 6-29-79 Date

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)

FROM

1/1/74

THROUGH

12/31/78

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3

\$2,659,305

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD

\$537,800

6. PERFORMANCE SITE(S) (See Instructions)

 Department of Genetics and
 Department of Pediatrics
 Stanford University School of Medicine
 Stanford, California 94305

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 15B)

9. APPLICANT ORGANIZATION(S) (See Instructions)

 Stanford University
 Stanford, California 94305
 IRS No. 94-1156365
 Congressional District No. 17

11. TYPE OF ORGANIZATION (Check applicable item)

 FEDERAL STATE LOCAL OTHER (Specify)

Private, non-profit

12. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE

 K.D. Creighton
 Deputy Vice President for Business & Finance
 Stanford University
 Stanford, California 94305

Telephone Number (415) 321-2300 X2251

10. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)

 Kathleen C. Butler
 Sponsored Projects Officer

 c/o Sponsored Projects Office
 Telephone Number (415) 321-2300 Ext. 2883

13. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)

01 School of Medicine

14. ENTITY NUMBER (Formerly PHS Account Number)

458210

15. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Per" signatures not acceptable)	A. SIGNATURE OF PERSON NAMED IN ITEM 2A (See also page 1)	DATE MAY 30 1973
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 10	DATE 5/30/73

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

LEAVE BLANK

PROJECT NUMBER

RESEARCH OBJECTIVES

NAME AND ADDRESS OF APPLICANT ORGANIZATION

Stanford University, Stanford, California 94305

NAME, SOCIAL SECURITY NUMBER, OFFICIAL TITLE, AND DEPARTMENT OF ALL PROFESSIONAL PERSONNEL ENGAGED ON PROJECT, BEGINNING WITH PRINCIPAL INVESTIGATOR

Joshua Lederberg, Professor and Chairman, Department of Genetics [REDACTED]
 Howard Cann, Associate Professor of Pediatrics, Dept. of Pediatrics [REDACTED]
 Norman Kretchmer, Professor of Pediatrics, Department of Pediatrics [REDACTED]
 Leonard Herzenberg, Professor of Genetics, Department of Genetics [REDACTED]
 Luca Cavalli-Sforza, Professor of Genetics, Department of Genetics [REDACTED]
 Luigi Luzzatti, Professor of Pediatrics and Community and Preventive Medicine,
 Department of Pediatrics [REDACTED]
 Clifford R. Barnett, Associate Professor of Pediatrics, Professor of Anthropology,
 Department of Pediatrics [REDACTED] *(CONTINUED BELOW)

TITLE OF PROJECT

GENETICS RESEARCH CENTER

USE THIS SPACE TO ABSTRACT YOUR PROPOSED RESEARCH. OUTLINE OBJECTIVES AND METHODS. UNDERSCORE THE KEY WORDS (NOT TO EXCEED 10) IN YOUR ABSTRACT.

A comprehensive program of basic and clinical research, uniting the efforts of the Department of Genetics and Pediatrics, will apply advances in analytical instrumentation to problems of genetic polymorphism and disease in man.

Program areas include:

Genetic errors of metabolism identified by computer-managed gas chromatography and mass spectrometry, applied to urine, blood and amniotic fluid;

Detection of fetal cells in maternal circulation;

Expression of genetic markers in fetal cells, linkage studies; antenatal diagnosis;

Polymorphisms involving specific binding to plasma proteins;

Psychological impact of genetic disease and counseling practices.

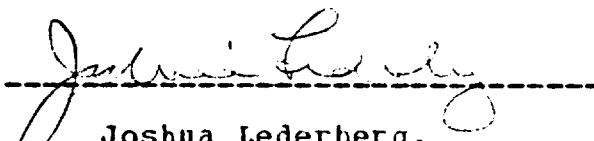
* Kenneth Tsuboi, Sr. Scientist, Department of Pediatrics [REDACTED]
 Alan Duffield, Research Associate, Department of Genetics [REDACTED]
 Wilfried Pereira, Jr., Research Associate, Department of Genetics [REDACTED]

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The undersigned agrees to accept responsibility for the scientific and technical conduct of this project and for provision of required progress reports if a grant is awarded as the result of this application.

MAY 30 1973

Date



Joshua Lederberg,
Principal Investigator

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SECTION I

Introduction and Program Summary

INTRODUCTION

The Genetics Research Center that would be enabled by this grant will be a new level of cooperative involvement of the Departments of Genetics and Pediatrics of the Stanford University School of Medicine in research in medical genetics and the application of such research to clinical aspects of medical genetics. We are requesting support for an interrelated set of basic and applied research projects involving members of both departments. Besides their importance as basic science, these investigations relate to genetic counselling, pathogenesis and diagnosis of genetic disorders and the mechanisms of human adaptation in genetic variation. In preparing this application, the two departments, labelled as basic science and clinical science respectively, have already enhanced the coordination of their activities for the benefit of patients with genetic disorders and their families.

Since 1959, the inception of the School of Medicine on the Stanford University campus, Genetics and Pediatrics each have been deeply involved in various aspects of genetic research. A number of ad hoc cooperative efforts have also evolved. Both departments are together teaching medical genetics to medical students. Informal communications in training graduate students and postdoctoral fellows have been excellent. Research projects undertaken in one department have involved consultation or collaboration with various members of the other department, and clinical problems have stimulated basic research activities in both departments.

In accordance with its clinical mission Pediatrics has focused on patients with genetic disorders, diagnosis, pathogenesis, and therapy; and the counselling of such patients and their families. Pediatrics has likewise emphasized perinatal biology, featuring a program of detection and management of high risk pregnancies in which the potential for morbidity or mortality of mother, fetus and/or newborn infant is high.

Genetics is well known for its basic research programs in molecular biology, population genetics, and immunogenetics, whose potential for clinical applications have long been recognized by members of both departments. As knowledge in molecular biology has increased and the necessary technology has permitted applications to the study of man and his disorders, it has become increasingly evident that relevant research efforts of both departments should converge on problems connected with clinical care. Both departments have begun to cooperatively design and carry out research applicable to genetic medicine and to plan for the application of the findings to the care of patients encountered on the various clinical services of the Department of Pediatrics.

The underlying theme that unifies the range of specific studies outlined below is genetic polymorphism in man. The pediatrician views this as the source of genetic disease; the basic scientist as an expression of gene mutation and evolutionary pressures. These are roles shared within as well as among individual investigators. To these challenges are brought a combination of clinical insights, experience with several aspects of basic genetics, and new analytical technologies -- the application of instruments like the mass-spectrometer, the computer and the cell sorter.

Besides the specific research projects to be funded under the Center grant, we work in an extensive context of genetic and related research -- and indispensable aspect of our own environment, and a set of activities to which the Center organization may also bring a new focus for developments that should advance both basic scientific knowledge and its application to human problems.

COMMENTS ON ORGANIZATION

The Stanford Genetics Research Center is organized as a cooperation of the Departments of Genetics and Pediatrics. Thus, it follows, rather than conflicts with existing departmental authority. This minimizes the need for new, formal arrangements that might be essential in other circumstances. It does reflect a deep-seated intention on the part of both departments to improve the application of new analytical methodology and of basic genetic science to clinical problems, and to relate clinical studies to enhancing knowledge of genetic polymorphism in man. The length of the following comments on organizational detail may lead one to believe that we are more interested in management than in scientific substance.

Exactly the opposite is the case, but we cannot afford the consequences of misunderstanding about how we will work together. Obviously, as at any other university, the actual character of our "organization" has a larger compass and bears little resemblance to the formal hierarchy of imputed power. Research is actually done in individual laboratories under the supervision of an autonomous faculty member. Most "direction" must be in the form of intellectual stimulation and criticism, and in the selection of key people for roles of autonomous responsibility.

Professor Joshua Lederberg, Chairman of the Department of Genetics, will serve as Principal Investigator and Director of the overall Genetics Center Program. Dr. Howard Cann, Associate Professor of Pediatrics, will serve as Associate Program Director with special responsibility for clinical research related to the Center's activities. (If this application is approved, and offers a funding basis for the step, it is anticipated that Dr. Cann will receive a joint appointment in the Genetics Department as well.)

The Director will have executive responsibility for the administration of the program, including the formulation of extended and new projects, their budgets, and reports to the NIGMS of progress under the grant, and internal functions of coordination, information and criticism.

The internal administration of the program is facilitated by the Director's position as Chairman of the Genetics Department, and by the enthusiastic support and participation of Dr. Irving Schulman, Chairman of Pediatrics, and of other senior members of both departments. In practice, the Director will be advised by, and will delegate subproject responsibility to, these colleagues in accordance with their special skills and interests.

The Director will also invite an informal visiting committee, principally from among his colleagues involved in similar lines of work at other West Coast institutions, to visit Stanford annually and to advise him of advantageous directions of

policy. This group may also help to identify new opportunities for inter-institutional cooperation and coordination, especially to further our emphasis on building complementary rather than competitive capabilities. These annual visits may also advantageously coincide with a local or regional conference on research progress concerning genetic polymorphism and disease.

The Director must play both an attractive and a critical role. That is he must encourage his colleagues to invest in the effort needed to orient their activities towards the common goals of the Center's research. At various times this attraction may be enhanced by the fiscal support of the Center's budget; on the other hand, it is hard to predict what the level of that support will be, and the level of discretion that will be delegated to the Director. Realistically speaking, then, his role is more inspirational than directive, at least with respect to the tenured fellow-members of the faculty. If the Center's budget is approved, and develops some de facto continuity, he may have greater formal authority -- but always with accountability to outside review groups and to the independent authority of other professors. The moral and intellectual influence that the Director can exert will obviously be greatest in relation to people in the same or closely co-functioning departments who already relate in many other ways. His main positive role will be to help bring in new ideas -- his own and others -- into the progress of the Center's research.

Conversely, he has responsibilities for negative functions -- especially to discourage the growth or even survival of projects that are inherently unsound, or become obsolete. Within a close-knit institution there are often serious personal and political obstacles to the exercise of objective scientific quality judgments. These limitations are both aggravated and compensated for by the limited discretion usually given to a director, i.e., the fact that final decisions about project items are generally made by peer review groups.

All of these problems would be much worse if the Director had the responsibility of coordinating every genetics-relevant activity in the school. He would have neither the budget nor the political mandate to do this well. In fact, the present proposal encompasses a serious and soul-searching effort to initiate a major Center program at a practically achievable and useful level of integration. It will also be the basis for less formal but equally important co-operations with other departments, whose policies and programs can be influenced but not directed in the exercise of our responsibilities.

Provision for Succession of Directorship.

Day to day deputization will be handled ad hoc in the same fashion as the duties of the departmental chairman. The question of possible succession, in the event of incapacity or transfer of the Director, poses a more difficult question. The Associate Director will serve pro-tem, in concert with the (acting)

chairman of each department; and a new director will be proposed by the Dean of the school after consultation with the visiting committee and with the participating faculty of the departments.

The Associate Director's role will be to maintain contacts with other clinical departments for patient referrals to Center projects, and to assure a high standard of clinical responsibility in all Center research activities that deal with patients. Stanford has long experience with Clinical Research Centers -- e.g. in medicine, in cancer therapy, in cardiovascular disease, and in premature infant research. (These facilities afford further research bed opportunities for protocols involving intensive study of selected cases without burdening the present proposal with inappropriate in-patient care charges). In addition, they have led to the institutionalization of formal procedures for the ethical review of research involving human subjects. Dr. Cann will be responsible for defending the present proposal before that review committee, and for assuring that all further research under this grant is properly submitted and reviewed according to established procedures.

An Application for renewal of a Genetics Research Training Grant was approved by Council for the period 1974-1979. This action was superseded, however, by the executive decision to wind down such programs and we are now operating on a decreasing budget to cover only the previously enrolled trainees.

That training grant renewal had, for the first time, provided for clinical research in genetics as part of our program. Lacking these funds, it is, of course, imperative that the research activities of the Center also double as training opportunities for graduate research assistants and postdoctoral fellows (research associates.) We will make every effort to fit each fellow into employment in the support roles of the Center. We cannot pretend, however, to be able to support the training functions at the level previously assumed without a compensatory expansion of the funding for these research programs. This is not reflected in the budgets submitted at this time.

The department faculties having agreed to the programs of the Center, there is much to do in substance, but little in organizational process, to coordinate our activities and insure meeting the Center's objectives. We are already in frequent, almost daily, communication in the pursuit of our other duties and socially. These contacts are promoted by the fact that our laboratories and offices are immediately adjacent in the Medical Center Building.

The Genetics Center Budget is intended to fund programs of new substance and scope or those for which other support (e.g. NASA) is no longer available. It goes into new territory beyond many on-going research projects which are identified in the material on each affiliated investigator. However, the coordinated funding and administration of the Center allows more careful planning and resource-sharing than would be possible for

a disparate set of individual proposals.

We intend to minimize the indefinite prolongation of autonomous projects merely to keep a research group intact. Instead we will promote an innovative search for new opportunities and on-going mutual scrutiny of projects.

For the continuity of a program as complex as the Center, we are requesting an award for 5 years. Yet it is obvious that many unforeseen obstacles and opportunities will arise in that interval to warrant changes of tactical direction and evolution of major strategies. The responsibility of the Director to manage this progression of emphasis is his most important executive role. It will be discussed with the visiting committee and reported to NIGMS from year to year, to assure the consistency of his decisions with the approved mandate of the Center grant.

This is not an assertion of unwarranted latitude. To the contrary, the political structure of a university ensures, if anything, a great deal of conservatism in changes of direction and support for individual programs, once approved.

The present proposal will also be a model for further developments to be initiated with the Departments of Gyn-Ob., Medicine, Psychiatry and Dermatology -- to mention only those with which we have had tangible discussions. For various reasons, potential projects with them are not yet ripe for formal submissions; these will be the subject of future requests, depending in part on the climate for funding expansion of genetic research. Meanwhile, we have working arrangements to assure their awareness of our activities and the provision of patient material for mutual advantage.

A brief description of the role of each participant in this program and of his research and/or clinical interests are presented here:

THE STANFORD ENVIRONMENT

Besides the Program Director (Professor Lederberg) and Associate Director (Professor Cann) the faculty and professional personnel associated with the Center, their roles and biographical detail are spelled out in Section IX.

In addition, we have included biographical sketches of a number of key members of the environment of the Department of Pediatrics and Genetics, although their research projects are not included in funding under the Center at this time.

Within one or two years after the activation of this program, we anticipate the direct participation of one or more colleagues from the Department of Obstetrics and Gynecology. A search for a chairman of this department is presently under way, and we expect that this individual will renew active research

here in fetal physiology and fetal monitoring. We look forward to interacting in this program with our obstetrical colleagues in various projects pertaining to antenatal detection of genetic disorders and selective abortion.

We have also consulted with the chairmen of the Departments of Medicine and of Dermatology, and have encountered great interest in the development of the Center, and assurances of cooperation in the referral of patients who would be pertinent to our screening technologies. Their position is best witnessed by the attached correspondence. At some future date, we may expect to formulate more specific proposals, as a consequence of recruiting now under way.

The Department of Psychiatry will be chaired by Albert Stunkard (now at University of Pennsylvania) beginning September 1973. He is personally well known to the Director (who served on the search committee for this appointment, which was chaired by Professor Schulman) and many other participants and we have strong assurances that the Psychiatry Department's interest in genetic etiologies of psychiatric disease will continue under his leadership. Dr. Stunkard has also voiced his concern that psychiatrists have not hitherto been more directly involved in problems of genetic counseling, like those reflected in Dr. Barnett's proposal here; and we are looking forward to closer cooperation on such issues after his assumption of his duties. For some time, Professor Cavalli-Sforza of the Genetics Department has been cooperating with Professor Barchas of Psychiatry on polymorphisms of biogenic amine metabolism.

Genetic counseling, per se, is a responsibility mainly of the Pediatrics Department -- diseases of adults only occasionally present serious problems of reproductive policy of the family. Genetic disease, of course, presents itself to all of the medical and surgical specialities. In recent years, these departments have not seen any requirement for a special organization to deal with genetic aspects of internal medicine or surgery -- and indeed these are fully and competently integrated into their overall teaching and practice. When special problems do arise, there are no impediments to consultation with pediatricians and with basic geneticists. Many members of those departments are already involved in immunogenetic and other genetic research.

DATE: May 25, 1973

To : Joshua Lederberg, Ph.D.

FROM : Daniel D. Federman, M.D. (Chairman, Department of Medicine)

SUBJECT: Genetics Research Center

Dear Josh:

I am not really clear about your timetable for submission of the proposal for a Genetics Research Center, but I thought I would make explicit several areas of potential involvement of the Department of Medicine.

1. Pharmacogenetics - We have a slot in Clinical Pharmacology for a new Assistant Professor. Stan Cohen and I have discussed finding someone with a particular interest in the area of pharmacogenetics, but at the present time, no particular individual has been chosen.

2. Endocrinology -

A. Hormonal control of gene expression. We will have at least one new person in the Endocrine Division in the next year and have placed as our first interest finding someone working in the area of the effective hormone on the expression of genetic information. This would provide an obvious link to a genetic center.

3. Early detection of hereditary endocrine disease. There are at least 80 endocrinopathies which show Mendelian segregation and a number more which probably have a major genetic control. Early detection of asymptomatic patients can be achieved by suitable provocative testing. This opens the way to family studies in which a) the hereditary mechanism can be defined, b) preventive or therapeutic intervention can be tried before irreversible damage has occurred, and c) the effects that intervention can be compared to the natural history of the disorder. The latter approach has important application to the management of "patients" discovered by multiphasic screening techniques.

4. Immunogenetics - This area of course is strongly represented in our Department and we could, I believe, contribute significantly to the activities of the genetic center in this area.

I should have liked to provide more specific information, but until particular people have been identified, it would be difficult to do so. I hope this is of some help -- I am certainly anxious for an active collaboration.

Sincerely,

DJF

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CLINIC FOR CHILDREN WITH PSORIASIS AND OTHER INHERITABLE SKIN DISEASES

Directors:

Eugene M. Farber, M.D.,
Professor and Chairman, Department of Dermatology

Alvin H. Jacobs, M.D.
Professor of Dermatology and Pediatrics

In the clinics of the Department of Dermatology there are about 12,000 total patient visits annually, of these approximately 3,000 are children seen in the Pediatric Dermatology Clinic. Over half of these children have either psoriasis or atopic dermatitis, both conditions with important heritable factors in their etiology. In addition many patients are seen with the less common genodermatoses, such as, the various types of ichthyosis, epidermolysis bullosa, and the many types of heritable neurocutaneous disorders. At the present time a special clinic is being established for the care and study of children with psoriasis and other inheritable skin diseases. The purpose of this clinic is not only to offer more complete care for these types of patients, but to study the genetic aspects of these conditions and offer genetic counselling to the patients and their families.

The Department of Dermatology already has an unmatched reservoir of material for genetic study in its Psoriasis Life History Research files; a data-bank of approximately 8000 patient histories. Not only is this computerized information a source of information on the epidemiology of psoriasis, but has served as the basis for several published follow-up studies on the genetics of psoriasis including family and twin studies.

At present several genetic studies are under way. For example, Dr. Farber and associates are studying the association between H-LA antigens and psoriasis. Drs. Jacobs and Chan are studying and developing techniques for accurate counting of melanocytes in pigmented and depigmented macules in order to predict the development of certain genodermatoses, such as neurofibromatosis, tuberous

sclerosis etc.

Studies are also under way to properly classify the various genetic types of ichthyosis and in cooperation with Dr. Howard Cann we are planning to investigate the x-linked blood type in the families with x-linked ichthyosis.

It is hoped that in the future, with the vast resources available in Genetics, Pediatrics, and Dermatology at Stanford, an investigation could be launched into the genetic aspects of atopic dermatitis, one of the commonest skin problems of childhood.

CLINICAL FACILITIES

The Stanford Medical Center, located midway between the cities of San Francisco and San Jose on the campus of Stanford University, consists of the School of Medicine, the University Hospital and the Stanford Clinics. Stanford is the only university hospital in the Regional Medical Program (RMP) Area III and functions as a tertiary care center. RMP Area III consists of eleven counties in mid California consisting of 2.6 million people. There are 60 acute hospital facilities, and 4000 physicians practice in RMP Area III. Approximately 90 percent of patients admitted to Stanford University Hospital live in this area. Additionally, patients for the clinics and university hospital are also drawn from other areas in and out of California. RMP Area III and its population form a base from which is drawn numerous patients with diseases resulting from major genes or chromosomal abnormalities or with a significant genetic component. Most of these patients are seen primarily on one of the services of the Department of Pediatrics or are referred for diagnostic evaluation and/or genetic counselling from other clinical services at the medical center. Clinical teaching services at other hospitals* affiliated with the Department of Pediatrics add to the source of patients with medical genetic problems.

As the Genetics Center activities evolve and research developments become available for clinical application, we anticipate that more patients will be referred to this medical center for genetic counselling and evaluation and management of genetic disease. The number of patients seen annually by the combined clinical facilities of the Department of Pediatrics for genetic disorders is estimated to be about 200. We predict that this number will double during the course of the Genetics Center grant.

Other clinical divisions of the Medical Center, of course, also see many adult patients with genetic disorders. Historically, Genetics has had weaker ties with them than with Pediatrics, although several joint projects connect with Medicine (H. McDevitt) and with Psychiatry (J. Barchas; S. Kessler). The appointment (effective February 1) of a "genetic endocrinologist", Dr. Daniel Federman, as new head of Medicine should remove administrative obstacles to extensions of Center-related programs.

Patients with genetic disorders are seen in the following pediatric subspecialty clinics at the Stanford Medical Center.

*The Children's Hospital at Stanford and the pediatric services at the Santa Clara County Hospital (San Jose) and at the Kaiser-Permanente Medical Center in Santa Clara.

Genetic Counselling Clinic (Director, Dr. Howard Cann, Associate Professor of Pediatrics)

Approximately fifty patients and their families are referred annually for genetic counselling. These include patients from outside of the Stanford Medical Center and from various clinical services at the Medical Center. About 50% of families seen are counselled for disorders determined by major genes at single loci. The remaining 50% of patients represent more complex counselling problems, e.g. possible sporadic cases and phenocopies, multifactorial threshold traits, undiagnosed familial disorders, and excessive exposure to radiation. Counselling includes confirmation of the diagnosis of the disorder and identification of heterozygotes among asymptomatic relatives at risk (autosomal recessive and X-linked recessive disorders). Families are always screened for the possibility of applying ante-natal diagnostic techniques. The motivation for and expectations from genetic counselling are explored by Dr. Cann and, for selected families, by a social worker, in order to plan for effective communication with each family.

Birth Defects Clinics (Director, Dr. Luigi Luzzatti, Professor of Pediatrics)

Approximately 100 new patients are seen in the Birth Defects Clinics annually for diagnostic evaluation and comprehensive and interdisciplinary management of congenital defects. There are about 400 return visits per annum in the clinics. In addition, about 50 newborn infants are seen annually for evaluation of congenital defects in the Stanford Hospital Nurseries. Most children seen have multiple congenital defects of unclear etiology, about 50% of these are screened for chromosomal abnormalities, about 25% are patients with disorders determined by major genes at single loci, and about one third are seen for structural malformations which are multifactorially inherited threshold traits. This is the clinic where most of the patients with mucopolysaccharidoses, mucopolysaccharidoses, simply inherited disorders of bone, Down's syndrome and other chromosomal abnormalities, cleft lip and/or palate and congenital defects of neural tube development are seen. Where appropriate, genetic counselling is provided to families of patients enrolled in the Birth Defects Clinics. In addition about 50 families are referred each year specifically for genetic counselling. An average of about 100 karyotype analyses are done annually in the Cytogenetics Laboratory in probands with congenital defects and/or in family members as indicated. To date diagnostic amniocentesis has been performed at 12-16 weeks gestation on approximately 25 women referred to the clinic for either advanced maternal age or a documented family history for an X linked gene.

Pediatric Hematology Clinic (Clinic Director, Dr. Herbert C. Schwartz, Professor of Pediatrics)

In this clinic approximately 100 new patients are seen annually for various disorders of blood. Between five and ten percent of these are children with inherited disorders -- sickle cell anemia and other hemoglobinopathies, thalassemia, congenital spherocytosis, glucose-6-phosphate dehydrogenase deficiency and other instances of congenital non-spherocytic hemolytic anemia and hemolytic disease of the newborn (feto-maternal blood group incompatibility). In this clinic workup of patients with genetic disorders includes identification of heterozygotes and homozygotes among first degree relatives of probands and subsequent genetic counselling.

The Stanford Nurseries (Director, Dr. Philip Sunshine, Associate Professor of Pediatrics)

The newborn nurseries of the Stanford Medical Center include facilities for uncomplicated births, an intensive care nursery and the Premature Infant Research Center. The research and clinical emphasis of these newborn units is on perinatal biology of the fetus, newlyborn infant and the mother. This emphasis has led to the development of a program dealing with high risk pregnancies, in which mother, infant or both are at high risk for morbidity and mortality. Whenever appropriate the fetus is monitored and such monitoring includes diagnostic amniocentesis for evaluation of fetal maturity, for fetomaternal Rh incompatibility and early antenatal detection of genetic disorders. A study conducted in California in 1971 by the Bureau of Maternal and Child Health indicated that the Stanford Medical Center is the only facility in RMP Area III with an intensive care nursery (based on fairly rigid criteria, e.g. full time neonatologists and basic infant monitoring equipment available at all times), and this is evident by referrals of fetuses and their mothers and newborn infants. Approximately 700 infants are admitted per year to either the intensive care nursery of the Premature Research Center at Stanford and about 30% of these are born at other facilities. Approximately 5% of newborn infants receiving intensive care at Stanford are ill because they are heterozygous or homozygous for a major mutant gene, because of a chromosomal abnormality or because of a malformation with a significant genetic contribution. We anticipate a major expansion of perinatal activities involving detection and management of high risk mothers and fetuses with the appointment in the near future of a Chairman of the Department of Obstetrics and Gynecology. The search is presently underway, and one of the criteria for selection is competence in the areas of fetal monitoring and evaluation and fetal and maternal physiologic interactions.

Pediatric Neurology Clinic (Clinic Director, Dr. Judith Koehler, Assistant Professor of Pediatrics and Neurology)

Approximately 500-600 patients with various neurologic disorders are seen annually in this clinic. Many of these patients have genetically determined or inherited disease, e.g. learning disorders, familial myopathies, spinocerebellar degeneration, familial spastic paraparesis and other metabolic or degenerative disorders of the central and peripheral nervous system. The Division of Pediatric Neurology has special interest in clinical treatments of and research into the basic mechanisms of epilepsy. Some patients with seizure disorders have hereditofamilial determinants underlying their basic seizure problems. Additionally, response to medication and drug metabolism may be genetically determined. The Division of Pediatric Neurology also has special clinical and research interest in neuromuscular disease, much of which is hereditofamilial. Specifically for this purpose, a neuromuscular clinic is being established in conjunction with the Muscular Dystrophy Association. This clinic will see 200-300 patients, mostly children, annually. A diagnostic neuromuscular histochemistry laboratory is already in function to process nerve and muscle biopsies from these patients. Active research in neuromuscular disease is being carried out by several workers in the Department of Neurology which incorporates ultrastructure and basic physiologic studies.

Hemophilia Program (Clinic Director, Dr. John Gribble)

The Hemophilia Program provides diagnostic evaluation and comprehensive management for patients with disorders of blood coagulation. Approximately 100 new patients are seen annually; these patients represent the clinical spectrum of disorders of blood coagulation, the most frequent being hemophilia A, hemophilia B and von Willebrand's disease. The families of all patients with inherited disorders are provided with genetic counselling. A comprehensive care clinic is located at the Children's Hospital at Stanford in order to provide continuing care for patients with hemophilia and includes prophylactic treatment of patients with hemophilia A with Factor VIII preparations.

Pediatric Metabolic and Endocrine Clinic (Clinic Director, Dr. R.O. Christiansen, Assistant Professor of Pediatrics)

Seventy to eighty new patients are seen annually in this clinic which logs about 700 patient visits each year. Many of the patients may be classified as inborn metabolic errors and these include phenylketonuria, galactosemia, the various syndromes associated with adrenal hyperplasia, alcaptonuria, the various glycogenoses and defects in thyroxine biosynthesis. Diet therapy of patients with phenylketonuria and galactosemia is an ongoing activity of these clinics. Furthermore, a number of patients with hypophosphatemia (X-linked) are enrolled in this clinic. Genetic counselling is provided for the families of all patients with hereditary metabolic and endocrine diseases, and this includes attempts to designate heterozygotes among unaffected siblings of probands with inborn errors of metabolism.

Growth and Development Clinic (Director, Dr. Norman Kretchmer, Professor of Pediatrics)

This clinic deals with genetic and environmental problems in infants and children which affect their growth and development. Usually children are referred to this clinic because of failure to grow or abnormally slow growth.

Thus children with hereditary conditions which result in significant growth failure in the phenotype (e.g. isolated growth hormone deficiency, various aminoacidurias and various chondrodystrophies) comprise a proportion of this clinic's patient population. Approximately 150 new patients are seen annually.

The Children's Hospital at Stanford, located approximately 1/2 mile from the Medical Center on the Stanford campus, is affiliated with the Department of Pediatrics for teaching and postdoctoral clinical and research training. There are three clinical services relevant to a medical genetics program at this hospital: 1) Cystic Fibrosis Service (Director, Dr. Birt Harvey, Clinical Associate Professor of Pediatrics), 2) Clinical Immunology Service (Director, Dr. Vincent Marinkovich, Assistant Professor of Pediatrics) and 3) Pediatric Oncology Service (Director, Dr. Jordan Wilbur, Clinical Associate Professor of Pediatrics). These services have in-patient and out-patient facilities at the Children's Hospital at Stanford. Here patients are evaluated, diagnosed and treated for cystic fibrosis, hereditary immune defects and hereditary malignant tumors (e.g. retinoblastoma). Genetic counselling is provided by the staff of these services.

Research Facilities

Research facilities in the Departments of Genetics and Pediatrics available to this Genetics Center Program include some 15,000 square feet of laboratories, research support areas and offices. The research and administrative areas for each department are contiguous on the third floor (the Joseph P. Kennedy Jr. Laboratories for Molecular Medicine) of the Joseph D. Grant Building of the School of Medicine. In addition the Instrumentation Research Laboratory (Department of Genetics), in the basement of this building, comprises about 7,000 square feet of offices, laboratories, and shops. Thus biochemical laboratories, fully developed tissue culture areas, heavy equipment areas, cold and warm rooms and an electron microscope facility are available for this program. A cell separator, a gas chromatograph, a mass spectrometer and an electron microscope lead the long list of equipment which is available for the research projects to be carried out in this program. Other equipment also includes centrifuges, scintillation counters, lyophilizers, spectrophotometers, incubators, microscopes and coulter counters. Rather good computer facilities are available.

THE RESEARCH PROGRAM

The Center program consists of research projects which have direct relevance to medical genetics. Some of these projects will require blood, urine, amniotic fluid and cells from patients and others will require the patients themselves. In none of these research projects are we ready to test methods for use in the actual clinical situation; rather, we are developing methodology. The general areas of research are as follows:

- I. Screening and Characterization of Inborn Errors of Metabolism by Gas Chromatography/Mass Spectrometry Analysis of Body Fluids.
(Drs. Lederberg, Kretchmer, Cann and Duffield).
- II. Maternal Blood Stream - Another Source of Fetal Tissue for Pre-Natal Diagnosis of Genetic Disorders.
(Drs. Herzenberg and Cann).
- III. Polymorphic Genetic Markers in Amniotic Fluid.
(Drs. Cann and Tsuboi).
- IV. A Search for Genetic Polymorphisms and Variances of Specific Binding Proteins in Blood.
(Dr. Cavalli-Sforza).
- V. The Impact of Genetic Counseling Practices on Family Decisions and Behavior.
(Drs. Barnett, Cann and Luzzatti).

The arrangement is also reflected in the budget presentation.

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SECTION II

Screening and Characterization of Inborn Errors of
Metabolism by Gas Chromatography/Mass Spectrometry
Analysis of Body Fluids

Drs. Lederberg, Kretchmer, Cann, and Duffield

**Screening and Characterization of Inborn Errors of
Metabolism with Computerized Gas Chromatography
And Mass Spectrometry**

**Dr. J. Lederberg, Principal Investigator
Drs. N. Kretchmer, H. Cann, and A. Duffield,
Associate Investigators**

A. INTRODUCTION

A.1 Objectives

The objectives of this work are to develop the uses of gas-liquid chromatography (GC) and mass spectrometry (MS) instrumentation, under computer management, for the screening, diagnosis (pre and postnatal), and study of inborn errors of metabolism. The efficacy of these analytical tools has been demonstrated when applied to 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 more economical and routine diagnosis and screening of disease.

Specific goals include the application of GC/MS analysis capabilities to larger and more diversified populations to establish better defined norms, deviations, and control parameters necessary to relate GC/MS analysis results to identifiable disease states. In order to ease the problems in analyzing the prodigious amounts of information expected in this research, we will augment the existing GC/MS data handling system to provide for increased throughput and automation.

Two other on-going or pending research projects relate to the present application, each with distinctive aims:

1. DENDRAL (NIH: RR-00612; Principal Investigator, E. A. Feigenbaum) is concerned with the advancement of artificial intelligence (computer software) techniques for the automated interpretation of mass spectrometry data. These programs attempt to emulate human reasoning processes in constructing explanations for mass spectra from basic principles.

2. SUMEX (NIH: RR-00785 pending; Principal Investigator, J. Lederberg) is a comprehensive resource grant to establish a national facility for developing applications of artificial intelligence in medicine. Our own use of this facility will include the integration of DENDRAL software with HIGH RESOLUTION mass spectrometry instrumentation. Genetic screening can be made even more sophisticated by using these techniques for the corroboration of the structures of newly discerned metabolites. However, the present program can also operate stand-alone, if necessary, using low resolution MS on line with the GC.

A.2 Background and Rationale

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 genetic 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 already begun to reduce our GC/MS laboratory staff in response to these cutbacks. It is therefore appropriate that we seek NIH support to help maintain this existing laboratory to apply its capabilities to problems of characterizing genetic metabolic disease.

Our focus on mass spectrometry (ref 3c) 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.

The application of these instruments to genetic 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 both as genetic polymorphisms and as clinical problems of genetic disease in man.

The sample populations comprise mainly healthy (control) and problematical newborns already under intensive study in the Stanford Pediatrics Department. Other samples will be furnished by collaborative arrangements with physicians elsewhere and with other Departments at Stanford (e.g., Medicine, Psychiatry, Dermatology, etc.). These inputs will have been prescreened for conditions likely to relate to possible genetic etiologies or otherwise to exercise the analytical utility of GC/MS screening.

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:



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

ion where they are separated ratio. In sector instruments in quadrupole instruments by an light instruments by an y. The mass spectrum of an a table of positive ions of molecular structure (for teroatoms) determines the pture subsequent to ionization, rt, a characteristic mass rical isomers may show subtle e mass spectra owing to the

spectrometer into the analyzer reg according to their mass-to-charge this is done by a magnetic field, electric field, and in time-of-fli adjustable ion detection time dela organic compound thus consists of different masses and abundances. M instance number and location of he frequency with which bonds will ru thereby producing, for the most pa spectrum for each compound. Geomet differences within their respectiv

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 ("Biochemical Applications of Mass Spectrometry," edited by G. R. Waller, Wiley-Interscience, New York, 1972). 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 2).

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 components. 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 700 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. 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 1) 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.

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 3a, copy attached) and subsequently for the amino acid content of biological fluids (ref 3b). 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. The results are discussed later (see Methods of Procedure and figure 5).

The overall management of the system and the reduction and selective presentation of the large volume of data emanating from the analytical instruments is an important task of the control computer. Our experience in instrumentation comprises a good deal of computational software embracing real time instrument management, automated data reduction, and artificial intelligence (ref 4, appended to this application). It also requires considerable effort in electronic and vacuum technology for the instrumentation hardware, and a coherent system approach for the overall integration of these components.

Present GC/MS systems are designed mainly for laboratory research, incorporating great analysis flexibility but the ability to handle only a small number of samples. Such systems are not practical for large volume screening, but they can be adapted for the pilot studies contemplated here. A properly designed automated system could reduce these costs by as much as an order of magnitude, as would be essential for cost-effective applications to general health care.

The routine screening of normal and abnormal body metabolites, including drugs, in human body fluids (ref 5) is currently the object of several research programs. Various

non-specific methods, including thin layer (ref 6,7), ion exchange (ref 8,10), liquid (ref 9), and gas chromatography (ref 11-14 and 17b), 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 (15) 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) metabolic error where rigorous identification of relevant metabolites 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 15-18. 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 18).

As mentioned earlier, urine and other body fluids (e.g., serum, amniotic fluid, etc.) are complex mixtures. The separation (by gas chromatography) and subsequent identification (by mass spectrometry) of these components can be a difficult task. To simplify the separation problem, the body fluid under analysis is chemically separated into a number of fractions which permit analysis for acids, amino acids, and carbohydrates present in free or in conjugated form. Drugs and hormones, as well as their metabolites, will also appear in the chromatographic separations. The gas chromatographic analysis of each class of compounds presents a metabolic profile. Abnormal profiles (containing either novel peaks or peaks deviating from their expected amplitudes) are then assayed by mass spectrometry. The mass spectra recorded during the elution of each gas chromatographic peak then serve to identify the constituents present in that peak.

The importance of this diagnostic technology may be illustrated by a family seen for a fatal inherited disease at the Stanford Medical Center. Three male infants have been born to this couple and each of the offspring died in a very similar manner by 10 days of age. The first infant was not seen at Stanford, but the second infant was transferred from another hospital, in extremis. Aside from the striking family history, there were no diagnostic clues, and the second child was dead within 6 hours of arrival. During the period of observation, blood and urine were collected from the proband, and analysis of the latter by gas chromatography (at another institution) revealed an excess of orotic acid. This diagnostic clue, coupled with the clinical picture of relatively rapid demise after protein (breast milk) feedings were well established, resulted in a diagnosis of a hereditary deficiency of hepatic ornithine transcarbamylase, with resulting hyperammonemia (ref 19). This diagnosis was eventually confirmed when the third male infant of this couple behaved in a manner identical to that of his two deceased brothers and, despite attempts at therapeutic intervention which included low protein feedings, he developed documented hyperammonemia and died in this medical center. Study of the liver revealed the complete absence of ornithine transcarbamylase. It must be stressed that clinical observations of the first two infants and post mortem examinations were not helpful diagnostically, and it was the chemical identification of orotic acid which led to the understanding of the familial pattern of neonatal mortality in this family.

Most medical centers have access to amino acid analyzers in order to screen patients for metabolic abnormalities of the principal amino acids, but unless a special research interest exists, other inborn errors of metabolism cannot easily be studied. At this institution the GC/MS system provides us the opportunity to detect a wide variety of inborn errors which show accumulation of amino acids, fatty acids, and many other metabolites in urine, blood, amniotic fluid, and other biological fluids and tissues.

Another application of GC/MS pertains to pre-natal diagnosis of hereditary inborn errors of metabolism. Fetal urine contributes to the amniotic fluid by the twelfth week of gestation and it should provide information which is diagnostically relevant to the fetus (ref 20). Surprisingly little is known about the origin, fate, and components of normal amniotic fluid, although information is accumulating because of interest in pre-natal diagnosis and evaluation of the fetus (ref 21). The fetal cells of the amniotic fluid, cultivated IN VITRO, are used routinely for pre-natal diagnosis of genetic disorders (ref 22). The fluid itself has been used infrequently for diagnostic purposes, although there is increasing evidence for the utility of direct assay of this component of amniotic fluid for pre-natal diagnosis of heritable metabolic errors. Mahoney et al. (ref 23) have recently reported the first successful pre-natal diagnosis of methylmalonic aciduria (confirmed by study of the fetus and performed in time to elect abortion). Their

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.

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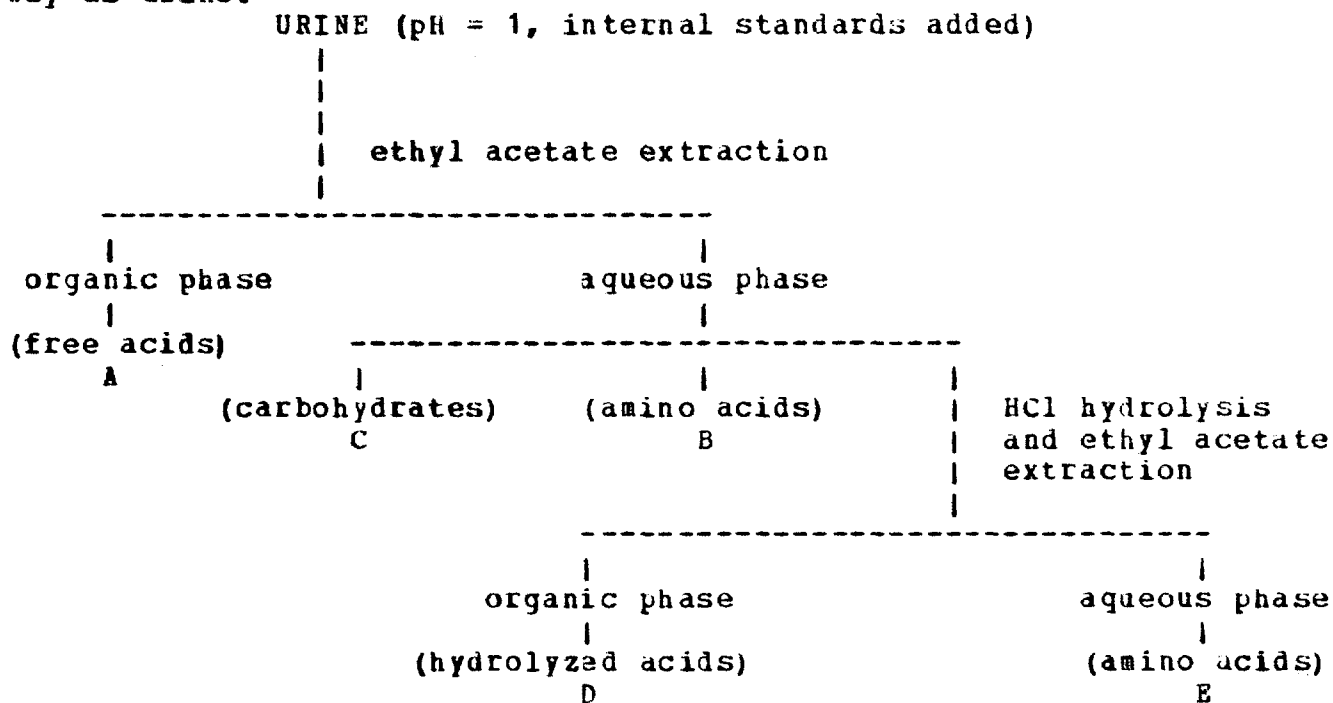
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- b) We plan to use GC/MS in the diagnosis of inherited metabolic abnormalities and in the detection and study of previously unrecognized metabolic disorders.

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 automatically 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 spectrometer. 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

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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).

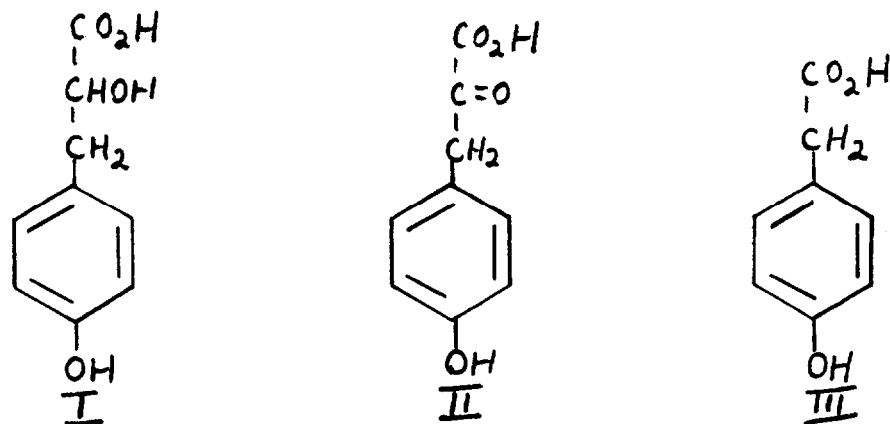
At least two authors (refs 33 and 41) have questioned the accuracy of the methods used for the detection and quantitation of BAIB in urine. The early work (refs 32 and 51), including the first isolation of BAIB from urine (ref 38), was completed using paper chromatography and reaction with ninhydrin 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 Montaj 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.

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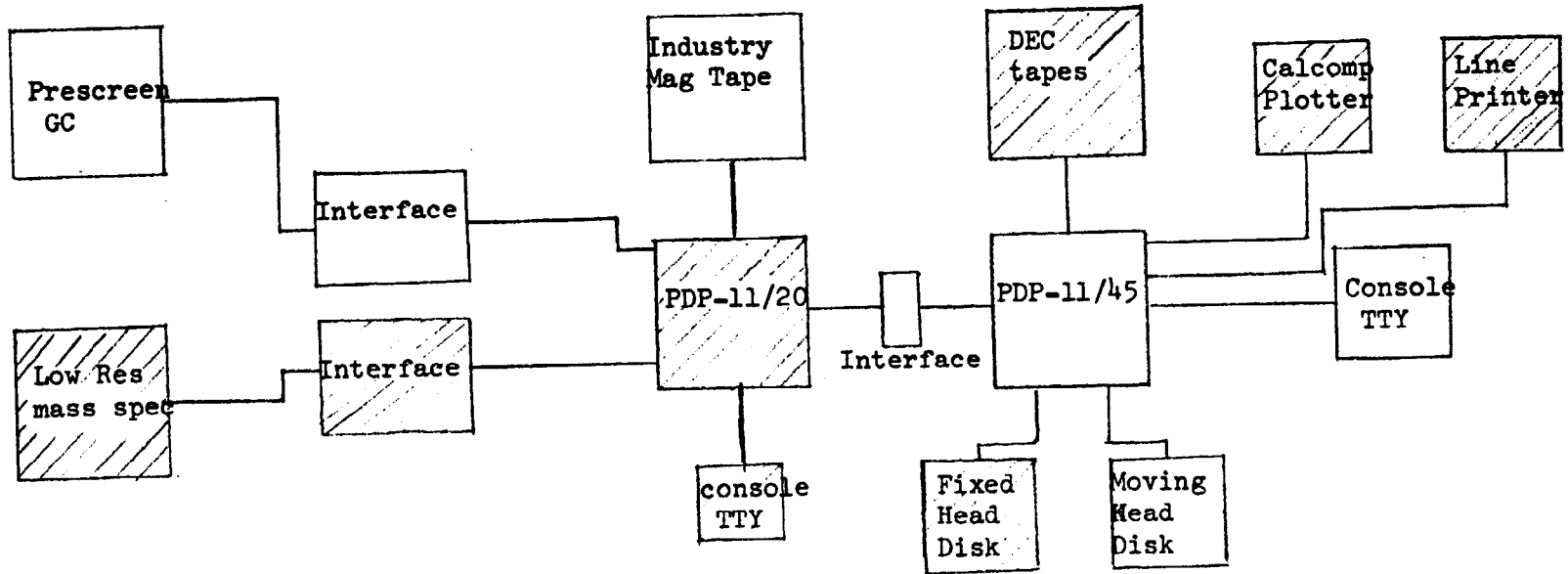
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Existing Equipment

FIGURE 1. PROPOSED DATA SYSTEM HARDWARE CONFIGURATION

P-48

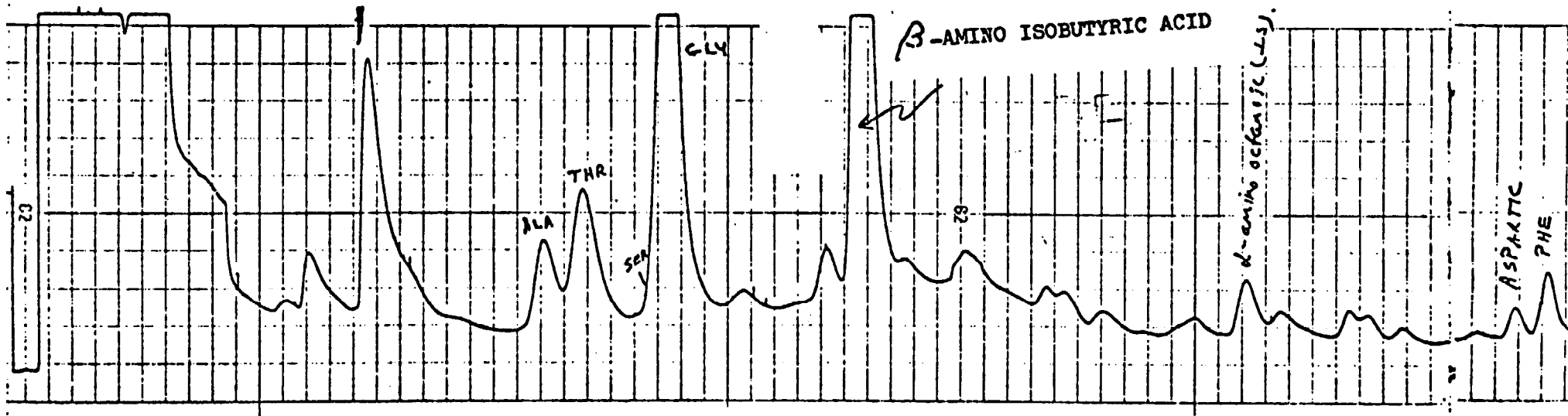


FIGURE 2

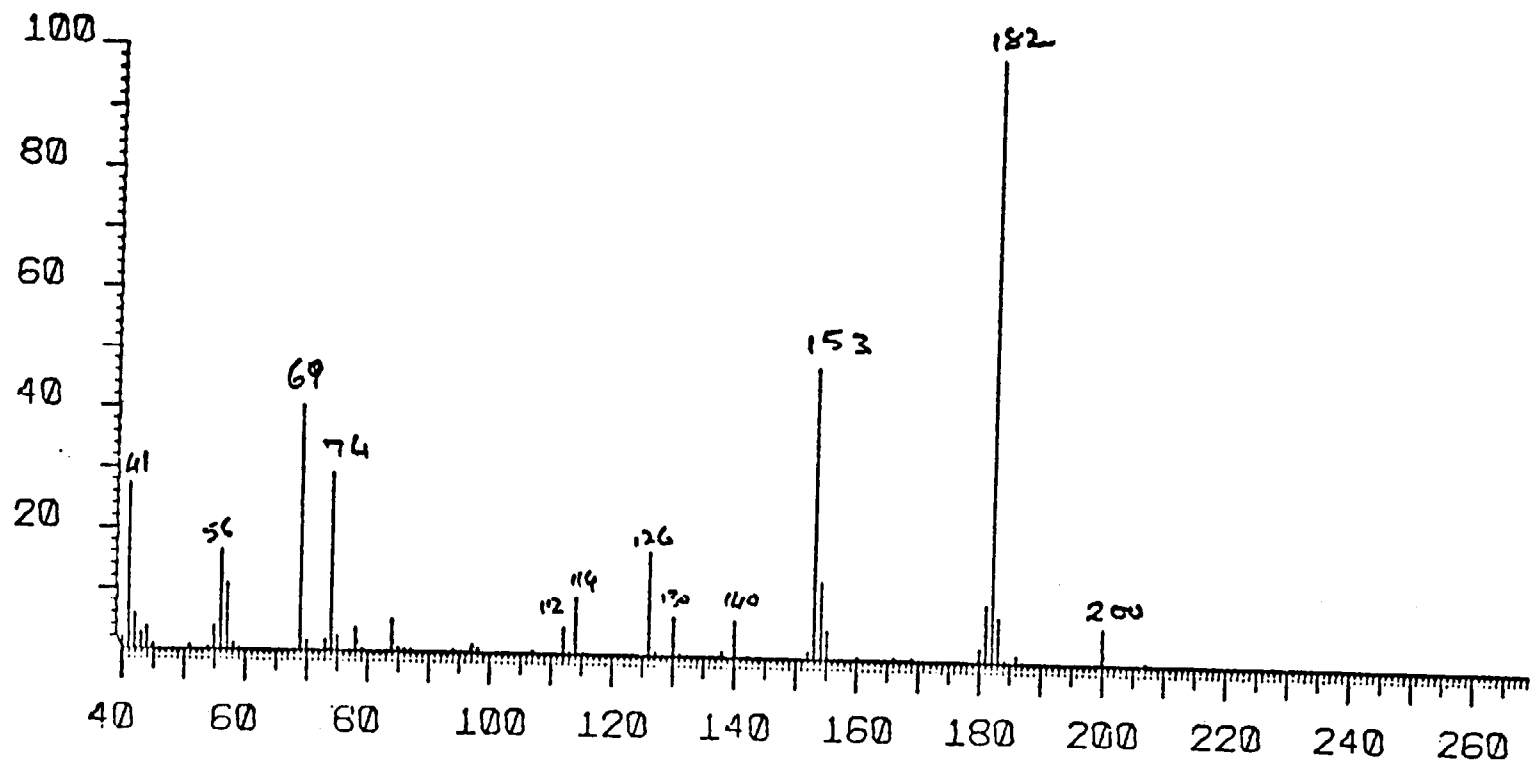


FIGURE 3

Mass spectrum of β -amino isobutyric acid

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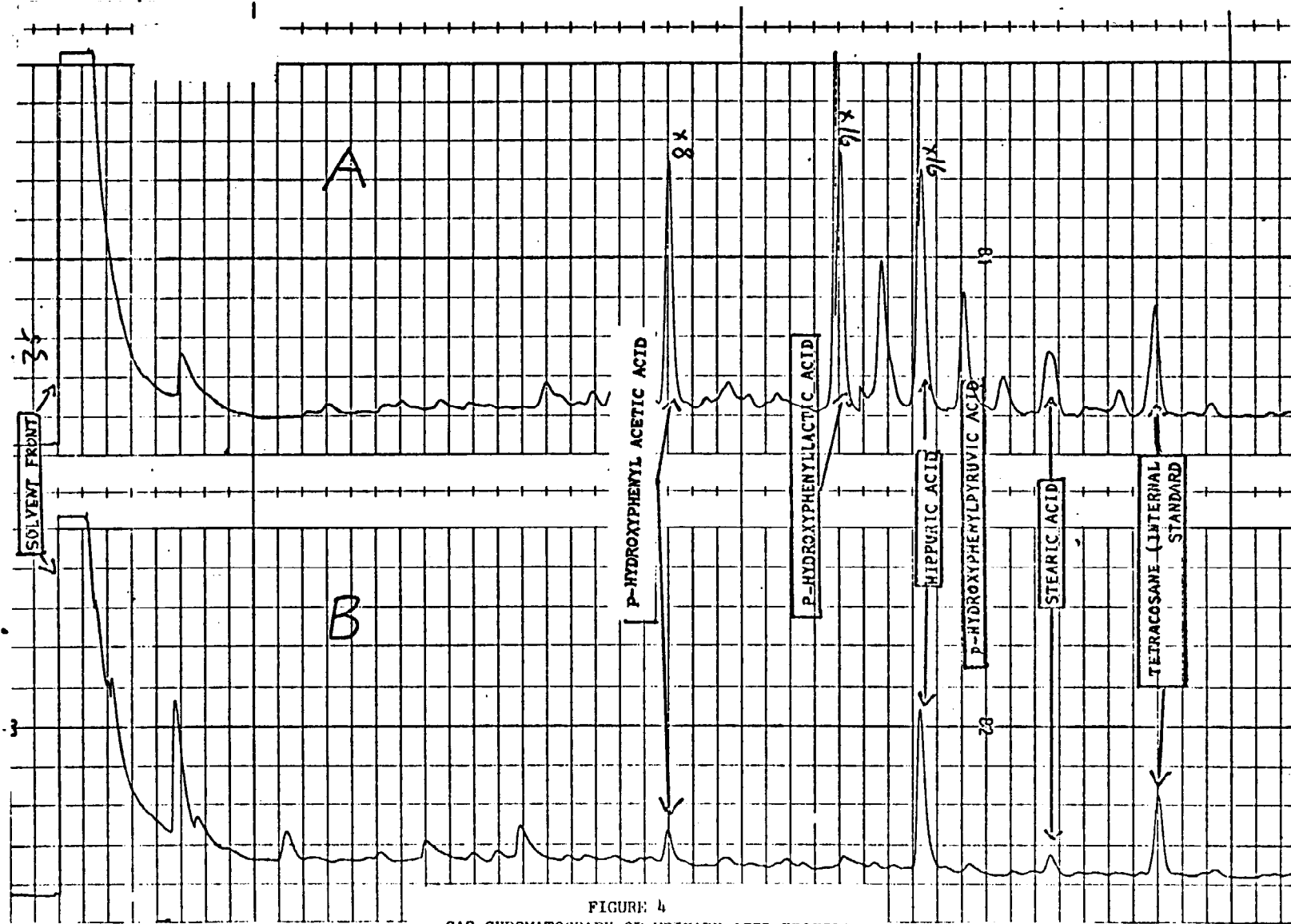


FIGURE 4
GAS CHROMATOGRAPH OF URINARY ACID PROFILE
A. PATIENT ACIDOTIC (2-17-73)
B. PATIENT NORMAL (3-10-73)

mass 140, amp	46.44						
mass 144, amp	89.93						
Amino Acid ALA	Masses 140/144	Areas	11349.2/ 21382.5	RATIO =	0.5308	Location Error =	0
mass 168, amp	7.23						
mass 176, amp	41.46						
Amino Acid VAL	Masses 168/176	Areas	1938.8/ 10947.3	RATIO =	0.1771	Location Error =	-2
mass 126, amp	22.65						
mass 128, amp	22.76						
Amino Acid GLY	Masses 126/128	Areas	6222.4/ 6054.7	RATIO =	1.0277	Location Error =	2
mass 182, amp	1.80						
mass 192, amp	14.23						
Amino Acid ILE	Masses 182/192	Areas	393.2/ 3653.7	RATIO =	0.1076	Location Error =	-1
mass 182, amp	3.50						
mass 192, amp	26.32						
Amino Acid LEU	Masses 182/192	Areas	835.1/ 6769.0	RATIO =	0.1235	Location Error =	2
mass 166, amp	26.34						
mass 173, amp	100.00						
Amino Acid PRO	Masses 166/173	Areas	7603.1/-33028.8	RATIO =	-0.2298	Location Error =	2
mass 153, amp	4.59						
mass 158, amp	10.31						
Amino Acid THR	Masses 153/158	Areas	1162.7/ 2722.9	RATIO =	0.4270	Location Error =	0
mass 139, amp	13.10						
mass 142, amp	12.32						
Amino Acid SER	Masses 139/142	Areas	3293.4/ 3104.7	RATIO =	1.0608	Location Error =	0
mass 148, amp	9.85						
mass 155, amp	32.24						
Amino Acid PHE	Masses 148/155	Areas	2920.1/ 8971.8	RATIO =	0.3255	Location Error =	-2
mass 240, amp	8.61						
mass 243, amp	20.71						
Amino Acid ASP	Masses 240/243	Areas	2281.8/ 5751.0	RATIO =	0.3968	Location Error =	0
mass 198, amp	58.15						
mass 203, amp	67.56						
Amino Acid GLU	Masses 198/203	Areas	16883.6/ 19353.8	RATIO =	0.8724	Location Error =	-1

Amino Acid # 12 location error

FINAL ANALYSIS (mc/100ml)

AMINO ACID	FOUND
ALA	1.32
VAL	0.52
GLY	3.51
ILE	0.23
LEU	0.42
PRO	-0.50
THR	0.76
SER	2.79
PHE	0.74
ASP	1.81
GLU	2.84

FIGURE 5
ANALYSIS OF 12 AMINO ACIDS IN URINE
USING MASS FRAGMENTOGRAPHY

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FINAL

THE SIMULTANEOUS QUANTITATION OF TEN AMINO ACIDS IN SOIL EXTRACTS
BY MASS FRAGMENTOGRAPHY

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The analysis of amino acids from terrestrial and extraterrestrial sources is becoming increasingly important (1-5). The need for a specific, sensitive and rapid method of quantitation is desirable. The methods currently employed for amino acid analysis involve ion exchange procedures (6,7) or gas chromatography (8-10). These techniques, although of immense value, are limited by their non-specificity for the absolute identification of any substance responsible for a gas chromatographic peak.

In the present communication we report an absolute, unambiguous method for the positive identification and quantitation of ten amino acids present in soil extracts using GLC-mass fragmentography. In mass fragmentography the mass spectrometer is used only to detect certain preselected ions known to be characteristic for each compound being quantitated, and the internal standard. The technique of mass fragmentography using sector mass spectrometers is usually restricted to the simultaneous monitoring of up to three integer mass values (11, 12), although with one instrument five ions were used (13). Using a quadrupole mass spectrometer up to eight ions have been selected and their respective analog signals monitored (14). We now wish to report the modification of the gas chromatography-quadrupole mass spectrometer-computer system previously described (15) for the simultaneous monitoring under computer control of the ion currents from 25 pre-selected integer mass values. *These values can range between masses 0 and 750 in contrast to the limited range available for mass fragmentography using sector spectrometers.* If required this number could be increased by suitable alteration of the computer control programs. Specifically we wish to report the application of this system to the quantitation of ten of the amino acids present in soil extracts.

METHODS

Reagents: A deuterated amino acid mixture was supplied by Merck Laboratory Chemicals (New Jersey). 1.25N HCl in n-butanol, 25% (v/v) trifluoroacetic anhydride in methylene chloride and Tabsorb (EGA on Chromat) column packing were obtained from Regis Chemical Co., Illinois. A standard amino acid solution was purchased from Pierce Chemical Co., Illinois.

Equipment: A Varian model 1200 gas chromatograph was coupled by an all glass membrane separator (16) to a Finnigan 1015 Quadrupole mass spectrometer which was interfaced to the ACME computer system of the Stanford University Medical School (15). GLC separations were conducted using a 6 foot by 4 mm. (I.D.) coiled glass column packed with Tabsorb (Regis Chemical Co.). The flow rate of the carrier gas (helium) was 60 ml/minute.

The uniqueness of the mass spectrometer instrumentation lies in the modified computer software (program) used. The hardware is the system previously described (15) and assumes an operating cycle of:

- (a) transmission of a control number, N, from the computer to an interface controller which sets the quadrupole mass analyser to a particular mass point in the m/e continuum.
- (b) an integration of the ion signal for a pre-set period, T (integration time = 8 milliseconds in our work), and
- (c) computer reading of the integration value with a twelve bit A → D conversion.

For the recording of normal mass spectra N is selected such that successive

cycles result in m/e values of 1,2,750. At the beginning of each day the instrument is calibrated using a reference compound. Idiosyncracies of the IBM 360/50 to IBM 1800 computer data paths dictate that the mass values be buffered into groups of 250.

For normal g.c.-m.s. procedures the operator is allowed to select a mass range of 1 to $n \times 250$ ($n = 1,2, \text{ or } 3$ buffers). For mass fragmentography n is set to zero and instead a "precision collect" buffer of 250 control-data acquisition cycles is employed. The operator must then enter the pre-selected m/e values he wishes to scan. When the precision collect buffer is constructed, 10 cycles are allocated to each m/e value selected. The first of the 10 cycles sets N to $N_m - 4$. The returned integrated ion measurement is discarded; this cycle serves only to slew the quadrupole electronics from anywhere in the m/e continuum to the mass region of interest. The additional 9 cycles are used with $N = N_m - 4 \dots N_m \dots N_m + 4$. The returned values represent a set of readings about the m/e value of interest ± 0.5 amu. The center three points are then smoothed with a five point quadratic function (17). The highest value of these three smoothed points is then selected, as the precision collect value. Thus small drifts in calibration are corrected and a signal average obtained. Finally, the abbreviated "spectrum" of 25 precision intensities for each m/e are filed on disc.

Such a "spectrum" is recorded every 2 seconds and a summation of all the ion intensities is used to construct the ion chromatogram shown in Fig. 2. Individual ion chromatograms can also be constructed if required (Fig. 3). A threshold is established from the ion currents before and after each gas chromatographic peak and a computer program performs integration of the ion currents under each peak.

Procedure

1 g of sieved, air-dried soil (Stanford University garden soil) was refluxed with 6N HCl (10 ml) for 20 hrs. The mixture was filtered and the residue washed with 1N HCl (5 ml). The combined filtrate and washings were extracted with chloroform (4 x 10 ml) and the aqueous phase evaporated to dryness. The residue is dissolved in water (5 ml) and passed through a column of "Ion Retardation Resin" AG 11-A8 (50-100 mesh, 1 x 21 cm). The amino acids were eluted with water (50 ml) and the eluate evaporated in vacuo to dryness. The residue is dissolved in water (5 ml) and placed on a column of

cation exchange resin (AG 50W-X12, 50-100 mesh, 1 x 21 cm) and washed with water (50 ml) to remove neutral and anion contaminants. The amino acids were eluted with 4N NH_4OH (80 ml) and the eluate evaporated to dryness. The residue was dissolved in water and made up to a volume of 4 ml. A portion of this solution (1 ml) was used for the amino acid analysis using an amino acid analyser. To another 2 ml of the processed solution was added 2 ml of the deuterated amino acid standard solution (100 mg in 100 ml of 0.1N HCl) and the mixture evaporated to dryness. The residue was refluxed with 1.2 N HCl in n-butanol (1 ml) for 30 min. and evaporated to dryness in vacuo. To the residue trifluoroacetic anhydride in methylene chloride (0.7 ml) was added and refluxed for 10 min. The solution was evaporated to dryness at room temperature and the residue dissolved in ethyl acetate (100 μl). An aliquot (1 μl) was injected into the injector port of the gas chromatograph and the oven kept at 100° for 1 min. when it was programmed at 4°/min. to 220°.

To each of 4 tubes containing 2 ml of the deuterated amino acid standard solution (100 mg in 100 ml of 0.1N HCl) was added 150, 200, 250 and 300 μl respectively of a standard amino acid solution (2.5 μmoles of each amino acid per ml). The solutions were mixed and evaporated to dryness. Each residue was derivatized by the above method and an aliquot of each (1 μl) injected into the gas chromatograph which was operated under the conditions described above. This procedure was used to construct a standard curve for the quantitation of each amino acid. A typical standard curve is shown (Figure 1) for glutamic acid.

RESULTS

The N-TFA, O-n-butyl derivative was chosen for the derivatization of amino acids for two reasons. Firstly, these derivatives have excellent glc separation characteristics ¹³(17) and secondly the selected characteristic fragment ions of the deuterated and non-deuterated derivatives do not interfere with each other, nor with other α -amino acids. Table I records the individual ions monitored for quantitation in the mass spectra of each of the deuterated and non-deuterated amino acids. ~~The computer integrates the intensity of the deuterated and non-deuterated ion currents with time and quantitation is achieved by calculation of the ratio of their respective peak areas.~~

Our results of a typical soil analysis are compared with those from an amino acid analyser in Table II. The higher value obtained with lysine by the amino acid analyser is due to a ninhydrin positive substance in soil interfering with the quantitation of lysine. In this respect mass fragmentography is superior to the amino acid analyser in that using a mass spectrometer as detector only characteristic pre-selected ions are detected and quantitated and any impurity present under the same gas chromatographic peak is not measured. A summation of 20 such characteristic ions was plotted as an ion chromatogram of a derivatized soil sample and is shown in Fig. 2.

Preliminary experiments showed that when the deuterated amino acid mixture was added directly to the soil sample extensive hydrogen-deuterium exchange occurred during acid hydrolysis of the soil extract. The removal of the isotopic label was catalysed by the hot mineral acid in presence of excess mineral used in the soil hydrolysis step. Fox

and collaborators have reported (4) a similar finding concerning the decomposition of amino acids in soil upon direct acid hydrolysis. In the present work the deuterated amino acid mixture was added just before derivatization (i.e. after hydrolytic extraction of the soil) in order to avoid this problem. However, in cases where it is necessary to quantitate the free amino acid content of complex mixtures, such as in serum or urine samples, the deuterated amino acid mixture may be added directly to the sample before processing without any deleterious effects ¹⁹ (18).

Although only ten amino acids present in soil were quantitated the method can be extended to all the normal amino acids found in protein. The deuterated analogs of arginine, histidine, serine, threonine and tyrosine are commercially available. Appropriate deuterated analogs of methionine, tryptophane, cysteine and cystine would have to be chemically synthesized from the appropriate precursors. In these instances at least two deuterium atoms should be incorporated in non-exchangeable positions so that for the characteristic ion chosen the P + 2 peak is separate from the ¹³C isotope contribution of the unlabeled amino acid. Furthermore, the deuterium substitution need not be quantitative (>90%) provided the same characteristic ion of that deuterated analog is used for the construction of a standard curve such as Figure 1.

In our experience, the use of a single mass value for each amino acid together with the g.c. retention time is sufficient for accurate identification and quantitation. The chemical work-up specifically yields a basic fraction thereby eliminating acid and neutral compounds which could possibly co-elute and interfere with the determination.

Instrument analysis time is approximately one hour and with our system we have been able to achieve accurate quantitation with samples containing as little as 10 nanograms of an amino acid.

SUMMARY

A specific and sensitive method for the identification and simultaneous quantitation by mass fragmentography of ten of the amino acids present in soil has been developed. The technique uses a computer driven quadrupole mass spectrometer and a commercial preparation of deuterated amino acids is used as internal standards for purposes of quantitation. The results obtained are comparable with those from an amino acid analyser. In the quadrupole mass spectrometer-computer system used up to 25 pre-selected ions may be monitored sequentially. This allows a maximum of 12 different amino acids (one specific ion in each of the undeuterated and deuterated amino acid spectra) to be quantitated. The method is relatively rapid (analysis time of approximately one hour) and is capable of the quantitation of nanogram quantities of amino acids.

ACKNOWLEDGMENTS

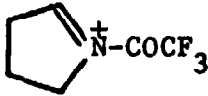
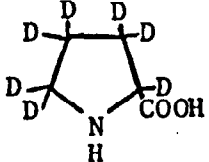
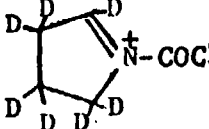
This research was funded by the Planetology Program Office, Office of Space Science, NASA Headquarters under grant NGR-05-020-004.

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Table I. CHARACTERISTIC FRAGMENT IONS SELECTED FOR MASS FRAGMENTOGRAPHY OF UNDEUTERATED AND DEUTERATED N-TFA-O-n-BUTYL-AMINO ACIDS.

Amino Acids	Fragment Ion	Deuterated Amino Acids	Fragment Ion
ALA	$\text{CH}_3\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 140)	$\text{CD}_3\text{CD}(\text{NH}_2)\text{COOH}$	$\text{CD}_3\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 144)
VAL	$1-\text{C}_3\text{H}_7\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 168)	$1-\text{C}_3\text{D}_7\text{CD}(\text{NH}_2)\text{COOH}$	$1-\text{C}_3\text{D}_7\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 176)
GLY	$\text{CH}_2=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 126)	$\text{NH}_2\text{CD}_2\text{COOH}$	$\text{CD}_2=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 128)
ILEU	$\text{C}_2\text{H}_5\text{CH}(\text{CH}_3)\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 182)	$\text{C}_2\text{D}_5\text{CD}(\text{CD}_3)\text{CD}(\text{NH}_2)\text{COOH}$	$\text{C}_2\text{D}_5\text{CD}(\text{CD}_3)\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 192)
LEU	$1-\text{C}_3\text{H}_7\text{CH}_2\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 182)	$1-\text{C}_3\text{D}_7\text{CD}_2\text{CD}(\text{NH}_2)\text{COOH}$	$1-\text{C}_3\text{D}_7\text{CD}_2\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 192)
PRO	 (m/e 166)		 (m/e 173)
PHE	$\text{C}_6\text{H}_5\text{CH}=\text{CHCOOH}]^+$ (m/e 148)	$\text{C}_6\text{D}_5\text{CD}_2\text{CD}(\text{NH}_2)\text{COOH}$	$\text{C}_6\text{D}_5\text{CD}=\text{CDCOOH}]^+$ (m/e 155)
ASP	$\text{BuOOCCH}_2\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 240)	$\text{HOCCD}_2\text{CD}(\text{NH}_2)\text{COOH}$	$\text{BuOCCD}_2\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 243)
GLU	$\text{HOOCCH}_2\text{CH}_2\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 198)	$\text{HOCCD}_2\text{CD}_2\text{CD}(\text{NH}_2)\text{COOH}$	$\text{HOCCD}_2\text{CD}_2\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 203)
LYS	$\text{CH}_2=\text{CHCH}_2\text{CH}_2\text{CH}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 180)	$\text{NH}_2(\text{CD}_2)_4\text{CD}(\text{NH}_2)\text{COOH}$	$\text{CD}_2=\text{CDCD}_2\text{CD}_2\text{CD}=\overset{+}{\text{N}}\text{HCOCF}_3$ (m/e 188)

LEGENDS TO FIGURES

Fig. 1. Standard curve for the quantitation of Glutamic acid.

Fig. 2. Typical ion chromatogram of soil amino acids.

Fig. 3. Mass Fragmentography^{MS} for Quantitation of Ala, Val and GLY.

Table II. ANALYSIS OF AMINO ACIDS IN SOIL ($\mu\text{g/g}$ SOIL)

<u>Amino Acid</u>	<u>Amino Acid Analysis</u>	<u>Mass Fragmentography</u>		
		<u>#1</u>	<u>#2</u>	<u>#3</u>
Ala	206.5	198.7	202.7	198.3
Val	148.3	151.0	150.5	149.9
Gly	215.4	196.8	201.6	201.3
Ileu	95.4	100.4	100.2	92.3
Leu	154.2	152.1	149.7	154.2
Pro	143.4	141.4	142.8	141.2
Phe	80.3	80.5	80.7	80.0
Asp	218.3	217.1	219.8	219.9
Glu	227.0	217.2	215.6	214.1
Lys	129.7	115.3	113.5	114.9

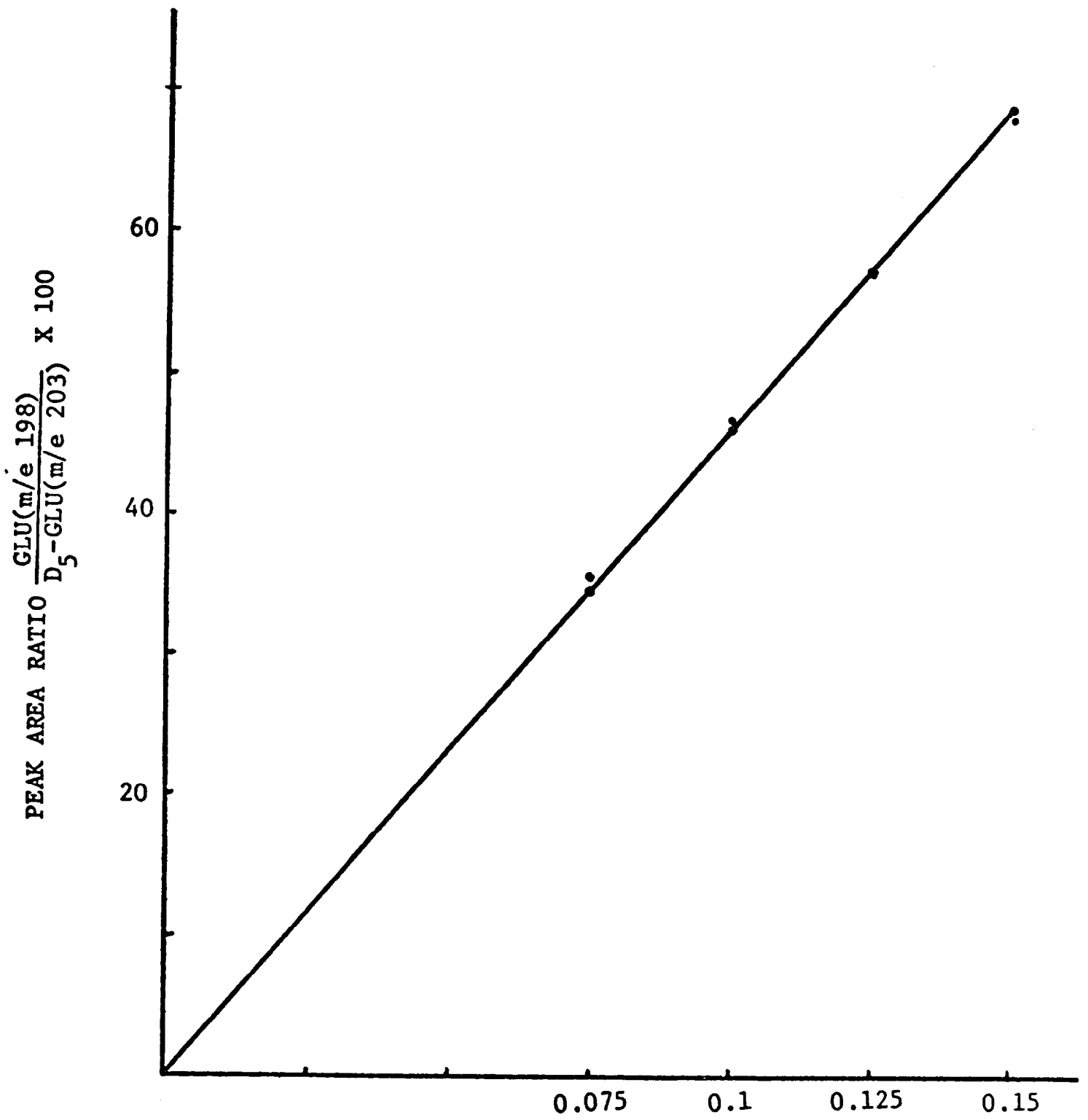


Fig. 1.

$\frac{\text{GLU}}{\text{D}_5\text{-GLU}}$ Added

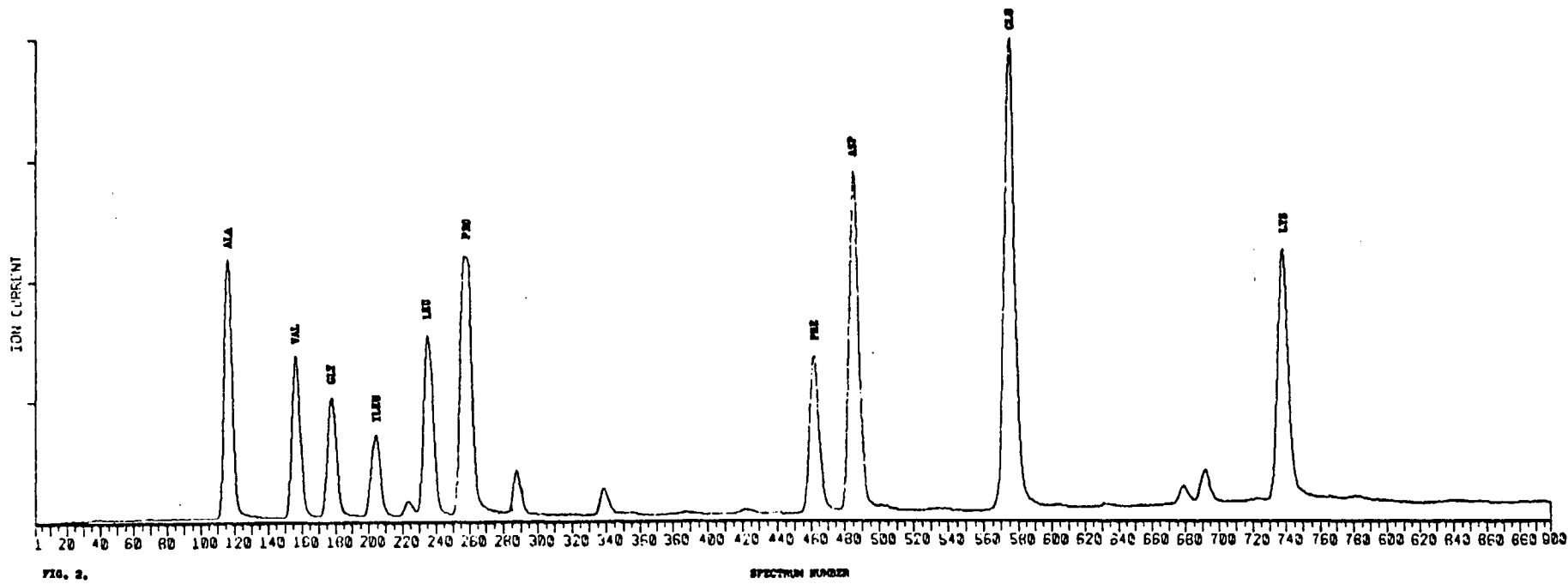


FIG. 2.

P-63

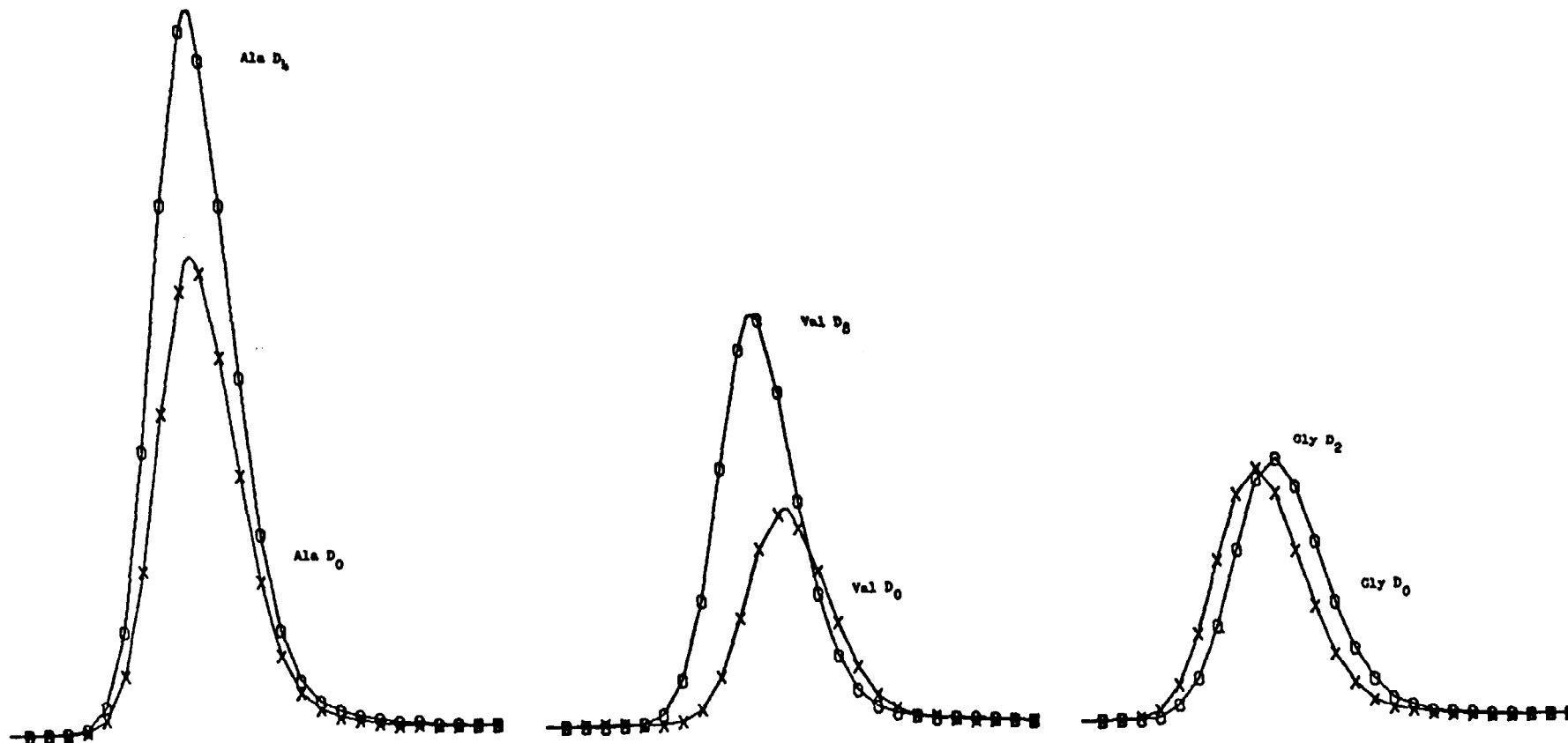


FIG. 3

A Computer Operated Mass Spectrometer System

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An integer resolution mass spectrometer-computer system has been developed in which the computer controls the "scan" of a mass spectrometer. In this system, the computer queries the user for operating parameters which are then translated into control functions which operate the mass analyzer. The spectral information acquired from the mass spectrometer is made available to the chemist within minutes in an on-line graphic system. Examples of the processing of GLC effluent are given.

THE USE OF MASS SPECTROMETRY has been hampered by the lagging development of a fast and convenient method of reducing the spectral output of the mass spectrometer (MS) to numerical data. Usually the operator must convert a MS chart recording, which is an analog plot of intensity *vs.* time, to a digitized plot of intensity *vs.* mass number. Because of instrument instabilities, wide range of signal amplitudes, large amounts of data, and other operational difficulties, (1, 2), it is often difficult and time-consuming to establish all the correct mass peak identifications. One aid is to use a reference compound (3) either prior to the run or as an internal standard with the unknown sample. By counting from known mass peaks, unknown spectral peaks can be identified. However, the processing of data by this technique is still a formidable task and it may take several days to accumulate all the information from a gas chromatograph-mass spectrometer (GLC-MS) run.

Several workers have demonstrated MS-computer systems in which the computer monitors and records digital data from a MS. In most of these applications the mass spectrometer has operated independently of the computer, scanning in some time dependent mode, measuring ion intensities at all points within the range of (500 to 5000 samples per second) and afterward performs the computations required to reduce the large amounts of digital data to useful information (4-7). Much instrument time and sampling effort is expended in the intervals between integer peak positions where there is little or no information. One system that improved

upon this latter inefficiency used step switches to step the scan from position to position (8).

We now describe a MS-computer system, suitable for routine laboratory use, in which the computer controls the operation of a quadrupole mass spectrometer (9, 10). In this system the "scan" is calibrated by relating known mass positions of a reference compound to a computer generated control voltage (V_c). R_c is generated as the result of a number N , sent from the computer to a Digital-to-Analog (D-to-A) converter in a MS-computer interface. The parameters of this V_c , or the N for each integer mass position, are determined by a computer program and stored in memory. The subsequent use of this information allows the computer-directed MS output to be recorded directly as mass-charge (m/e) *vs.* intensity. On request, this data is then made available to the operator in an on-line system.

The use of this computer-MS interaction, combined with the decision-making ability of the operator, permits a significant saving in data processing costs. Furthermore, a much larger duty cycle of analyzer "on peak" time is obtainable, resulting in the detection of more ions for a given mass position than is possible in conventional time based scanning.

The new MS-computer system has at least three unique features. There is a hardware control interface to connect the MS intimately with the computer; there is an improved efficiency of information acquisition from spectral peaks that are limited in ion production rates; and there is a user-oriented control and data presentation system that conceals the foregoing details from the operator, but presents the user with prompt and concise data which include normalized mass spectral plots.

The described system has evolved through three mass spectrometers, three computers, and two basic computer programs (11, 12). The later systems have greater range, sensitivity, and convenience, but they all have a common concept. Therefore the description that follows will be conceptual rather than specific to any one configuration.

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- (5) *Ibid.*, **40**, 1217 (1968).
- (6) R. A. Hites, S. Markey, R. C. Murphy, and K. Biemann, 16th *Ann. Conf. Mass Spectrometry Allied Topics*, ASTM E-14, Pittsburgh, Pa., May 1968.
- (7) R. B. Tucker, "A Mass Spectrometer Data Acquisition and Analysis System," IRL Report No. 1063, Instrumentation Research Laboratory, Department of Genetics, Stanford University School of Medicine, NASA Accession No. N68-25743, 1968.

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- (9) W. E. Reynolds, "A Small Computer Approach to Low Resolution Mass Spectrometry," Pacific Conference on Chemistry and Spectroscopy, Anaheim, Calif., November 1967.
- (10) W. E. Reynolds, T. B. Coburn, J. Bridges, and R. Tucker, "A Computer Operated Mass Spectrometer System," IRL Report No. 1062, Instrumentation Research Laboratory, Department of Genetics, Stanford University School of Medicine, NASA Accession No. N68-11869, Nov. 1967.
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- (12) J. Lederberg, E. Levinthal, and Staff, "Cytochemical Studies of Planetary Microorganisms Explorations in Exobiology," IRL Report No. 1076, Instrumentation Research Laboratory, Department of Genetics, Stanford University School of Medicine, October 1967 to April 1968. Appendix A is a published reprint of the above "Mass Spectrometers in a Time Shared Environment," NASA Accession No. N68-29546.

The present system is operating with a Finnigan 1015 quadrupole MS and a Varian Aerograph 600D chromatograph. The same computer programs and a similar interface were also operated successfully with a Bendix Time-of-Flight (T-o-F) MS (13) and an EAI quadrupole (14) MS. In all cases the GLC-MS, the teletypewriter, and the digital plotter were situated in a wet chemical laboratory.

A schematic diagram of the GLC-MS combination is shown in Figure 1. The effluent from the gas chromatograph, equipped with a flame ionization detector, first passes through a variable splitter that diverts between $\frac{1}{3}$ and $\frac{1}{2}$ of the flow through a Biemann separator (15) and into the MS. A solenoid-actuated valve in this line helps to keep the large initial solvent peak from entering the MS system. A reference gas reservoir containing a fluorine compound at a vapor pressure of approximately 3×10^{-7} Torr is also incorporated in the system and is connected to the MS by another solenoid valve. The computer, via the interfacing electronics, has direct control of gas valves, and can valve in or shut off the reference gas whenever it is needed for the calibration routine.

These valves were constructed in our shops in such a way that the back side is open to the vacuum system when the valve is closed. This avoids the common pressure burst when conventional valves are opened to a vacuum.

The right side of Figure 1 illustrates the major components and functions of the interface. This computer-MS interface was built in our Instrumentation Research Laboratory (16) and contains all the electronics not normally supplied with a standard configuration MS or computer. All of the operating parameters of the MS are, or may be, controlled by a digital word (binary number) sent from the computer. The principal control is via the "N" register to the D-to-A converter. The analog signal, V_c , from the D-to-A sets and holds the mass analyzer to pass ions of a predetermined m/e . Alternately the digital output may be coded to operate auxiliary control functions, such as actuate valves, set amplifier gains, the low speed multiplexer, or enable the digital plotter.

The characteristic method of controlling the m/e passband and taking measurements while the mass analyzer dwells upon a m/e value converts what is normally measured as a time dependent parameter, to a stationary signal. This statistically stationary property of the signal enables the employment of full integration to enhance signal to noise. Both the electrometer and the integrator are standard commercial FET operational amplifiers of the \$50.00 class. The time allowed for integration and the operation of the integrator reset are controlled by signals (numbers) from the computer to the "T" register. The output of the integrator is sampled, held, and read via the Analog-to-Digital (A-to-D) converter.

Auxiliary signal sensing is provided by the low speed multiplexer. This is useful to determine the automatic settings for self calibration, or may be used to record temperature, pressure, etc. These sense functions, plus some valve

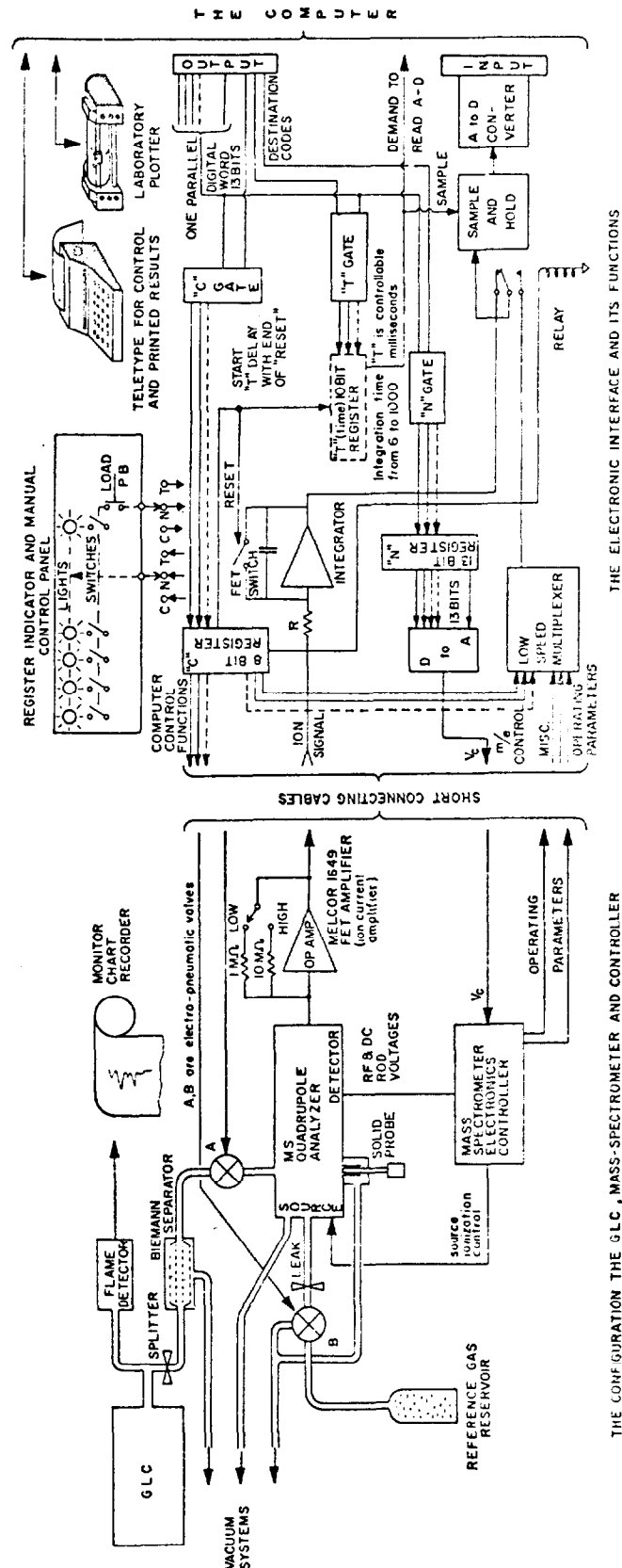


Figure 1. The GLC-MS instrumentation and the electronics interface to enable computer systems integration

control and checkout functions, are controlled by the "C" register.

There are no manual operator control functions in any of the above steps. The control is accomplished at the teletypewriter keyboard. This keeps the system flexible and makes it independent of the idiosyncrasies of individual computer

(13) D. B. Harrington and R. S. Gohlke, "High Resolution Time of Flight Mass Spectrometers," 10th Ann. Conf. Mass Spectrometry Allied Topics, ASTM E-14, New Orleans, La., 1962.

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COMPUTER DIALOGUE
(The user's response is underlined)

OPTION = collect
 EXPERIMENT# = test

T = 35

MODE = single

PLOT, TYPE, or FILE = plot

BY MASS, AMP, or ENTIRE = entire

FROM MASS = 1
 TO = 510
 QUICK = n

PLOT, TYPE, or FILE = /
 T = /
 EXPERIMENT# = MS26x15
 CONTINUATION = yes
 T = 6
 MODE = continuous

OF SPECTRA = 130
 FILED IN POSITIONS 940 to 1070
 T = /
 EXPERIMENT# = /
 OPTION = sum
 EXP# = MS26x15
 FROM SPECTRUM = 940
 TO = 1020
 FROM MASS = 40
 TO MASS = 500
 PLOT = yes

EXP# = /
 OPTION = plot
 EXPERIMENT# = MS26x15
 FROM SPECTRUM = 957
 TO = 957
 FROM MASS = 1
 TO MASS = 340

COMMENT

The user requests the data collection phase.
 A catch-all name, "test", is given; the spectrum will be used simply for a systems check.
 An integration time of 35 milliseconds per peak is requested.
 Only one spectrum will be taken (single mode).
 The data is acquired after this answer.
 The user can plot, type, or file the data collected; here a plot is requested.
 The user can plot selected masses, the highest intensities, or the entire spectrum within requested limits.
 The user indicates the limits.

A QUICK plot omits annotation, etc.
 Note that "y" and "n" mean "yes" and "no".
 The user completed the checkout and now wishes to proceed with the experiment.
 The "/" is used to backup through the conversation.
 An existing experiment name is given here.
 The user confirms that the spectra are to be added to the existing experiment file.
 The user requests that spectra be collected continuously until 130 are taken.

Data collection is complete.

The user then wishes to sum the elements of each spectrum to produce a "total ion" plot, analogous to a GLC trace.

Mass position 40 to 500 are summed for each of the collected spectra.

The "total ion" curve is now plotted.
 (Figures 6 and 7 are illustrative of this sample dialogue.)
 Guided by the total ion plot, the user will plot interesting mass spectra.
 Only the spectrum filed in position 957 is chosen.

The plot (Figure 17) is drawn and normalized to the base peak, $m/e = 31$.

Figure 2. An example of the user-computer dialogue during operation

interrupt lines and/or individual computer characteristics. This straightforward system definition makes the software design much like conventional computer programming rather than encouraging intricate techniques highly dependent upon the specific hardware.

Thus the system is not oriented specifically to any given computer. It has operated on an early model LINC (17) computer with 2K words of 12 bits. memory, and on a time-shared, locally programmed, IBM 360/50, buffered with an IBM 1800 (18). In all cases the computer was somewhat remote, separated by some 500 ft of cable from the rest of the instrumentation. The system is very economical of computer resources. Most of today's small general purpose computers would be able to operate the described functions if it were desired to avoid time-shared computer dependency. Some

sort of magnetic storage for object code programs and data storage is most desirable. DEC-type tapes have been used on the LINC system and disc packs on the IBM system.

THE SOFTWARE STRUCTURE

The objectives of the software are to operate and control the MS, acquire data from the MS, process and present this data in a manner useful to the chemist, and provide certain control and information to aid in maintaining and servicing the instrument.

With the program loaded into the computer, the user requests any one of several functions (see Table I) by typing the name of that function. The computer responds with a series of prompts (see Figure 2) to elicit user macrocommands. The computer then generates the detailed control functions to perform the assigned task. At the completion of the task, requests are made for new parameters. By striking the slash ("/"), the user can "backup" through any conversation to correct errors or to go to a different function. This conversation technique makes the system both flexible and reasonably self-instructing.

- (17) R. W. Stacy and B. Waxman, "Computers in Biomedical Research," Vol. II, Academic Press, New York, N. Y., 1965, pp 35-66.
 (18) W. J. Sanders, G. Breitbard, G. Wiederhold, *et al.*, "An Advanced Computer for Medical Research," *Fall Joint Computer Conf. Proc.*, ACM, Anaheim, Calif., 1967, p 497.

Table I. A List of Program Options

- (1) **CALIBRATE:** Creates an accurate *N* Table. The *N* numbers which correspond to the peaks in the reference gas are used as the end points of a piecewise linear interpolation procedure for calculating a complete *N* Table.
- (2) **COLLECT:** Is the primary data collection step. It is here that the 750 *N* Table values are sent to the MS and the 750 *m/e* intensities recorded. This operation can be repeated at five-second intervals as the data are filed on disk under an experiment name.
- (3) **TYPE:** Allows the user to print out spectral data by indicating what spectra in a given file are to be reviewed. The user can request that the amplitudes of particular *m/e* positions be typed; that a given number of the highest amplitudes be typed, or that a consecutive number of them over a given range be typed.
- (4) **PLOT:** Enables the user to have bar graphs produced by the computer controlled digital plotter. The amplitudes to be plotted can be selected with the same flexibility as described in TYPE.
- (5) **SUM:** Produces a plot of the total ion current over a series of gathered spectra. All responses of a spectrum are summed to produce one datum point on the plot. This plot corresponds closely with the GLC output when running with the GLC.
- (6) **TRACE:** Produces a record of a spectrum similar to the normal chart recording output. The analyzer is sampled at all *N* values (about 10 per amu) over a given range and the result is plotted as a "broken line." (Used for system check out)
- (7) **MONITOR:** Provides for inspecting the peak profiles by sampling the spectrum around a given *m/e* position. The gathered data are then typed out. (Normally used for system service or service log)
- (8) **DISPLAY:** Enables the user to display a given mass position (or *N* number) in the center of the console oscilloscope. (Used in the adjustment of the mass spectrometer)
- (9) **GAS:** Allows the user to remotely turn the reference gas on or off. This is helpful when operating the system from a remote position.

The example of a user-computer conversation given in Figure 2 represents the day-to-day computer-researcher dialogue given to direct the system's operation. Deeper level programming may be done at the terminal to redefine these functions or to add new modes. Additional system development may be done by the chemist-user, or his programmer in a manner typical of general purpose computer software. In normal daily practice, the user first requests the calibrate function and then proceeds to data acquisition, analysis, and presentation. Usually the calibration is done once every four hours.

It is this calibration subsection of the program that assigns to each integer mass position a value *N* which when sent to the D-to-A converter in the interface, will set the mass analyzer to pass that particular species of *m/e*. During this calibration phase a reference compound (perfluorotributylamine, FC-43), is introduced into the MS. The calibration procedure in addition to determining the *N* values, makes data available that will aid the operator in making qualitative judgments about the stability, sensitivity, and resolution of the MS. Also a service or maintenance record plot is available (see Figure 3), that, at least indirectly, shows these and other important instrument conditions. Figure 3 is actually 12 traced segments of a complete spectra, each segment covering a span of about 4 amu and each taken at a different integration time (gain). The *m/e* value, its position, and a parameter indicating the gain is automatically printed below each peak. The date and time is printed by the computer, but at present the operator must insert the sample pressure and ionization parameters. The file of these plots represents an excellent record of the instrument's serviceability. The calibration is automatic and its use less complicated than the description. It takes about 5 minutes, after which the reference gas is pumped out of the system. In the IBM 360-1800 system, the time is used to compile the main program.

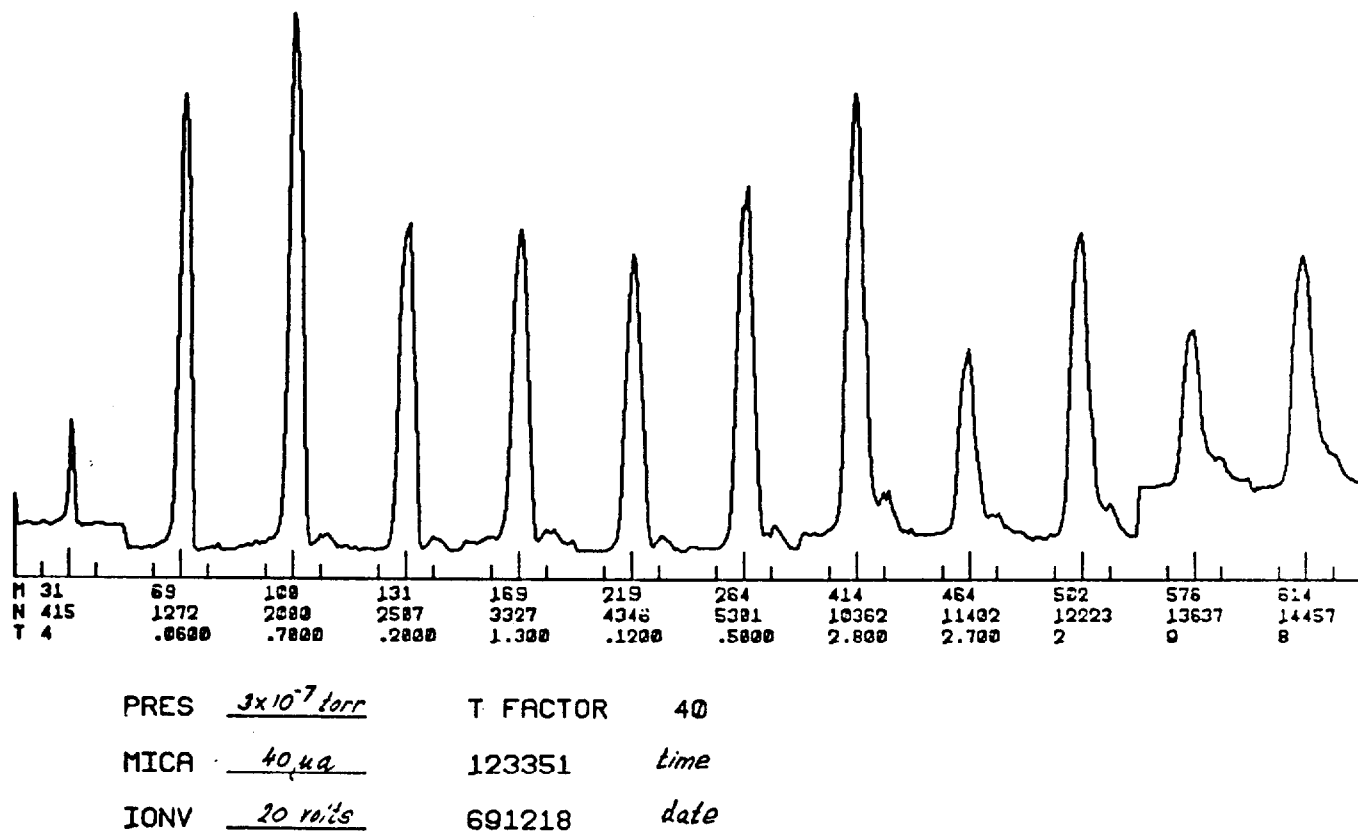


Figure 3. A monitor plot indication of instrument serviceability

Total spectral data acquisition time depends upon the number of m/e positions measured and the integration time allowed per position. It may be calculated:

$$\text{Spectrum acquisition time} = P \times (T_i + T) \quad (1)$$

where P is the number of m/e positions to be measured (they do not have to be contiguous or sequential), T_i is a transition time (2 msec in our system), and T is the integration time per peak (nominally 6 to 17 msec, but we have usefully used 1 to 1000 msec).

Normally data are collected at each integer m/e position 1 through 750. The 750 N values are sent through the D-to-A converter to the MS and the 750 responses (a full spectrum) are recorded by the computer via the A-to-D converter. This process can be repeated approximately every 5 to 10 seconds for an arbitrary number of times. The spectra thus gathered are stored by the computer on magnetic disks or tapes. Program changes may be made to measure any subset of the 750 m/e positions and thus achieve faster repetitive spectra. Conversely more measurements may be made at any specific peak position, a technique which may be used for accurate isotopic ratio measurements.

Since many spectra are taken and stored during a GLC run or a solid probe experiment, the user requires fast methods to evaluate the data. The more useful data abstracting programs we use are:

THE MATRIX SEARCH. The user specifies which group of spectra, what range of mass values in each spectra, and how many large peaks he wants abstracted from each spectrum. An abstract of these highest peaks is then typed out and in many cases this abstract contains useful chemical information or at least indicates the spectrum of interest.

THE TIME PRESENTATION (PLOT). This is a computer drawn plot of certain peak intensities or a sum of all peak intensities of each spectrum (total ion current) plotted against time. The latter gives a good reproduction of the GLC curve and also indexes the spectra of interest (19).

NORMALIZED SPECTRUM PLOTS. Conventional bar graphs of mass vs. time, normalized and annotated, are routinely available.

All data outputs are in the laboratory and are available immediately after data acquisition. All spectra are filed and may be recalled at a later time or date and reprocessed in any way desired.

Involved programs of these magnitudes are specifically dependent upon the language of a given computer. The logic may be easily transferred, but in general the specific program may not. We have about 4 man-years of programming invested in this system.

THEORY OF OPERATION

During spectrum data acquisition, the computer directs the mass analyzer to a program-selected mass position and reads the output intensity of the MS. The mass analyzer is not swept in a conventional sense. As indicated in Figure 1, it is controlled by a voltage (V_c) such that

$$m/e = f(V_c) \quad (2)$$

where m/e is the mass/charge ratio and $f(V_c)$ is a monotonic function characterized by the MS. For every M_i , ($M = m/e$), to be passed by the mass analyzer, the computer has (according to the prior run calibration program) a digital number N_i

which is transformed by the D-to-A converter to the voltage V_c .

The determination of these values, N_i , is accomplished by the calibration program. The value of N for 12 key peaks of the reference compound are known to approximately 1 amu from prior calibrations. The actual N value for the centroid of each of these peaks is then determined by detailed examination of the m/e continuum in each of these areas. Sufficient detail is obtained by designing the D-to-A resolution to be 10 or more values per peak width.

After determining these exact 12 N values, linear interpolation, superimposed upon the analytical function, $m/e = f(V_c)$, is used to expand the list of 12 experimentally determined values to a full table of 750 entries. (The analytical function of m/e to control voltage is linear for the quadrupole and parabolic, $m/e = k(V_c^2)$, in the case of the T-o-F MS.)

Thus the procedure to measure the intensity at any M_i is as follows: *a.* The number N_i which corresponds to the selected m/e ratio (M_i) is loaded from the computer into the D-to-A converter. This sets the control voltage, V_c , to the mass analyzer. The output of the mass spectrometer is proportional to the quantity of ions, M_i , passed from the sample.

b. An analog circuit, reset and released by the computer, integrates the output of the MS.

c. Several milliseconds after the integrator is released. (the choice of integration time was initially supplied by the user upon program request), the computer samples the output of the integrator by means of an A-to-D converter. This digital value is stored as the intensity of M_i .

Steps (*a*) through (*c*) are repeated to acquire a complete spectrum.

The fundamental restraint upon this system is the drift of the function $m = f(V_c)$ following calibration. Our experience with the Finnigan 1015 and a Bendix T-o-F instrument and our interface, is that this drift causes an error in N of less than $1/3$ the value from one N entry to the next in a 1-hour period. This is sufficiently small to allow an unambiguous mass identification.

Table II contains comparisons of signal-to-noise ratios and the following defined figures of merit. The comparisons are made between the described control system, a linear scan in time, a parabolic scan in time such as the T-o-F, and the exponential time scan characteristic of magnetic instruments.

Uniform conditions are used to give realistic values for comparison; it is assumed that in each case the peak shapes are uniform if scanned in time, and that they are gaussian, and that the resolution is commensurate with the 10% valley (5% points on a single peak side) definition (3). In order to give typical comparison figures, it is further assumed that a spectrum will be taken from mass 50 to 500 in 4500 milliseconds.

The first column in Table II is the time the mass analyzer is on or about the mass position. In the case of the computer control system, the 4500 milliseconds is divided equally into 450 periods of 10 milliseconds each. Two milliseconds are allowed for each transition, and the mass analyzer will dwell on the peak position for 8 milliseconds. In the case of a conventional linear scan, the analyzer will enter a peak area and leave it 10 milliseconds later. By the 10% valley convention, this means the time from the beginning 5% level to the end 5% level of a single peak. However for the parabolic case (the T-o-F) it will be found that the resolution of the instrument will have to be set for the work case, peaks 499 and 500. It will be found that there is 6.6 milliseconds between these peak

(19) R. A. Hites and K. Biemann, "Advances in Mass Spectrometry IV," Elsevier Publishers, Amsterdam, 1968, p 37.

Table II. A Comparison of Attributes Affecting Signal-to-Noise Efficiencies

Typical Operation Condition: Scan from m/e 50 to 500 in 4500 Milliseconds

Type of scan	Time on, or between 5% points, of a peak, msec	Time constant of amplifiers, msec	Ions detected: (Peak intensities of n ions/msec)	Effective noise bandwidth, Hz	Figure of merit: $1000/n \times$ detected ions/bandwidth
Control and integrate	8	N/A	$8.0 n$	40	200
Linear scan $m = kt$	10	2	$5.1 n$	80	64
Parabolic scan $m = kt^2$	6.6	1.3	$3.4 n$	120	28
Exponential scan $m = m_0 e^{kt}$	3.9	0.8	$2.0 n$	200	10

centers, and the theoretical 5% heights must be at the midpoint. Since all peaks are similar, all peaks of the scan will be just 6.6 milliseconds from 5% point to 5% point. During 34% of the scan time, the analyzer is not in the area of any peak position at all; but is mostly between peaks in the low mass range. The case of the exponential scans is similar. In this case it will be found that the peaks are 3.9 milliseconds wide and 61% of the time no information can get through the instrument.

The next column indicates the time constant (τ) of the amplifier channel appropriate to the scan parameters. The control system uses a full integrator, so the entry is not applicable. In the conventional scanning system, the time constant is usually chosen as large as skewing permits to integrate signal and discriminate against noise. The relationship between τ and the 3-db bandwidth (f_{3dB}) of an amplifier is simply $\tau = 1/(2\pi f_{3dB})$. If τ is chosen to be large, peak skewing and broadening as illustrated in Figure 4 will occur. If τ is chosen small, the bandwidth with its attendant noise is excessive and there is little integration of the signal.

This is the dilemma always faced by the user of linear amplifier circuits: the desire to limit amplifier bandpass to smooth the signal, as opposed to the need for a wide bandpass to pass

the signal without distortion. Since the purpose here is to compare our described amplifier and integrator system with conventional linear amplifiers, a τ of 0.2 is assumed for the conventional case. This τ is still large enough to cause degradation of resolution in the conventional output signal (25 to 35% depending upon the definition used). It is felt that this choice represents a fairly typical operational parameter. The assumption of a rigorous lower value would result in an unnecessary, and perhaps unrealistic, comparison advantage for the described control and integrate signal system.

The column "Effective Noise Bandwidth" is the f_{eq} for the time dependent scans. However an equivalent 3-db bandwidth is not as well defined for the integrator. It can be shown that for an integration interval, T , (8 milliseconds in this example) an f_{eq} may be determined such that a linear amplifier of bandwidth f_{eq} would pass the same amount of "white" noise as the integrator. The actual bandpass of an integrator is a $\sin(x)/x$ type function.

The white noise power passed by either system may be expressed as an integration of the white noise model, e^{-x^2} , (20)

(20) W. B. Davenport, Jr., and W. L. Root, "Random Signals and Noise," McGraw-Hill, New York, 1958, p 88.

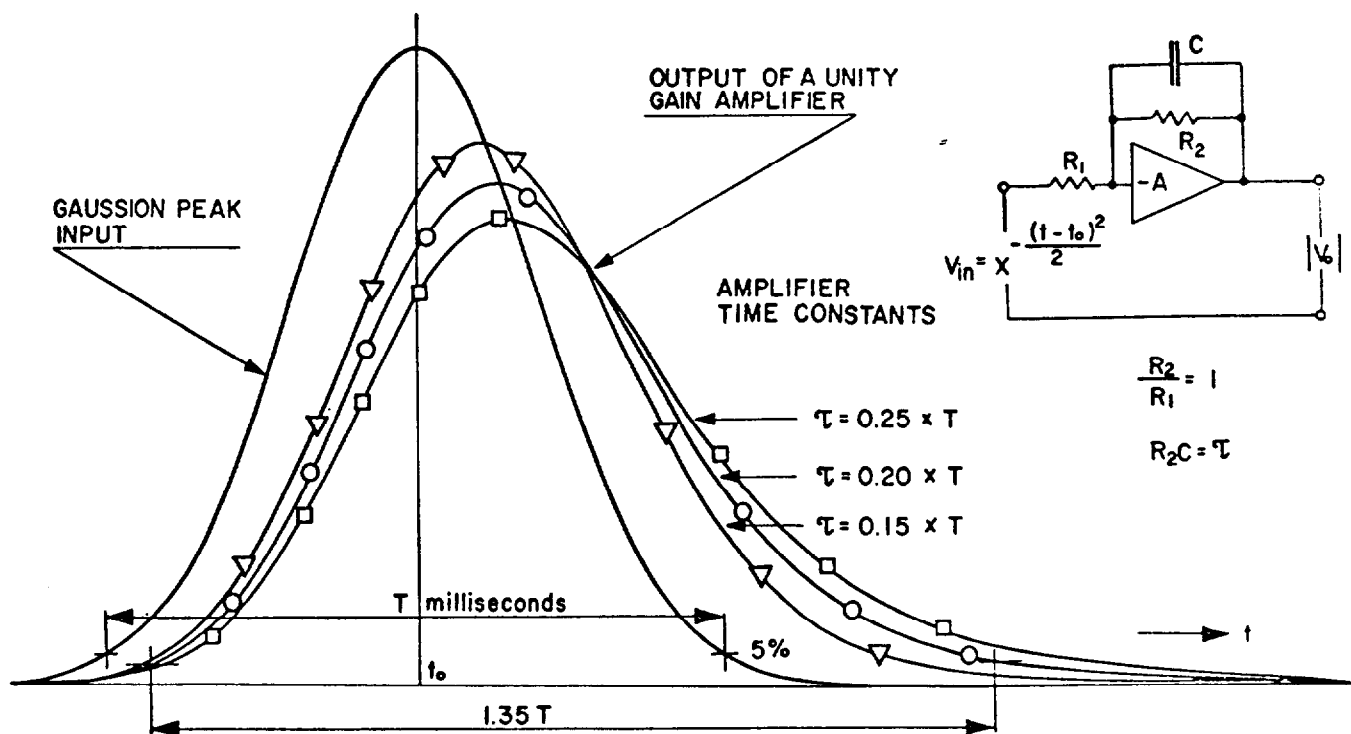
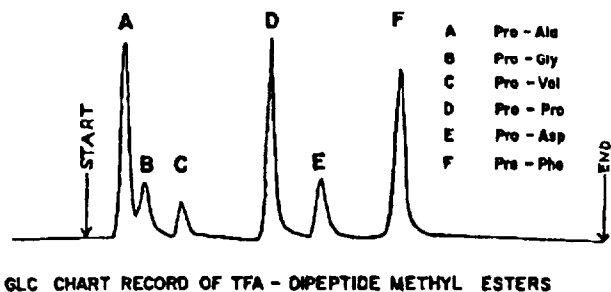
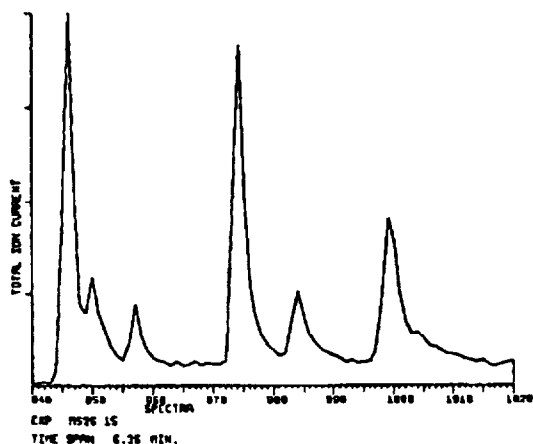


Figure 4. Peak broadening and skewing effect of narrow bandwidth amplifiers



GLC CHART RECORD OF TFA - DIPEPTIDE METHYL ESTERS



A COMPUTER PLOT OF THE SUMMED PEAKS (IONS) OF EACH SPECTRUM

Figure 5. The above trace is a GLC flame ionization chart of an experiment. Below is a total ion plot, created, and plotted by the computer, that indexes the individual spectra

```

OPTION = ?TYPE
EXPERIMENT# = ?1526 15
FROM SPECTRUM = ?955
TO = ?960
BY MASS, AMP, or ENTIRE = ?AMP
FROM MASS = ?330
TO = ?750
NUM'BER = ?9
SPECTRUM = ?955
MASS 31 42 43 46 57 69 139 166 167
AMPL 103 22 22 26 22 30 23 37 27
SPECTRUM 956
MASS 31 45 46 69 70 96 139 166 167
AMPL 117 25 25 32 33 27 33 64 44
SPECTRUM 957
MASS 31 43 45 69 70 72 139 166 167
AMPL 124 38 39 57 64 52 47 102 76
SPECTRUM 958
MASS 31 43 45 69 70 72 139 166 167
AMPL 114 31 32 36 31 34 32 60 48
SPECTRUM 959
MASS 31 43 56 69 70 96 139 166 167
AMPL 116 26 24 26 27 25 29 51 39
SPECTRUM 960
MASS 31 32 45 46 69 70 72 166 167
AMPL 113 22 24 23 25 24 21 36 30
  
```

Figure 6. The principal peak listing from the matrix search

times the circuit transfer function over the frequency range in ω . The first shown below (on the left side of the equation) represents the white noise retained by a full integrator during a time interval T . The second expresses the white noise passed by a simple amplifier of bandpass, $f_{eq} = 1/(2\pi\tau_{eq})$.

$$\int_0^{\infty} \left| \int_0^T e^{j\omega t} dt \right|^2 d\omega = \int_0^{\infty} \left| e^{j\omega t} \frac{1}{1 + j\omega\tau_{eq}} \right|^2 d\omega \quad (3)$$

A solution for τ_{eq} may be made by numerical methods:

$$\tau_{eq} \cong 0.496 T \quad (4)$$

Using this value, it is found in the example that $f_{eq} = 40$ Hz.

A last analytical benefit of the system is the efficiency of detecting ions. In any MS with electronic ion detection, an ion is detected or not detected and there is no "5% detection."

There is a 5% probability an ion generated in the source will pass the analyzer and hit the detector. Or more accurately, if when a peak is "centered," n ions register upon the detector each millisecond, it may be expected that at the appropriate low side of the peak, $n/20$ ions will register each millisecond. The control method is always "centered" upon the peak, hence the total expected ions will be $T \times n$ ions. However, in scanning a gaussian peak, it can be shown that for an equivalent time, from the first 5% point through the maximum and out to the last 5% point, the total expected ions will be approximately $0.51 \times T \times n$, where now T is the time from 5% point to 5% point. These numbers of ions expected to register upon the detector are tabulated as "Ions Detected" in Table II. Many small, but important, peaks will have an n of 1 to 10 ions per millisecond under the operating conditions imposed by some modern research requirements.

A figure of merit for comparing the systems may be defined:

$$\text{Figure of Merit} = \frac{1000}{n} \times \frac{\text{Detected Ions}}{\text{Bandwidth (Hz)}} \quad (5)$$

This is the final tabulation in Table II. Our use of the system has verified these results, that introducing computer control to the quadrupole or T-o-F MS can enhance the useful sensitivity by a factor of 3 to 8. The greater convenience offered to the user is not at the expense of performance, but offers these intrinsic advantages.

One other practical benefit is possible by reviewing the attributes of resolution. The idealized gaussian peak considered here, which has a width commensurate with the 10% valley resolution criteria, has a contribution of only 0.06% at the neighboring integer mass position. Since in the control method, only the cross contribution at the integer positions is harmful for some experiments, the resolution may be degraded until a 0.5% or 1% cross contribution is observed. If the physics of the particular instrument are exploited, it will be found that this will greatly increase the portion of generated ions that will be passed by the analyzer.

Some consideration should be given here to the software and its interaction with the user and the instrument. A mode of conversation has been programmed into the system to prompt the user for necessary parameters, and then expand upon these parameters to conduct the necessary detailed operation of the instrument. In retrospect we find that we have very closely followed the concept of R. J. Spinard (21). A similar problem occurs with the presentation of information, which is often too voluminous for complete display. To overcome this, the bulk of the information is held in the computer, and is made available by similar conversational techniques.

It should be realized that the present software is in an embryonic state of development compared to its eventual potential. The system of computer, interface, and MS may be defined in terms of computer syntax to a systems developer in a manner comparable to the definition of a specific data processing task to a system programmer. There is an enormous potential in the implemented syntax of this instrumentation system for the chemist who may have special requirements and who understands fully the principles of the augmented instrument. The user can then program, or have programmed, efficient solutions to many of his instrumentation problems.

The features that do allow the foregoing benefits, do themselves impose certain limitations. The control system does not normally return any information about doubly ionized odd

(21) R. J. Spinard, *Science*, **158**, 3797, 55 (1967).

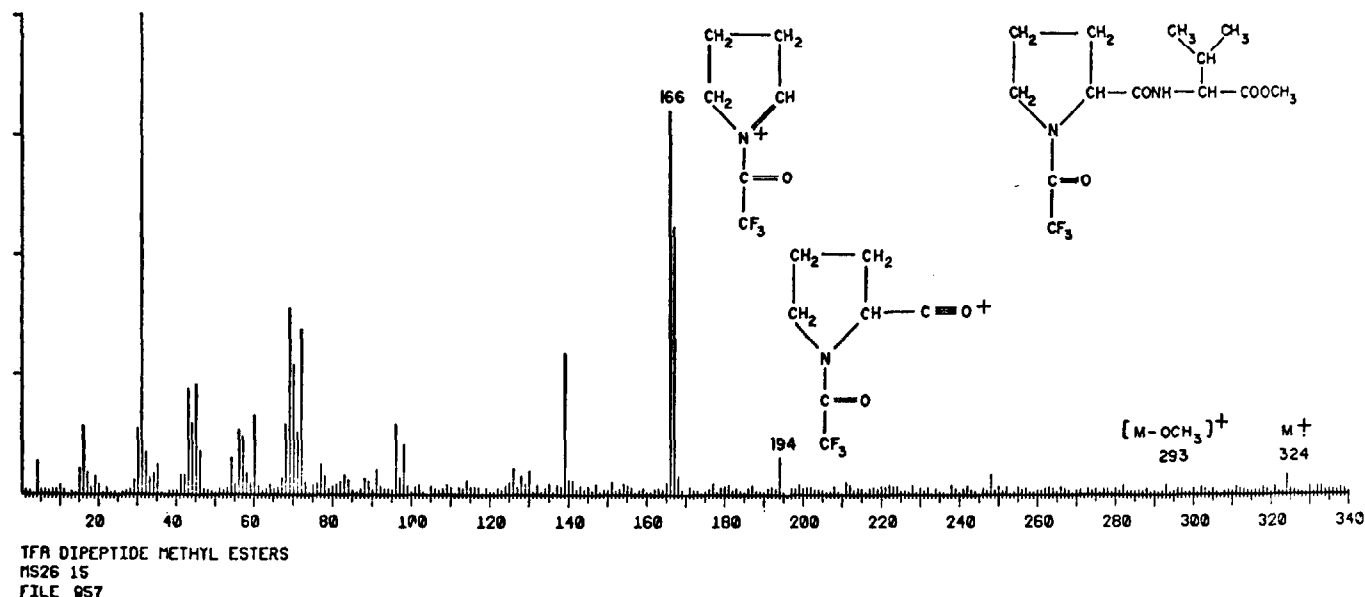


Figure 7. The on-line, computer plotted spectrum

mass values (peaks at 0.5 integer position), wide metastable peaks, or the value of mass defects. It also might be expected that mass defect deviations, especially at high m/e values, would cause difficulty. In practice there have not been any problems attributed to this latter case, the high m/e defect. The resolution and other operational factors have masked this inherent difficulty. If operational use should be hampered by this problem, we have proposed techniques of concurrent calibration on the unknown peaks themselves.

For example, the major peaks in the unknown compound, identified to their nominal integer value (less mass defects), would be used to calibrate the N table. After a programmed computer decision to explore a peak area, similar to those illustrated in Figure 3, the computer would direct the "scan" to that area. Data acquisition time would be about 100 msec for 10 data points over the peak profile. Centroid identification and recalibration might be another 100 msec. If this were repeated for 8 or 10 key peaks, total recalibration would be in the order of 2 sec. This technique could also be employed to verify the linearity above the last reference peak (614 with our system) or below the first. (We have paid little attention to m/e below 12 or 18.)

As a final minimal operating mode, the scan could be programmed to simulate any present mode of time-based scan and data collection, with comparable attributes of performance. As an example, doubly ionized masses or metastable peaks might be identified and measured. Of course the MS used must have sufficient resolution and/or sensitivity, also the total spectrum acquisition time would be increased. Such changes of operational mode would be by program only; hence they could be established or removed in milliseconds with no hardware or switch changes of any nature.

RESULTS

In a typical experiment a mixture of TFA-dipeptide methyl esters (22) was injected into the GLC-MS system, and 130 complete mass spectra were collected and recorded by the computer. Eighty-one of the individual spectra were summed to obtain the total ion plot (Figure 5) (19). This may be compared with the GLC flame ionization chart record on the top. The mass spectral output from peak "C," for example, in the

(22) B. Halpern and J. W. Westley, *Biochem. Biophys. Res. Commun.*, **19**, 361 (1965).

region of scans 955 to 960, was then abstracted by the TYPE routine and the results are shown in Figure 6. This data shows that Peak C is homogeneous and that a satisfactory normalized plot can be obtained from scan No. 957. Figure 7 is the plot as produced in the laboratory. The background in the mass spectrum is primarily due to the GLC column bleed. Only the chemical notation was added by hand.

The taking of spectra, in this computer-compatible form, opens the way to many types of further processing: searching through each stored spectrum to pick out the amplitude at a specified mass position and plot this data to show the variation of the ion current of a specific m/e with time; check the homogeneity of each GLC peak; resolve the mass spectra of simple mixtures into those of the individual components; and subtract background signal from a mass spectrum.

The system is also well suited to the recording of spectra from solid samples introduced directly into the ion source. Because of the fast scanning speed and the recording of many spectra, there is no need to establish a constant vapor pressure in the mass spectrometer and any contamination or decomposition of the sample is readily detected.

CONCLUSION

It is feasible to build a limited purpose computer and a quadrupole MS as one unit, forming a compact instrument with features similar to the system presented in this report. In addition, some of the software could be embodied in hardware circuits. Such units would be more costly than those using commercial general purpose computers, but could be uniquely well adapted to space or other special physical environments. Alternatively, the system can be interfaced with a larger time-shared computer.

RECEIVED for review March 23, 1970. Accepted June 15, 1970. This research was aided by grants from the National Institutes of Health, Public Health Service, AM 12797-01 and FR-00311-04. The LINC computer was provided under NIH grant FR-00151-01. The work is a part of a program of instrumentation research oriented toward automated planetary experiments, supported by the National Aeronautics and Space Administration, research grant NGR-05-020-004. Principal investigator, Joshua Lederberg; program director, Elliott C. Levinthal.

P-7;

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER		
		FROM 1/1/74	THROUGH 12/31/74			
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT	AMOUNT REQUESTED <i>(Omit cents)</i>			
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION	%/HRS.	TOTAL			
Lederberg, J.	Principal Investigator or Program Director	20	SCREENING FOR INBORN ERRORS OF METABOLISM			
Kretchmer, N.	Prof. of Peds.	10				
Cann, H.	Assoc. Prof. of Peds.	10				
Duffield, A.	Research Associate	100				
Pereira, W.	Research Associate	100				
Summons, R.	Post.Doc. Fellow	100				
Rindfleisch, T.	Research Associate	20				
Tucker, R.	Computer Programmer	75				
Veizades, N.	Research Engineer	33				
Steed, E.	Research Engineer	33				
Pearson, D.	Elect. Tech.	40				
Wegmann, A.	Senior Res. Assist.	10				
Open - Genetics	Grad. Res. Assist.	50				
Open - Genetics (continued)	Grad. Res. Assist.	50				
TOTAL →			\$ 157,208			
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$ -			
3. EQUIPMENT <i>(Itemize)</i>			\$ 110,400*			
see attached list						
4. SUPPLIES			\$ 16,200			
See attached list						
5. STAFF TRAVEL <i>(See Instructions)</i>	a. DOMESTIC 2 East Coast (1000), 1 Mid-west (300), 1 West Coast		\$ 1,500			
	b. FOREIGN (200)		\$			
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>			\$			
7. ALTERATIONS AND RENOVATIONS			\$ 1,800			
Relocate equip., power, etc. for GC/MS Data System						
8. OTHER EXPENSES <i>(Itemize per instructions)</i>			\$ 9,100			
Mini-computer maintenance		\$6,000				
Freight on capital equipment		\$ 500				
Office supplies, telephone, repro., postage, publication costs, etc.		\$2,600				
9. Subtotal - Items 1 thru 8 →			\$296,208			
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			\$		
	a. STIPENDS	PREDOCTORAL	No. Proposed _____			\$
		POSTDOCTORAL	No. Proposed _____			\$
		OTHER <i>(Specify)</i>	No. Proposed _____			\$
	DEPENDENCY ALLOWANCE					\$
	TOTAL \$TIPEND EXPENSES →					\$
b. TUITION AND FEES			\$			
c. TRAINEE TRAVEL <i>(Describe)</i>			\$			
11. Subtotal - Trainee Expenses →			\$			
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i> →			\$296,208			

continued

1. PERSONNEL	TITLE	% TIME
Wyche, M.	Lab. Tech.	100
Boswell, M.	Lab. Tech.	100
Jamtgaard, R.	Administrator	15
Allen, M.	Secretary - Genetics	25
Murray, R.	Secretary - Peds.	25
Harlow, W.	Machinist	10
Open - Genetics	Lab. Glasswasher	25

3. EQUIPMENT

4. Column Gas Chromatograph		\$14,400*
Mini-Computer System:		
PDP-11/45 (8k memory)	20,700	
Memory (16k)	5,200	
Floating Point Hdw.	5,200	
Prog. Clock	600	
Bootstrap Loader	600	
Industry Tape	10,500	
Disk System	11,700	
Lab Interface Units	4,600	
Interproc. Comm.	800	
	<u>59,900</u>	59,900*
Amino Acid Analyzer		32,500*
Event Counter		2,700*
Digital Voltmeter		900*
		<u> </u>
	Total Equipment	\$110,400*

4. SUPPLIES

Chemicals, glassware, and lab apparatus	\$5,000	
GC supplies (columns, phases, etc.)	1,100	
Dry ice and liquid nitrogen	500	
Data recording media (GC/MS, Calcomp, etc.)	1,800	
Mini-computer supplies (start-up & continuing)	1,600	
Mass spectrometer repair parts & supplies	2,300	
Electronic parts & supplies	1,900	
Amino Acid Analyzer supplies	2,000	
	<u> </u>	
	Total Supplies	\$16,200

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	157,208	168,338	215,892	230,758	246,632		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>							
EQUIPMENT	110,400*	22,500*	17,600*	5,300*	6,000*		
SUPPLIES	16,200	16,900	20,200	21,400	22,800		
TRAVEL	DOMESTIC	1,500	1,800	1,900	1,900	2,000	
	FOREIGN						
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS	1,800						
OTHER EXPENSES	9,100	11,500	12,000	12,700	13,500		
TOTAL DIRECT COSTS	296,208	221,038	267,592	272,058	290,932		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →					\$ 1,347,828		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget Explanation attached

BUDGET EXPLANATION

The early phases of the present study have been funded by NASA but that agency is rapidly severing its involvement in biological projects. It has already severely reduced its fiscal support of our program in anticipation of total withdrawal within one or a few years. We have started to reduce laboratory staff in response to those cutbacks and will not be able to maintain this laboratory without new funding (like the present proposal). Since we cannot predict the level of funding we will receive for each of these proposals, we simply note the overlap and will negotiate a suitable joint effort for system development and operation, depending on funds available.

The following is a detailed explanation of the budget for this subprogram. This budget covers only the indicated segment, with an indicated allocation for the time of a number of participants who also appear in other segments. These allocations are integrated into the overall proposal budget summarized later (see Table of Contents for location).

It should be noted that support for Professor Lederberg's time is shown at 20%. This includes not only his participation in this subproject but also his role in overall program direction as Principal Investigator.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

YEAR #1 (Metabolic Screening only)

PERSONNEL: Support is requested for 10% of Professor Cann in Pediatrics (Professor Kretchmer will devote 10% of his effort without grant salary support) for clinical and diagnostic inputs to the program. Drs. Duffield, Periera, Summons, and Ms. Wyche are responsible for the derivatization and chemical analysis of urine samples including GC/MS operations. Support for an operator (Ms. Boswell) for the prescreening amino acid analyzer is included. Mr. Steed will maintain the glasswork aspects of the GC/MS system. Messers Rindfleisch and Tucker will be responsible for system design and software implementation respectively and Mr. Veizades, supported by Mr. Pearson, will design the electronics. Ms. Wegmann operates the GC-High Resolution Mass Spectrometer in the Department of Chemistry and will be available to run high resolution mass spectra on critically important samples. Other part-time personnel (machinist, secretaries, and administrative support) act in necessary supporting roles including assisting with liaison work with collaborating physicians at Stanford and elsewhere.

CAPITAL EQUIPMENT: We request a PDP-11/45 mini-computer system as an extension of the data system for our low resolution mass spectrometer. This system will initially have 24K words of memory and use a 1.2M word disk system for monitor, program, and spectral data storage. Removable data storage for raw data and archival spectrum recording is provided by the requested industry compatible magnetic tapes. The terminal, loader, clock, instrument interface hardware, etc. complete the system. Funds to maintain the computer system under manufacturer contract are included under "OTHER." A detailed computer equipment list is included in the "First 12 month budget detail."

We also request a 4 column gas chromatograph and an amino acid analyzer which will increase our urine prescreening capacity thereby alleviating the bottleneck at the mass spectrometer. The latter instrument is particularly pertinent to the larger scale prescreening of samples from the Kaiser Hospital, the Santa Clara Valley Medical Center, the Children's Hospital at Stanford, and the LA County Hospital. The amino acid analyzer will also allow reliable continued access to screening facilities for routine clinical studies during times when the GC/MS may be tied up for more sophisticated studies, or may be down for maintenance, repair, or re-engineering. The reliability offered by this back-up will help resolve the problem faced by any clinician when he attempts to investigate new techniques while committed to offering the highest standard of care. Once an analytical technique is coupled to patient care, it cannot ethically be disengaged - a priority that puts great strains on any system also intended for further research. In the present context, once a metabolite has been identified, it may be efficient to monitor its level, in repetitive samples, by techniques like the amino acid analyzer.

We have access to both low resolution and high resolution mass spectrometers (physically located in the Genetics and Chemistry Departments respectively) and do not request augmentation of this costly hardware for the present proposal.

SUPPLIES: Supplies are requested for sample preparation and equipment operation. These include chemicals, glassware, laboratory apparatus, GC supplies, amino acid analyzer supplies, dry ice and liquid nitrogen, recorder paper for the GC/MS instruments and Calcomp plotters, and computer operation supplies. In addition, we request supplies and parts for the maintenance (by our own hardware personnel) of the GC/MS system and electronic equipment as well as for the implementation of necessary data system interface hardware.

TRAVEL: Travel costs are estimated at round trip expenses for two professional meetings on the east coast and 1 trip each to the mid-west and west coast.

ALTERATIONS AND REMOVATIONS: We will need to modify the electrical service and relocate some equipment in order to install the new data system and gas chromatograph and to allow for future equipment we are requesting. This estimate is based on a similar modification just made in another laboratory.

OTHER EXPENSES: Office supplies, telephone service, postage, publication costs, etc. are requested based on operating experience in our laboratory. Computer maintenance and equipment freight are estimated for the proposed data system augmentations.

YEAR #2

PERSONNEL: Personnel are unchanged in year 2.

CAPITAL EQUIPMENT: As the data system adaptation reaches completion after year 1, we request additional machine features to facilitate operational use simultaneously for GC prescreening and for MS analyses. These include a block of fast memory (1K words) to speed up the machine, an additional disk drive for larger volume on-line storage requirements and a high speed display device for system status and processing result feedback. Appropriate equipment maintenance increases are included for the expanded data system.

SUPPLIES, TRAVEL, AND OTHER: These items are increased by 6% to reflect inflation and the needs of increased throughput of urine screening.

YEAR #3

PERSONNEL: We anticipate an increased volume of samples to be processed by the third year as our baseline studies progress and more extensive clinical screening is undertaken. This implies the need for additional support both at the clinical and laboratory level. For this reason we have added 50% time of an Assistant Professor of Pediatrics and 100% time for a laboratory technician for sample preparation and equipment operation to the budget.

CAPITAL EQUIPMENT: An additional prescreening GC is requested to allow larger population analyses along with the appropriate computer interface equipment.

SUPPLIES, TRAVEL, AND OTHER: The budget for supplies and equipment maintenance are increased consistent with personnel and equipment additions. Also provision is made for inflation and longer daily operation.

YEAR #4

PERSONNEL: Personnel remain unchanged in year 4.

7-11

CAPITAL EQUIPMENT: We request a system for the vacuum distillation of aqueous samples for specimen preparation and derivatization as well as miscellaneous test equipment which will require replacing.

SUPPLIES, TRAVEL, AND OTHER: Increases are requested to cover expected inflation.

YEAR #5

CAPITAL EQUIPMENT: A small provision is made for replacement test equipment.

SUPPLIES, TRAVEL, AND OTHER: Increases similar to year 4 are requested.

SECTION III

Maternal Blood Stream - Another Source of Fetal
Tissue for Pre-Natal Diagnosis of Genetic
Disorders

Drs. Herzenberg and Cann

The Maternal Bloodstream - Another Source of Fetal Tissue for Prenatal Diagnosis of Genetic Disorders

Dr. L. A. Herzenberg, Principal Investigator
Dr. H. Cann, Associate Investigator

A. INTRODUCTION

A.1 Objectives

We propose to study methods of labelling, detecting and isolating fetal lymphocytes which pass into the maternal circulation during gestation. Further data on the time, extent and conditions of occurrence of these cells during normal human pregnancy could lead to non-invasive methods for prenatal diagnosis of genetic disease.

A.2 Background and Rationale

Fetal lymphocytes have been reported to be present in the mother's blood in the majority of pregnancies; they may be present in the maternal circulation in all pregnancies. The frequencies of fetal cells found, that is the percentage of fetal lymphocytes in the pregnant woman's blood stream, have ranged from 0.05% to as high as 1.5% in two studies. In one, by Walknowska, Conte and Grumbach (1), the presence of 5 small acrocentric chromosomes was sought in lymphocytes obtained from 10 milliliters of maternal blood, stimulated by phytohemagglutinin (PHA) and examined at metaphase. From one to three metaphases out of many hundreds, suggested a male fetus in 21 pregnancies. This diagnosis was checked at birth and 19 males were correctly predicted. Three of these mothers were studied at 20 weeks or less of gestation. In the second study (2), quinacrine fluorescent-staining Y bodies were looked for in interphase lymphocytes obtained from blood (20 milliliter specimens) of women in the second and third trimesters of pregnancy. Here eleven male infants were predicted and seven were verified at birth. Since the method used by Walknowska et al. is relatively insensitive, we suggest that fetal lymphocytes are present in the maternal bloodstream in frequencies of about 0.1% or more in all pregnancies at least from 14 weeks of gestation on.

Fetal lymphocytes possess HL-A antigens perhaps as early as 12 weeks of gestation (3). The enormous amount of polymorphism noted in Caucasian populations (4) is such that most individuals will be heterozygous at one or both HL-A sub-loci and the likelihood of mates differing at one or both sub-loci is high. These facts suggest that fetal lymphocytes may be separated from the maternal bloodstream by taking advantage of the HL-A differences existing between parents. A cytotoxic antibody directed toward the maternal HL-A antigen which the fetal lymphocytes do not carry, will, in the presence of complement, lyse the maternal leukocytes sparing those of the fetus. Or,

indirect labeling of the fetal lymphocytes coated with antibodies to the paternal HL-A type with fluorescein conjugated anti-human globulin will render these fluorescent in a sea of non-fluorescing maternal cells.

The fluorescent cell-separator perfected by engineers working under the direction of one of us (Herzenberg) can separate fluorescent from non-fluorescent cells with high efficiency, viability and yield (Figure 1; also reference 5). It can detect fluorescent-labeled cells present in frequencies lower than 10^{*-5} and can separate and thus enrich by a factor of 10^{*3} . In model experiments with human erythrocytes, Rh+ cells stained with anti-D and fluorescein-conjugated anti-human immunoglobulins were detected about one time in two at a frequency of one positive cell per 10^{*5} Rh negative cells. Post-partum blood samples of Rh- mothers who just gave birth to an Rh+ child were found to have from 1/163,000 to 1/5,000 Rh+ cells indicating fetal blood leakage, probably at birth, of from 20 to 680 microliters (6). Thus, it should be quite feasible to enrich fetal lymphocytes from the reported frequencies of 10^{*-3} to close to purity after the fetal cells are stained by immunofluorescence for paternal HL-A antigens.

We recognize the potential of the cell separator to separate fetal red blood cells from the maternal bloodstream on the basis of heritable erythrocyte antigens. Such methodology could lead to the development of prenatal diagnostic tests for sickle cell anemia, possibly thalassemia, and other hereditary disorders of the erythrocyte. We have decided, however, to emphasize isolation of fetal lymphocytes in this research project because of the reports (1,2) suggesting that these cells are detectably present in the maternal bloodstream in most, if not all, pregnancies. The literature suggests that fetal red blood cells are detectable in the maternal circulation in 5-10% of pregnancies at 20 weeks of gestation or less (7,8). Whether the application of the cell separator to detection and separation of fetal red blood cells will improve these figures, is a question we have been discussing. We believe that more data on the frequency of mothers showing fetal red blood cells in their circulations and the frequency of the cells themselves at various times in pregnancy are needed, and the cell separator could be applied to this research question. At present, our estimates of the amount of fetal red blood cells we can isolate, based on the performance of the cell separator and on the amount of fetal blood reported in the maternal circulation, indicate that our yield will be too low (perhaps 100 times too low) to permit diagnostic studies based on incorporation of radioactive label into globin synthesized by fetal cells (9,10). For these reasons too, we are persuaded to work with fetal lymphocytes. However, since we developed and used the methodology to detect Rh- fetal cells in post partum Rh+ maternal blood specimens (6), we are planning preliminary experiments designed to answer questions as to amount, frequency, and requirements, raised above. We are collaborating with Dr. Herbert Schwartz, Professor of Pediatrics and Chief of the Pediatric Hematology Service at Stanford, in this preliminary

work. Dr. Schwartz will review the morphology of and determine whether globin synthesis can be measured in cells separated from the maternal circulation. Our preliminary work will be aimed at ways to increase yield and/or decrease amount of fetal blood required for prenatal detection tests. At this time, we are not asking for budgetary support in this area, although we may, should preliminary work show promise.

The remainder of this proposal will deal only with the separation of fetal lymphocytes from the maternal circulation.

B. SPECIFIC AIMS

We propose to use heritable surface antigens as a basis for separating fetal lymphocytes from the maternal bloodstream during gestation. These separated cells will be used to diagnose various genetic abnormalities in the fetus.

C. METHODS OF PROCEDURE

C.1 Immunoselection of Lymphocytes

(a) POSITIVE IMMUNOSELECTION WITH THE FLUORESCENT CELL SEPARATOR. Specific fluorescent staining for a variety of HL-A antigens will be developed. In most cases, we will use the indirect procedure, i.e. anti HL-A whole serum followed, after washing, by fluorescein or rhodamine conjugated anti-human immunoglobulin (F or R anti-Ig). In pilot studies for this project, we have already obtained staining sufficiently bright to achieve excellent cell separation with a polyspecific and two specific HL-A antisera. In order to decrease non-specific staining (of lymphocytes lacking the antigens to which the antibodies are directed) we have had to remove aggregated proteins of antiserum by centrifugation at 100,000 g for 2-3 hours.

Lymphocytes are prepared by ficoll-Isopaque isopycnic centrifugation (11) followed by filtration through nylon wool to remove monocytes and many B-lymphocytes. Since these latter have surface immunoglobins, they stain with the fluorescent reagents, although much more dimly than the HL-A stained cells. With these virtually pure lymphocyte population, of which greater than 90% are thymus-derived (T) lymphocytes, we have obtained bright speckled fluorescence by staining at 0 degrees C. When we have passed positive and negative (stained for a particular HL-A type) cells separately through the cell separator, the fluorescence intensity distribution has shown two clear populations with very little overlap (Figure 2). Thus, good separation can be obtained.

Initially, reconstruction experiments will be performed using various mixtures of cells positive and negative for an HL-A antigen or antigens. In addition to numerical evidence (from the fluorescence intensity distribution) for successful separation, we will use male and female cells so that sex chromatin, karyotyping and quinacrine staining for Y bodies (2) can be used as confirmatory markers.

In isolations from maternal blood, antibodies directed to paternal and not maternal antigens will be used followed by F anti-Ig.

(b) **NEGATIVE IMMUNOSELECTION WITH COMPLEMENT MEDIATED LYSIS.** As an alternate procedure to or a step preliminary to fluorescent cell separation, (maternal) cells with particular HL-A antigens will be killed by incubation with antiserum and complement. Antisera directed against maternal antigens not shared by the father will be used when possible. With separate aliquots of lymphocytes, antisera negative for one or the other set of paternal antigens will be used.

C.2 Fetal Lymphocyte Antigens as Alternates to HL-A for Immunoselection with the Fluorescent Cell Separator

Several fetal antigens have been described (12) but, so far, none of lymphocytes. If an antigen could be found to be present on early fetal lymphocytes but absent, or in greatly diminished amount, on adult lymphocytes, it would be considerably more convenient to use than HL-A antigens. There would be no problem of typing the father and having to select for each of the paternal haplotypes.

We will attempt to find a fetal antigen by direct immunization of other species with lymphocytes from aborted fetuses. Any antisera obtained will be absorbed with adult cells, including lymphocytes, to render it specific. Of course, we cannot predict in advance if this will succeed. Another means to look for fetal lymphocyte antigens is to use antisera raised against leukemias. Tumors often have fetal or embryonic antigens; whether leukemias do is unknown. We will test for them with fetal and normal adult lymphocytes.

C.3 Mitogens Preferentially Stimulating Fetal Cells.

It is possible that some mitogens will be relatively more stimulatory to fetal than adult lymphocytes. If so, this would decrease the importance of maternal contamination of separated fetal cells. We will screen all the available lectins including PHA, PWM (pokeweed mitogen), concanavalin A, wheat germ agglutinin, etc. for mitogenic activity with cells from aborted fetuses of several ages and cells from adults. One precedent for differential mitogenesis is PHA which stimulates fetal mouse thymocytes 10 times more than adult mouse lymphocytes (13). Evidence of differential response to PHA and PWM by lymphoid cells of human newborns (cord blood) and adults suggests that this approach may be feasible (14).

C.4 Lymphocyte Culture and Karyotyping.

Separated lymphocytes will be grown in micro-culture plates with phytohemagglutinin (PHA) for 48 hours, colcemid added and chromosome spreads made 16-20 hours later. In preliminary experiments we have obtained high percentages of metaphases with

50,000 cells cultured in 250 microliters of medium. Prior staining with anti HL-A does not seriously decrease (less than 2-fold) the mitotic index. We have been successful in obtaining high quality chromosome spreads by standard techniques with these small numbers of cells in micro-cultures. We believe we could culture (in 10 microliters medium) and spread as few as 1,000 lymphocytes. However, that is yet to be tried.

To check our ability to separate, grow and karyotype fetal lymphocytes, we will predict the sex of the fetuses and compare with the sex observed at birth.

C.5 Cell Separations

Fluorescent cells will be separated in the fluorescent cell sorter we have built (see Figure 1). Cells suspended in medium pass in single file in a stream past 2 laser light sources and 2 photodetectors. One laser and detector observes cell fluorescence and the other light scatter which is a measure of cell volume. When a cell with the desired fluorescence level and of the right volume is detected, a charge is applied to the stream with a delay of about 100 microseconds. The stream is broken into 40,000 uniform droplets per second downstream of the observing point. Charges are applied when droplets containing the desired cells are just being formed so they become charged. They are then deflected by constantly charged deflecting plates into collecting tubes.

The number of cells processed per unit time is generally inversely related to the enrichment obtained. With a frequency of desired cells of $10^{(-3)}$, 10^{+4} cells per second can be processed to obtain about 100% purity of wanted cells. A yield of at least 20,000 desired cells per hour should be obtained. This is certainly enough to culture and karyotype. If the frequency of fetal lymphocytes in the maternal bloodstream is 10-fold lower, i.e. $10^{(-4)}$, then only 2,000 cells per hour would be obtained. This is probably a marginal number for culture. Furthermore, there would probably be prohibitable losses with so few cells. However, there is no need to seek high purity. When looking for male karyotypes, or later for gross chromosome abnormalities, only a few successfully spread cells of the selected type are needed. If we predict 5% purity, we need to screen about 100 cells to be quite certain of seeing a few metaphases from the selected cells. Thus, preliminary analysis of maternal samples will be used to determine the amount of cells to be put through the cell sorter.

With the $10^{(-4)}$ frequency, which is at least 10 times lower than the published estimates, $2 \times 10^{+7}$ lymphocytes from maternal blood will be processed per hour. This approximately represents the number in 10 milliliters of blood. For a routine, non-objectionable procedure, we feel this (or perhaps 20 milliliters) is about the limiting amount of blood which could be used. Thus, for screening purposes, the minimum fetal lymphocyte frequency is probably slightly less than $10^{(-4)}$. We will

establish what the real frequencies are in a reasonable number of pregnant women at various stages of gestation. If the published values are correct, we will not be limited by fetal cell frequency. (For high risk pregnancies, it may be that even with a frequency of less than 10^{*-4} , it would be preferable to do the antenatal cytogenetic analysis with 50-100 milliliters of blood rather than by amniocentesis).

C.6 Strategy (and Summary)

(a) HL-A specific staining by immunofluorescence will be developed. This is presently in progress.

(b) Preliminary enrichment by cytotoxicity with HL-A antisera and complement will be assessed.

(c) Separation from artificially mixed populations will be carried out using sex markers to confirm.

(d) Fetal lymphocyte frequencies will be determined at various stages of gestation.

(e) Fetal lymphocyte antigens will be sought.

(f) Selective fetal mitogens will be sought.

(g) If the frequency in late first trimester or early second trimester pregnancies is about 10^{*-4} , separations, culture and karyotyping will be done. Accuracy of prenatal sex prediction will be used to confirm separations.

Time Table: Development of the method with artificial mixtures and maternal blood will take between one and two years. Assessment of frequency of fetal lymphocytes in maternal blood as a function of gestational time will take about six months.

Subsequent steps involve developing enough reagents for a significant trial at detecting chromosome abnormalities using high risk pregnancies. This stage will also require setting up a cooperative effort involving patients at Stanford and other nearby institutions (e.g. the University of California Medical Center, San Francisco; Dr. C.J. Epstein). This will involve comparing results from the cell separator technique with those from amniocentesis. We anticipate applying for supplementary funds after 3 years if this pilot project is successful.

D. SIGNIFICANCE

A routine method of antenatal diagnosis using 10-20 milliliters of maternal blood would permit mass screening for genetic disorders, especially those involving chromosomal aberrations. The methods we propose could result in avoiding the use of amniocentesis even in high-risk pregnancies (amniocentesis would be difficult to apply for mass screening).

The cost of separation and karyotyping could be brought into the range of a few dollars per test with the development of semi-automated micro-cultures and computer assisted karyotyping (Dr. C.J. Epstein, personal communication). The cost of the 0.5-1% of congenital chromosome abnormalities among births is far higher than the cost of such screening. It is impossible to assign a uniformly acceptable cost in total human terms, but the actual costs to the family and/or society must be in excess of 100,000 dollars for each infant with Down's syndrome born. Screening to detect an extra chromosome 21 would yield 1 case in 600 at a cost of probably from 1,000-10,000 dollars. Thus, the cost would likely be justified even for only this one condition.

Further work could permit biochemical and particularly cytochemical tests (15) for inherited disease to be performed on the separated fetal lymphocytes either before or after culture.

We wish to stress that the use of maternal blood rather than amniotic fluid samples provides a non-invasive method of antenatal diagnosis. This methodology can eliminate the risks to fetus and mother which are encountered in amniocentesis.

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TITLES OF FIGURES

Figure 1. Diagram of fluorescent cell separator.

Figure 2. Demonstration of almost complete separation of HL-A stained and unstained cells.

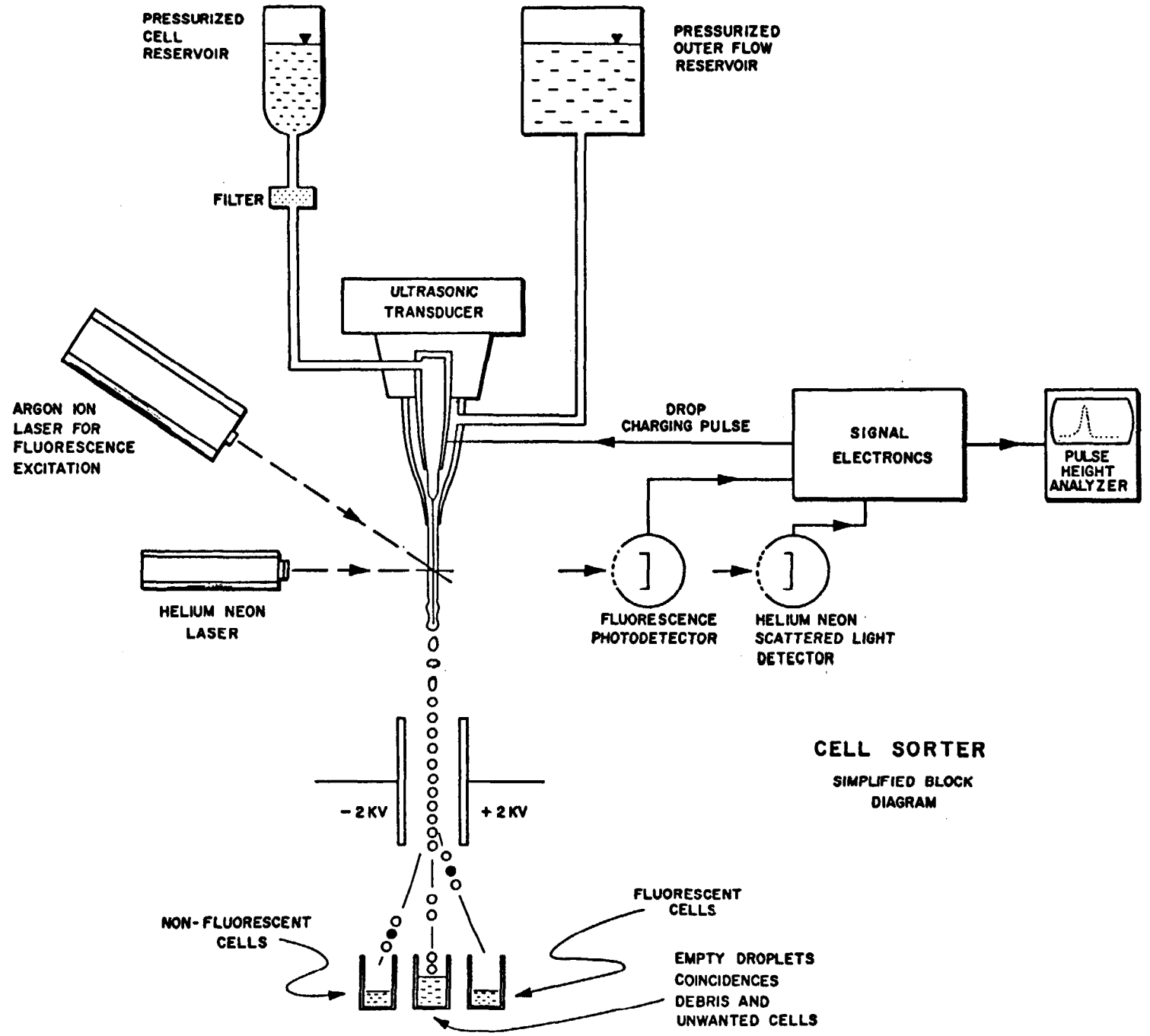


FIGURE 1

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Frequency Distribution

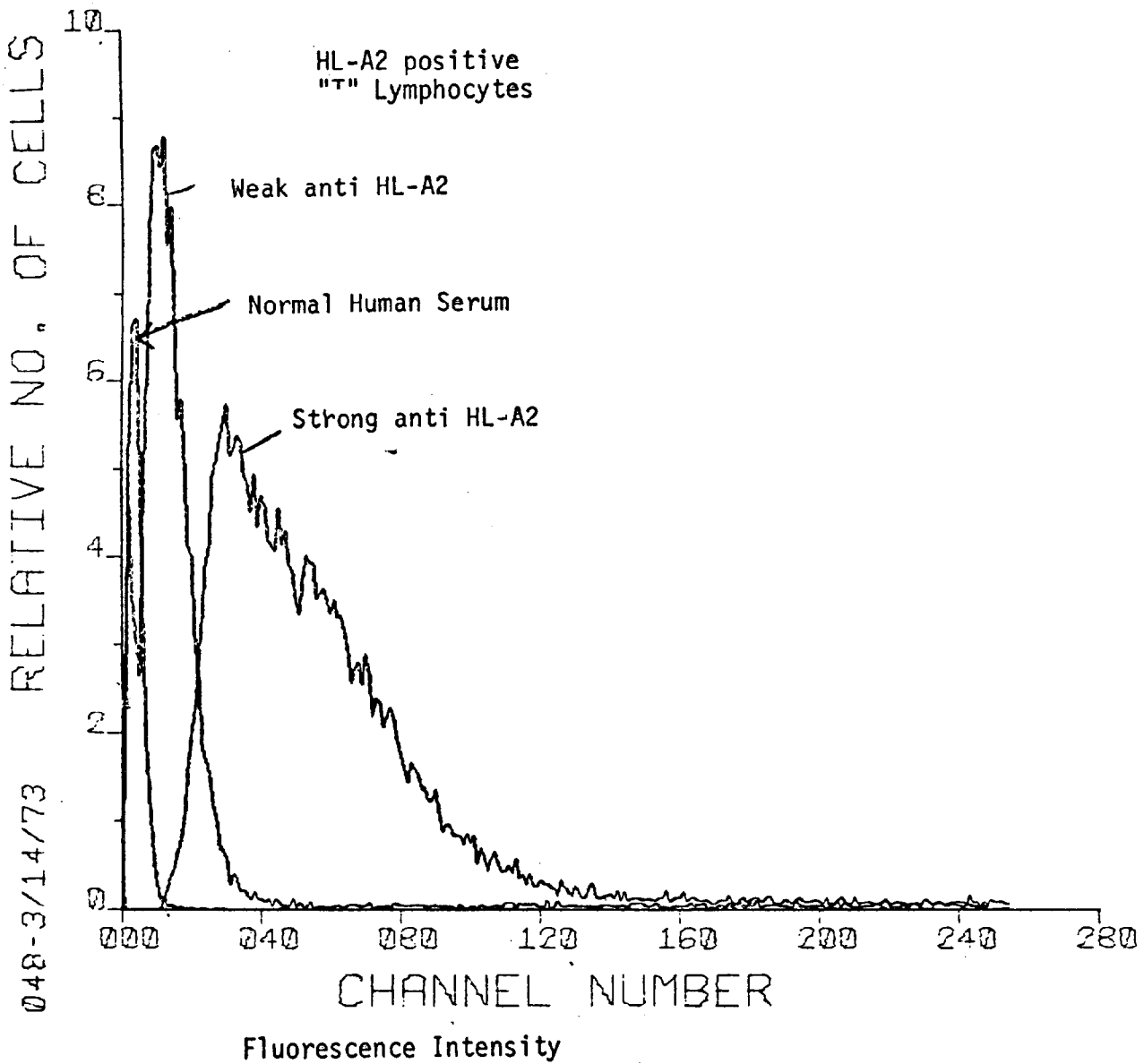


FIGURE 2

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM 1/1/74	THROUGH 12/31/74	
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT %/HRS.	AMOUNT REQUESTED <i>(Omit cents)</i>	
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION		TOTAL	
Herzenberg, Leonard	Principal Investigator or Program Director	10%	FETAL CELLS IN MATERNAL CIRCULATION	
Cann, H.	Assoc. Prof. Peds.	10%		
Open - Pediatrics	Research Associate	100%		
Hulett, H.	Research Associate	20%		
Waters, Ruth	Research Assistant	100%		
Sakaguchi, S.	Research Assistant	50%		
Open - Genetics	Engineering Tech.	50%		
Meyering, P.	Secretary	10%		
TOTAL →			\$ 54,782	
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$	
3. EQUIPMENT <i>(Itemize)</i>	Carbon Dioxide Incubator	\$2,500	\$ 12,500 *	
	Laminar Flow Hood	1,000		
	Fluorescence Microscope	9,000		
4. SUPPLIES			\$	
Tissue culture supplies, (filters, trays, etc.), dishes, media, pipets, sera, etc.			\$ 7,000	
5. STAFF TRAVEL <i>(See Instructions)</i>	a. DOMESTIC	1 East Coast meeting	\$ 500	
	b. FOREIGN		\$	
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>			\$	
7. ALTERATIONS AND RENOVATIONS			\$	
8. OTHER EXPENSES <i>(Itemize per instructions)</i>			\$	
Office supplies, telephone, repro., postage, publication costs, etc.			\$ 1,000	
9. Subtotal - Items 1 thru 8			\$ 75,782	
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>		\$	
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
	TOTAL \$TIPEND EXPENSES →			\$
b. TUITION AND FEES			\$	
c. TRAINEE TRAVEL <i>(Describe)</i>			\$	
11. Subtotal - Trainee Expenses			\$	
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>			\$ 75,782	

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	54,782	58,659	62,704	67,021	71,629		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>							
EQUIPMENT	12,500*						
SUPPLIES	7,000	7,500	7,900	8,400	8,900		
TRAVEL	DOMESTIC	500	600	600	600	700	
	FOREIGN						
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS							
OTHER EXPENSES	1,000	1,100	1,200	1,200	1,300		
TOTAL DIRECT COSTS	75,782	67,859	72,404	77,221	82,529		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →					\$ 375,795		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget explanation attached.

BUDGET EXPLANATION

The budget covers 10% each of Professor Herzenberg's and Professor Cann's time in relation to this subproject. A full time Research Associate is required to obtain, prepare, and fluorescent label the lymphocytes for separation and then to culture and perform cytogenetic studies on the separated cells. The part time (20%) Research Associate is to be responsible for the actual separations. Two Research Assistants, one part-time (50%) for cytogenetic studies and one full-time for serological studies, as well as a part-time (50%) cell separator operator are required to assist in these procedures. Support is also budgetted for 10% of Professor Herzenberg's secretary.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The laminar flow hood is essential for aseptic culturing and protection of personnel from possible infectious agents in human blood. The carbon dioxide incubator is required for culturing lymphocytes. The fluorescent microscope will be used to detect interphase fluorescent Y bodies and the Y chromosome at metaphase. In the later stages of this study, the fluorescence microscope will be used to study the karyotypes of lymphocytes of fetuses with chromosomal aberrations.

The budget for this project represents the MINIMUM ADDED COSTS to Dr. Herzenberg's and Dr. Cann's other programs (cell separator project, in particular) required for this work to be performed.

SECTION IV

Polymorphic Genetic Markers in Amniotic Fluid

Drs. Cann and Tsuboi

Polymorphic Genetic Markers in Amniotic Fluid

Dr. H. Cann, Principal Investigator
Dr. K. K. Tsuboi, Associate Investigator

A. INTRODUCTION

A.1 Objective

We propose to study amniotic fluid (AF) and cultured amniotic fluid cells for the expression and variation in the expression of various polymorphic genes. As the loci of those polymorphic genetic markers which are expressed in AF are shown to be closely linked to loci of genes determining disease, we will use the specific syntenic relationship for prenatal detection of the disease in the fetus.

A.2 BACKGROUND AND RATIONALE

Surprisingly little is known about the origin of AF, and it appears that various sources contribute to the 98% water and 2% solids making up this biological fluid of the fetal environment (1). Fetal urine, amniotic epithelium, cells of the fetal respiratory tract and, perhaps, the umbilical cord are thought to contribute at different times during gestation (2). The fetal cells suspended in the AF probably represent exfoliation from the skin, umbilical cord, urinary tract, oropharyngeal mucosa and amnion (3). Erythrocytes which are seen following the majority of amniocentesis procedures are almost certainly maternal in origin. The soluble and cellular constituents of AF provide the opportunity to study a sample of the genome of the unborn fetus, especially genetic systems of antigens and enzymes. Detection of certain of the latter in cultured AF cells, of course, forms the basis for prenatal diagnosis of some of the hereditary metabolic errors (4). It is possible to study some polymorphic genetic systems in the fetus from AF analysis (5,6,7), although a systematic search for expression of human polymorphisms in AF has not been undertaken. The emphasis on prenatal diagnosis of hereditary disorders has, understandably, focussed on detection in AF of enzymes whose deficiencies cause disease, and these genetic markers are usually idiomorphs (8). The variation among individuals implied by polymorphic genetic systems adds a dimension to the research design which allows certain areas of genetic significance to be explored. Comparison of expressions in the fetus of differing alleles at the same locus can be made. The investigator can pursue the effects of fetomaternal incompatibility on expressions of various alleles.

It is clear that a significant scientific advance has been encountered in the successful exploitation of amniotic fluid for the prenatal diagnosis of genetic disorders. The ability to isolate cells of fetal origin from AF and successfully culture them is the basis for monitoring "high risk" pregnancies to detect fetuses with various chromosomal aberrations and some

inborn errors of metabolism (4). The technique of prenatal diagnosis removes the uncomfortable uncertainty from genetic counseling and, coupled with selective abortion of affected fetuses, joins the armamentarium of preventive medicine. As with most desirable techniques, there are limitations. Considerable time may elapse between amniocentesis and diagnosis in order to obtain sufficient numbers of cultured AF cells required for a particular test, usually biochemical in nature. Sufficient cells must be available for testing by (approximately) 20 weeks of gestation, i.e. the deadline for performing a therapeutic abortion. With reference to this limitation, the development of methodology which accurately tests the AF, or AF cells, at once is desirable. Another and more fundamental limitation presently confronting prenatal diagnostic activities arises from the inability to detect phenotypes at the cellular level. For some diseases, although the biochemical phenotype is known, absence of the normal protein from AF cells prevents their detection in the fetus; sickle cell anemia, hemophilia, phenylketonuria and ornithine transcarbamylase are some of these prenatal diagnostic "orphans". For other hereditary diseases, sufficient information on the underlying biochemical defect is not available, and numerous autosomal recessive, autosomal dominant and X-linked pathologic characters can be cited for this category. Cystic fibrosis (although this disorder may soon be liberated from this category), X-linked ichthyosis, neurofibromatosis and Huntington's Chorea are just a few. While we can optimistically look forward to increasing progress in the elucidation of basic mechanisms of these hereditary disorders, a waiting period before application to prenatal diagnosis of these conditions is not necessarily implied. In other words, even though a biochemical or other suitable cellular phenotype may not be available, it may still be possible to diagnose with considerable accuracy some of these conditions in the fetus. Genetic linkage may offer this possibility.

Human linkage (syntenic) groups are relevant to prenatal diagnosis of inherited disorders because the detection of the phenotype of a genetic marker which is closely linked to the locus of an allele determining disease provides the possibility of genotyping the fetus. This is already possible for myotonic dystrophy, a disease expressed in heterozygotes for an allele at a locus which is linked to the ABH secretor locus, the recombination fraction being 0.04 (9). ABH substances are secreted early in gestation by the fetus into amniotic fluid, forming the basis of determining its secretor status (5). Thus, if the coupling phase is known for an individual who is heterozygous at both loci and married to a non-secretor, detection of the secretor status of the fetus will predict presence or absence of the allele determining myotonic dystrophy. The magnitude of error in this prediction, 8%, is slightly greater than the recombination fraction (9).

The genetic map of man is growing. More and more linked loci are being found, autosomal linkage groups containing more than two loci are recognized and loci are being assigned to visible

autosomal chromosomes (10). The majority of the newer linkage groups involve polymorphic biochemical or serological markers rather than inherited disease markers. This is understandable because ascertainment of infrequent diseases in families is by-passed and accumulation of data is facilitated. Furthermore, linkage analysis by in vitro, Sendai virus mediated, interspecific somatic cell hybridization (11) does not require polymorphic gene markers so long as differences between interspecific allozymes or other homologous markers can be detected. Still, for prenatal diagnosis, synteny involving a locus with a disease determining allele is essential.

At present, a small number of autosomal syntenic groups involving clinically significant loci are known. The loci of five autosomal dominant disorders have been shown by family studies to be linked to polymorphic loci (reviewed in reference 10):

MAIN LOCUS	TEST LOCUS	DISTANCE CENTIMORGANS	95% PROBABILITY LIMITS OF MAP DISTANCE CENTIMORGANS	COMMENTS
Congenital total nuclear cataract	Duffy	0	0-17	Probably syntenic with the two loci for pancreatic amylase. Assigned to chromosome #1.
Elliptocytosis	Rh	3	2-7	Syntenic with loci for 6-phosphogluco- natedehydrogenase & phosphoglucomutase (first locus), and peptidase C. If these loci are correctly assigned to chromosome #1, they are syntenic with loci for the Duffy system, con- genital total nuclear cataract and pancreatic amylase. Linkage studies with Rh have detected genetic hetero- geneity for ellipto- cytosis.
Nail-Patella	ABO	13	8-21	
	Adenylate Kinase	0	0-6	
Sclerotylosis	MNS	4	0.3-19	
Myotonic Dystrophy	Secretor	4		

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The biochemical basis of almost all autosomal dominant disorders, including those listed above, is unknown, denying us a rational foundation for prenatal diagnostic testing. Genetic linkage affords a potentially useful strategy for antenatal detection of autosomal dominant disorders. This also applies at present to some clinically significant loci on the human X chromosome:

MAIN LOCUS	TEST LOCUS	DISTANCE CENTIMORGANS	95% PROBABILITY LIMIT OF MAP DISTANCE CENTIMORGANS	COMMENTS
Hemophilia A (Factor VIII deficiency)	Glucose-6- phosphate dehydrogenase	4	0-16	Polymorphism at the test locus in black, Mediterranean & some Asiatic populations
Ichthyosis	Xg	18	11-31(*)	
Ocular Albinism	Xg (references 12,13 and 14, respectively) (*) - 90% probability limits	18	7-34(*)	

With respect to the above autosomal syntenic groups it should be pointed out that the ABO types of secretor + fetuses and secretor types have been detected from AF (5), and that 6-phosphogluconate dehydrogenase (6-PGD), of the Rh syntenic group, has been detected in cultured AF cells (7). The electrophoretic phenotype of glucose-6-phosphate dehydrogenase (G-6-PD) has also been detected in cultivated AF cells (7). Thus, the potential exists for diagnosing the nail patella syndrome, myotonic dystrophy, hemophilia A and perhaps elliptocytosis in fetuses at risk by virtue of syntenic relationships.

It is clear that, at present, there are limitations in the use of syntenic groups for prenatal diagnosis of inherited disorders. The method will be useful only for informative families, i.e. those with a doubly heterozygous (at test and main loci) parent for autosomal dominant traits, a doubly heterozygous mother for X-linked recessive characters and both parents doubly heterozygous for autosomal recessive disorders. Meiotic crossing over between the marker (test) and main loci will always provide the possibility of error in predicting the fetal genotype, and the magnitude of this error is provided by the recombination frequency. The smaller the distance between these loci, the lower this error will be. Still this error will be, in general, much less than the 50% "error" that now attends genetic counseling based on detecting a male fetus at risk for an X-linked disorder.

Another limitation of utilizing linkage information for prenatal diagnosis is the small number of syntenic groups involving clinically significant loci. At present, there is good reason to be optimistic about mapping the human chromosomes. Ruddle has indicated that each of the chromosomes will have a known linkage assignment within "the next several years" (15). Family studies and interspecific somatic cell hybridization are providing data for new syntenic groups, assignment to recognized groups and to visible chromosomes, and these in vivo and in vitro techniques for linkage analysis are also confirming each other's findings (e.g. reference 16). We should inject a slightly less optimistic comment concerning the application of genetic linkage groups detected by somatic cell hybridization to prenatal diagnosis of inherited disorders. The genetic markers used in these studies with interspecific hybrids are usually enzymes which are not necessarily polymorphic characters, as classified by electrophoresis. This may be especially true for autosomal recessive genes determining various metabolic errors. For prenatal detection of autosomal recessive disorders, both carrier parents must also be heterozygous, preferably for codominant alleles, at the linked test locus, an unlikely expectation if the test locus is not polymorphic. Thus, this restricts the number of marker loci and the frequency of couples at risk which are informative for application of linkage for the prenatal diagnostic test. The situation is only slightly improved for X-linked loci because of the paucity of X-linked polymorphic systems (Xg, Xm and in some populations, glucose-6-phosphate dehydrogenase; color blindness is a recessive trait).

Another problem with linkage data collected from analysis of somatic cell hybrids resides in the inability to estimate the frequency of recombination between test and main loci. For purposes of prenatal detection, the recombination frequency is an estimate of the error in the prediction of the fetal genotype and phenotype. The limitation in observing polymorphism at a number of the (enzyme) loci which will be linked by this technique suggests that for some linkages, confirming family studies will not be forthcoming because informative families will be rarely encountered.

Despite these reservations, the more linkages determined between loci and between loci and identifiable chromosomes, the more likely this knowledge can be applied to prenatal diagnosis of various inherited disorders. Certainly we can expect assignment of polymorphic loci to linkage groups and visible chromosomes, and detection of polymorphism in a number of presently monomorphic or idiomorphic systems is more than a reasonable prediction. The amount of genetic variability in terms of polymorphism, has surpassed estimates of a mere decade ago, and, indeed, it is not unreasonable to consider the possibility that almost all loci are polymorphic in man (17). Thus, we can expect assignment of a number of clinically significant loci to syntenic groups.

A final limitation to the use of synteny for prenatal diagnosis stems from the relatively small amount of information available on the expression of polymorphic genetic markers in AF. Should additional, clinically relevant, syntenic groups be detected, we still must be able to test for the expression of the marker loci in AF in order to infer the fetal genotypes. In addition to providing information pertaining to the biology of the fetus and AF, a systematic study of polymorphisms in AF can provide the information necessary to use syntenic relationships for the prenatal diagnosis of inherited disorders.

B. SPECIFIC AIMS

1. We propose to seek in AF and cultured AF cells the expression of known polymorphisms. Among the polymorphisms to be studied will be erythrocyte antigenic systems, the HL-A system and systems of various intracellular enzymes.

2. We will delineate variation in expression of allelic markers in AF and attempt to understand the basis of the variation.

3. We will delineate expression of polymorphic markers in AF in terms of gestational age.

4. We will compare the expression of polymorphic markers in AF with their expression in other tissues (e.g. blood, cultured fibroblasts, etc.).

5. As we come to understand the variation encountered in the expression of polymorphic markers in AF and the limitations of the tests we use, we shall begin to employ or help others employ those markers involved in a syntenic relationship with clinically significant loci for prenatal detection of affected fetuses.

C. METHODS AND PROCEDURE

1. AF Samples

AF samples for these studies are being obtained from appropriate patients by staff physicians with full-time or clinical faculty appointments to the Department of Obstetrics and Gynecology. These physicians provide 10-15 AF samples each year for prenatal diagnosis of chromosomal disorders (primarily trisomy 21); these specimens are usually obtained at 12-16 gestational weeks. We expect the numbers of AF samples sent for prenatal diagnosis will gradually increase as more obstetricians take advantage of the service provided by the Cytogenetics Laboratory at Stanford. One problem in this source of AF samples which concerns us pertains to maternal age. Most of the samples obtained for prenatal diagnosis are taken from women over 35 years of age. The effect of maternal age on expression of polymorphic markers in AF is a variable about which there is no information.

The obstetricians have been cooperative in providing us with AF samples collected at the time of therapeutic abortion; these specimens are usually obtained at 18-20 weeks of gestation. About 2-3 therapeutic abortions are performed each week at Stanford University Hospital.

2. Cultivation of AF CELLS

We shall work with cultivated AF cells in order to assure ourselves that we are working with only fetal cells. After amniocentesis AF usually contains a mixture of maternal blood cells (probably resulting from the procedure) and fetal cells. Although it is possible to remove most of the maternal erythrocytes by lysis (18), other nucleated cells from maternal blood may remain. Cultivation results in dilution of maternal blood cells by the replicating fetal cells.

The use of polymorphic markers and of markers of fetal sex will enable us to decide on the origin of the cells growing in culture (fetal or maternal). Obviously cultured cells carrying a fluorescent interphase Y marker (19) or showing no sex chromatin (Barr) bodies are of fetal origin. For cells which do show evidence of the XX karyotype, we will compare the phenotypes of the polymorphic markers we employ with the phenotypes in the mother and father. In most instances these precautionary procedures will help us avoid mistaking maternal for fetal cells in AF.

We have been cultivating AF cells for the past year. Our culture technique is similar to that used by other investigators in the field. Usually 10 ml of AF (more, if obtained before instilling a hypertonic saline solution for therapeutic abortion) are centrifuged at 100 g for 5-10 minutes. The supernatant AF is removed and saved (see below) and the sedimented cells are suspended in 0.5-1.0 ml of fetal calf serum. We carefully place drops of the serum-cell suspension on the surface of a small plastic Petri dish or on a cover slip in a Petri dish and then incubate the cells at 37 degrees C in a 5% CO₂ and air environment. After 6-18 hours, during which time cells have attached to the surface of the dish (or coverslip), we add tissue culture medium (F10-Grand Island Biological Co.) with 30% fetal calf serum. This medium is changed every other day. With this procedure we can see cell growth within one week. There are sufficient cells in a dish or on a coverslip for the first subculture (0.05% trypsin solution) within 2-3 weeks. After the first subculture, we find that we can perform the second subculture within a week. At this time the cultivated AF cells are growing well.

The cells with which we are dealing at this time usually are epithelioid cells. It is our experience that these cultivated AF epithelioid cells will grow well for about 4-5 subculture passages after which their growth will cease. Within these limits we have been able to cultivate large quantities of these epithelioid cells in roller culture bottles. This will be important for the study of polymorphic enzyme markers in cultivated AF cells.

Less frequently we have noted fibroblasts growing in cultures. These cells grow for longer periods than the epithelioid cells. For instance, one AF fibroblast culture which we are propagating and using for various investigations (7B2B) is now in its 14th subculture passage. We know this line is of fetal origin because it does not show Barr bodies and possesses the XY karyotype; this is a diploid culture. It is our impression that more time is required for fibroblastic growth to be evident initially than for epithelioid cell replication. We will use either morphological type for our studies of cultured AF cells, although for purposes of prenatal diagnosis epithelioid cells may be more relevant.

Aliquots of replicating cells from each AF culture are frozen in 10% DMSO (10**6 cells per ml per vial) and stored in liquid nitrogen for future studies with live cells. Aliquots of 10**7 cells which have been washed, sedimented and drained of supernatant liquid are stored in liquid nitrogen for future electrophoretic and enzyme activity studies.

3. The Supernatant AF Fluid

After AF cells are separated from the supernatant AF by centrifugation, we again centrifuge the latter (2,900 r.p.m. for 10 minutes in an angle head International Clinical Centrifuge) to

remove any remaining cells or large pieces of cell debris. The supernatant AF is then transferred to another container and stored at -20 degrees C. This fraction of AF is used for ABH hemagglutination inhibition studies and will be used for studies on fetal Lewis substances (6).

4. Controls for Expression of Polymorphic Markers

Whenever possible blood samples will be obtained on both parents of the fetus under study. These samples will be typed for the various polymorphic markers being studied in AF. In addition umbilical cord blood specimens will be collected at birth of those fetuses whose AF was studied and who were not aborted. These specimens will also be typed for the various polymorphisms which were studied in the AF. In those instances in which fetuses are aborted, we shall attempt to use appropriate material from the abortuses to type for polymorphisms. The collections of the various specimens just listed are meant to provide us with material which will serve as controls for the observations we make on AF. Working with polymorphic systems permits us to predict the phenotype of the fetus, provided we know maternal and biological paternal phenotypes. A high frequency of discordance between observed (from AF) and expected phenotypes will signal us to examine our testing procedures, to consider that changes are occurring in cell culture or that interesting variations in interallelic expression are occurring. The direct controls of studies of expression of polymorphisms in AF will be the results of testing cord bloods or aborted material.

5. The ABO Secretor and Lewis Polymorphisms

Fetuses which are heterozygous or homozygous for the gene which determines secretion of ABH substances (Se) secrete these soluble blood group antigens which appear in AF (5,6). The secretor type of the fetus and the ABO type of secretor + fetuses can be detected by studying the AF in early gestation free of cells, for hemagglutination inhibition of appropriate detector systems. Inhibition by AF of A, B, or H hemagglutinins indicates that one (or two) of these blood group substances have been secreted by the fetus. In this case the fetus is secretor +, and his ABO type is determined directly by specific inhibition of the A, B or H hemagglutinin. If the AF does not inhibit agglutination the fetus is a non-secretor and the ABO type cannot be determined. About 25% of Caucasian fetuses will be non-secretors (20).

We are already determining in our laboratory secretor status and ABO types of ABH secretor + fetuses. AF is serially diluted for nine or ten doubling dilutions in three series of tubes. In one series of tubes the AF dilutions (including undiluted AF) are incubated at room temperatures for 1 hour with an equal volume of single donor, non-commercial, anti-A hemagglutinin. In the second series of tubes the AF dilutions are incubated with single donor, non-commercial, anti-B and in the third with anti-H (*Ulex europaeus*). A single dilution of each hemagglutinin is used

throughout the determination, this being determined from the anti-A or anti-B titer of the serum; we do not dilute the anti-H reagent which has a very low titer (commercial preparations or our own preparation). After the incubation of AF and hemagglutinins, we add A, B and H erythrocytes to the first, second and third series of tubes, respectively. Following thorough mixing of cells and reagents (and AF), the tubes are centrifuged and are then observed for gross hemagglutination. As a control for each AF determination, agglutination inhibition is carried out in a similar manner using salivas containing A, B and H substance.

As of the date of preparation of this application, we have standardized (for our laboratory) the conditions of the hemagglutination inhibition procedure (e.g. titer of hemagglutinins, volumes of reagents, incubation times, centrifugation times and speeds) and tested five amniotic fluids:

WEEK OF GESTATION	FETAL ABO	TYPE SECRETOR	RECIPROCAL HEMAGGLUTINATION INHIBITION TITER IN AF		
			A	B	H
19 weeks	B	+	0	64	0
17 1/2 weeks	?	-	0	0	0
16 weeks	A	+	8	0	0
14 weeks	A	+	16	0	0
16 weeks	O	+	0	0	4

We believe that we will be able to distinguish between A1 and A2 secretor fetuses on the basis of inhibition of anti-H; A2 individuals secrete more H substance than do A1 individuals (20). We are continuing to type AF specimens for ABH hemagglutination inhibition and to compare the inhibition titer with that of the saliva controls. We plan to check the ABO types of secretor + fetuses by typing cord blood specimens and ABH secretor status with saliva collected in the newborn period.

In a manner analogous to the methodology just described, secretion of Lewis a, Le(a), and Lewis b, Le(b), substances will be studied (6). Non-secretor fetuses do secrete Le(a) substance.

We are planning to decrease the amount of serological reagents and AF used in the ABH and Le typing procedures. We will attempt to adapt these hemagglutination inhibition tests to tissue typing plates (21) which require only 1 microliter each of AF dilution, antiserum and erythrocytes. These plates can be "centrifuged" on a serological rotator and hemagglutination observed microscopically. Direct reacting (complete) antibodies must be used in this microtechnique. Fortunately, ABH hemagglutinins and some antibodies to Le(a) and Le(b) do not require anti-human globulin (Coomb's reagent) to agglutinate erythrocytes.

6. Other Polymorphic Systems of Erythrocyte Antigens

Recently, the expression of the blood group P has been detected on cultured cells and interspecific cell hybrids by complement fixation (22). If this finding can be confirmed, a technique might be available for detecting the expression of various blood group polymorphisms on cultured AF cells. ABO, Rh, MNS, Duffy, P and Xg specificities will be sought on cultured AF cells by complement fixation. Other serological methods which we will explore for the detection of these substances on AF cells include absorption of specific antisera and mixed agglutination. Fuchs et al. (23) used the latter technique to detect ABO types of uncultured AF cells.

Although there is no indication whatsoever of secretion of blood group antigens other than ABH and Le substances, (except for Sd(a), see reference 20), we shall examine AF for hemagglutinin inhibition activity for the above mentioned erythrocyte antigens.

7. The HL-A System

We have been able to type for HL-A antigens cells in the fifth passage of the fibroblastic euploid culture (7B2B) derived from AF obtained at 19 1/2 weeks of gestation. Cultured AF cells grown to confluence in a plastic tissue culture flask (75 square centimeter growing area) were released from the surface of the flask with 3 ml of 0.05% trypsin solution. The cells were exposed to trypsin for approximately five minutes. They were washed and resuspended in medium F10 without fetal calf serum; the cell concentration of this suspension was 10^{*6} cells per ml. These cells were prepared and typed for HL-A antigens in Dr. Rose Payne's laboratory, Department of Medicine, Stanford, as follows: Aliquots of the cell suspension were incubated with fluorescein diacetate (FDA 2 micrograms in 0.1 ml tissue culture medium added to each 1 ml aliquot of cell suspension) for 30 minutes and the cells were separated from the FDA by centrifugation. Examination of the FDA-treated, cultured AF cells under the fluorescence microscope revealed masses of fluorescent cells. Aliquots of 1,000-2,000 of these cells were then added to approximately 60 HL-A antisera, representing 25 antigenic specificities, on tissue plates and incubated in the dark at room temperature for 30 minutes. Rabbit serum, absorbed with cultured human fibroblasts, was used as a source of complement; an excess of complement was added to each well of the tissue typing plate containing cells and antibodies. Following an incubation of 1-4 hours at room temperature in the dark, the cells were observed with a fluorescence microscope for evidence of cytotoxicity. Cells which remained fluorescent failed to react with antibodies and thus did not carry the HL-A antigen which the antibodies detect. Fewer or absent fluorescent cells indicated cytotoxicity mediated by the antigens recognized by the antibodies with which the cells had been mixed. Cytotoxicity was graded as 4+ when no fluorescent cells were seen, 3+ few fluorescent cells, 2+ more fluorescent cells and 1+ no decrease in fluorescent cells. This method has been used by Dr. Payne and her associates to detect HL-A antigens on cultured cells (24).

Typing of the 7B2B cells by this method revealed HL-A first series antigens, HL-A9 and W29, and the second series antigen HL-A7. We are especially confident of the detection of HL-A9; most of the anti HL-A9 reagents in Dr. Payne's sublist of antisera reacted with these cells. Although more than one of the antisera for W29 and for HL-A7 reacted with the 7B2B cells, there were some which did not. We are about to type this AF cell culture again, now at passage 14. We are especially interested in the gain or loss of HL-A specificities, especially involving W29 and HL-A7. The genetic structure of the HL-A system (2 closely linked loci with a low frequency of recombination between them, reference 25) serves as a control for our typing results. If we are correctly detecting the HL-A phenotype of the fetus, we should find no more than two first series and two second series antigens on the cultured AF cells. Unfortunately, this fetus was aborted (by hypertonic saline injection), so that we cannot compare the HL-A antigenic type of the cultured AF cells with that of (aborted) fetal tissue or of lymphocytes in cord blood.

We look forward to studying variation in expression of HL-A antigens in cultured AF cells. The genetic structure of the system will permit us to look for variation within sub-series as well as between series (i.e. do the antigens of one sub-series tend to be expressed more frequently in cultured AF cells?) Furthermore, we will study the evolution of expression of HL-A antigens on AF cells throughout the life of the cell culture as well as at different weeks of gestation. Finally, shifts in expression of cross-reacting antigens (26) will be sought.

8. Polymorphic Enzyme Markers of Cultured AF Cells

The demonstration of the electrophoretic phenotypes of G-6-PD and 6-PGD in cultured AF cells by Nadler (7) raises the possibility of detecting other polymorphic enzyme markers. It is not clear from Nadler's report that he considered polymorphism for G-6-PD as the source of the variation he observed in the cultured cells from different AF samples. Nor is it clear that he studied sufficient samples to note variation for 6-PGD in cultured AF cells which he reported as failing to show variation. Three to eight percent of Caucasian individuals show variant 6-PGD electrophoretic phenotypes (27). Harris and Hopkinson (28) have listed some 20 loci determining enzyme structure which have been found by electrophoretic studies to be polymorphic in Europeans, and G-6-PD can be added to this list for Black populations. Of these enzyme polymorphisms we can test extracts of cultured amniotic fluid cells for seven by starch gel electrophoresis: phosphoglucomutase locus 1 (PGM1), phosphoglucomutase locus 3 (PGM3), adenylate kinase (AK), adenosine deaminase (ADA), 6-PGD, hexokinase and G-6-PD. Furthermore, we believe we will be able to develop procedures for five additional systems: Peptidase A, C and D, glutamate-pyruvate transaminase (soluble - SGPT) and galactose-1-phosphate uridyl transferase (gal-1-P-tfase).

Cultured AF cells will be tested for total enzyme activity (per unit weight of cell protein) and for the electrophoretic phenotypes of the systems listed above (many of the procedures which we will use are provided in references in 28). Quantitative determinations and qualitative observations will be made throughout the lifetime of the same AF cell cultures and on cultivated cells from AF specimens taken at different times in early gestation. We will attempt to ascertain all common phenotypes of each polymorphism. Over a three year period we will probably have had the opportunity to encounter the expressions of common alleles for most if not all of these phenotypes.

We shall use standard cell cultures with reference phenotypes to control our electrophoretic procedures. These cultures will be obtained from cell culture repositories (e.g. The Mammalian Genetic Mutant Cell Repository, Institute for Medical Research) or from biopsies (after informed consent procedures) from individuals who have been typed for various enzyme electrophoretic phenotypes.

9. Applications to Prenatal Diagnosis

As this study progresses we will become familiar with the behavior and the limits of variation of expression of various polymorphic markers in AF. This information will serve as a base for using syntenic relationships for prenatal diagnosis. We envision a cooperative effort with a number of other centers involved in genetic counseling and prenatal diagnostic activities in finding families who are at risk for having affected fetuses and to which the methodology discussed herein can be applied for prenatal diagnosis of those fetuses. Such families will be typed for the test marker in order to determine that one or both (for autosomal recessive traits) parents are heterozygous and to work out the coupling phase. We will then seek the expression of the syntenic marker in the AF from those informative and suitable pregnancies. Certainly, families with hereditary disorders seen at Stanford will be screened for the possibility of applying the methodology presented in this application, but we believe we will need access to a larger number of families to apply this prenatal diagnostic technique.

D. SIGNIFICANCE

1. This project will enable us to infer information about well known polymorphic markers in the developing fetus. This includes the development of the expression of these markers in early antenatal life. The project gives us the opportunity to find fetal forms of these markers. By understanding differential expression of polymorphic alleles in fetal life, we may be able to make inferences about the nature of selective forces which may be acting on these polymorphisms.

2. This project could ultimately result in expanding the number of hereditary conditions amenable to prenatal diagnostic techniques. This is especially true for autosomal dominant

disorders for which little information on basic biochemical mechanisms is available to fashion appropriate techniques for prenatal detection of the fetal phenotype.

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SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM	THROUGH	
		1/1/74	12/31/74	
1. PERSONNEL <i>(List all personnel engaged on project)</i>			TIME OR EFFORT %/HRS.	AMOUNT REQUESTED <i>(Omit cents)</i> TOTAL
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION			
Cann, H.	Principal Investigator or Program Director	20%	POLYMORPHIC GENETIC MARKERS	
Tsuboi, K.	Senior Scientist	20%		
Van West, B.	Research Assistant	100%		
Makk, G.	Research Assistant	100%		
Open - Pediatrics	Research Assistant	50%		
Cusumano, M.	Laboratory Diener	50%		
Murray, R.	Secretary	25%		
TOTAL →			\$	52,330
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>				\$
3. EQUIPMENT <i>(Itemize)</i>				
Eppendorf Microfuge and accessories \$500				
Buchler Power Supply (constant voltage/current) \$600				\$ 1,100 *
4. SUPPLIES				
Chemicals, antisera, tissue culture supplies, glassware, etc.				\$ 5,000
5. STAFF TRAVEL <i>(See Instructions)</i>	a. DOMESTIC	1 East Coast Meeting		\$ 500
	b. FOREIGN			\$
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>				\$
7. ALTERATIONS AND RENOVATIONS				\$
8. OTHER EXPENSES <i>(Itemize per Instructions)</i>				
Office supplies, telephone, repro., postage, publication costs, etc. \$400				
Central computer usage and terminal rental \$1600				\$ 2,000
9. Subtotal - Items 1 thru 8			\$	60,930
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
	DEPENDENCY ALLOWANCE			\$
	TOTAL \$TIPEND EXPENSES →			\$
b. TUITION AND FEES				\$
c. TRAINEE TRAVEL <i>(Describe)</i>				\$
11. Subtotal - Trainee Expenses			\$	
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>				\$ 60,930

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION		1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
			2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS		52,330	56,035	59,897	64,022	68,424		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>								
EQUIPMENT		1,100*						
SUPPLIES		5,000	5,300	5,700	6,000	6,400		
TRAVEL	DOMESTIC	500	600	600	600	700		
	FOREIGN							
PATIENT COSTS								
ALTERATIONS AND RENOVATIONS								
OTHER EXPENSES		2,000	2,100	2,300	2,500	2,600		
TOTAL DIRECT COSTS		60,930	64,035	68,497	73,122	78,124		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →						\$ 344,708		

REMARKS: Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)

Budget explanation attached.

BUDGET EXPLANATION

Professor Cann's and Dr. Tsuboi's time are each budgetted at 20% in support of this project. The project also requires two full time and one part time research assistants. The part time research assistant will assist in typing cultured cells from amniotic fluid for HL-A antigens. One full time assistant (Mrs Van West) will assist in the development of serologic tests on amniotic fluid and amniotic fluid cells. The other research assistant (G. Makk) will assist in culturing amniotic fluid cells and in performing electrophoresis on cultured cell extracts. The laboratory diener will provide the needed support for cell culture work (washing, sterilization of equipment, etc) and also for general laboratory work. We are also requesting 25% of secretarial time to support the activities of both professionals in this project.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The microfuge will be used to centrifuge small amounts of cultured cells. The power pack will be used for electrophoresis. Necessary supplies are budgetted to support laboratory work including chemicals, glassware, antisera, etc.

We are requesting \$100 per month for computer time and data file storage and \$400 per year for our share of the rental of a computer terminal. Access to computation is necessary for cataloging of cultured cells from AF specimens, automatic filing and storage of test results on various cell cultures and amniotic fluid samples, filing and storage of data on phenotypes of parents, relating parental data to fetal data, monitoring lists of expected dates of delivery of fetuses we have studied and data analysis.

SECTION V

A Search for Genetic Polymorphisms and Variances
Of Specific Binding Proteins in Blood

Dr. Cavalli-Sforza

Genetic Polymorphisms and Variants
of Specific Binding Proteins in Blood

Dr. L. L. Cavalli-Sforza, Principal Investigator

A. INTRODUCTION

A.1 Objective

To search for new medically significant genetic variants and genetic polymorphisms of specific binding proteins in blood.

A.2 Background and Rationale

The recognition of genetic differences by electrophoretic studies of proteins is by now a widely applied procedure. Methods employed for detecting proteins after electrophoresis range from unspecific protein staining to the identification of specific proteins by a great variety of techniques. Harris and Hopkinson (1972) have recently summarized the evidence collected in electrophoretic studies of specific enzymes in Caucasians. Out of 7 enzymes analyzed, about 1/3 were found to be polymorphic. Certain other categories of enzymes may be subject to a lower rate of polymorphism (Omenn, Cohen and Motulsky, 1971).

One method of labeling a variety of proteins is already established but has not been tested systematically for its capacity to identify polymorphisms or rare variants. It consists of adding to serum (or other biological fluids) a radioactive ligand molecule and following its binding to a specific protein (or proteins) by electrophoresis followed by autoradiography. This method was used (Giblett, Hickman and Smithies, 1959) to amplify the classical notion that transferrin binds iron and that electrophoretic variants of this molecule retain the capacity to bind iron. The physiological function of this protein is fairly well known and its important role in the organism well ascertained (for details see Giblett, 1969). Polymorphism is well documented and many rare variants are also known. Other proteins known to specifically bind metals (e.g. copper: ceruloplasmin, see Giblett for details) and other substances (e.g. thyroxine; see Heinonen et al., Fialkow et al., and Penfold et al.) have also been studied.

The suggestion advanced in the present application is to test systematically samples of human blood for proteins binding specific substances, taking advantage of the fact that many physiologically important ligands are available in a radioactive form. Two aims could be thus obtained: 1) increase the present wealth of polymorphisms, a very desirable aim (see Cavalli-Sforza, 1973); 2) a functional basis for each specific polymorphism or rare variant could be sought, given that the nature of the substance would usually suggest possible advantages or handicaps of the variants.

B. SPECIFIC AIMS

(1) Search for polymorphisms in proteins binding specific substances available in radioactive form. This may enable us to look for a large number of new polymorphisms, allowing us to considerably increase the genetic map and to find new linkages.

(2) In all cases in which polymorphisms are detected, test for developmental behavior of the proteins in question, and for the chemical specificity of the ligand.

(3) In cases of toxicity or idiosyncratic drug reaction, test for variation or absence of proteins binding the specific poison or drug.

C. METHODS AND PROCEDURE

The experimental methodology has already been developed on the basis of experience accumulated in another research line. In the analysis of platelet proteins binding certain neurotransmitters (in particular, serotonin and norepinephrine) a method of electrophoresis followed by autoradiography was set up. In order to test the method for its capacity to reveal serum proteins binding specific substances, it was applied to lead-binding proteins. A short description of the method follows: Pb(210) at an appropriate concentration is incubated at 37 degrees C. for 30 minutes with human serum. 0.1 ml of the incubated serum is electrophoresed on acrylamide gel. Bromophenol blue is used as a tracking dye and electrophoresis stopped when the dye is at the bottom of the column. (The procedure can be used both on columns and on slab gels). At the end of electrophoresis, the gel column is cut longitudinally and the flat surface applied on an X-ray Kodak film for 4-10 days in conditions guaranteeing close adhesion and maximum efficiency of arrival to the plate of the electrons emitted by the decaying radioactive atoms. A diagram of a developed autoradiogram is shown in Figure 1.

Four different individuals are shown. There is considerable binding of Pb to albumin which forms the large thick band. Albumin is known to have a high affinity for many substances. In addition, there are other lighter bands of Pb-binding proteins in the globulin region. Some of these correspond to thin protein bands visible by staining. Two proteins in the beta globulin region have been constantly found in all individuals tested so far. In the gel at the extreme left, two bands are visible near the origin (the top of the figure) while there is only one band at the same location in the other three individuals. The double band may be due to heterozygosis but so far only one individual with a double band has been found on the limited number of individuals tested. It is planned to continue the testing on at least a hundred normal individuals, to establish if a polymorphism really exists, to test the families of individuals showing electrophoretic differences, and to test all cases of plumbism which may come to our attention by the cooperation of

the hospital staff. Some of these individuals may be idiosyncratically sensitive to lead; or, alternatively, may show physiological responses to lead intoxication. Other disease states suspected of affecting plasma protein distributions will also be examined.

The extension of this work to substances other than lead is the subject of the present grant application. The substances to be tested for binding to serum proteins can be many, the main limitation being that the organic substances must be labelled with C(14) or otherwise produce beta radiation of similar energy. Pb emits also gamma-radiation, so that exposed plates must be shielded; this, however, is no serious limitation since contact of the experimenter with the radioactive material is very short and his or her exposure controlled by the supervision ordinarily carried out in the laboratory (film badges, routine checks of benches and equipment). Results with H(3) labelled material have so far been unsatisfactory due to the short range of the weak electrons emitted in H(3) decay. C(14) labelled material is entirely satisfactory, as shown by the previous experience with platelets.

A shortened list of the substances to be tested includes the following:

- (1) Elements which have radioactive isotopes suitable for the test.
- (2) Amino acids, peptides
- (3) Nucleotides, nucleosides
- (4) Sugars
- (5) Lipids
- (6) Hormones
- (7) Vitamins
- (8) Drugs, including antibiotics, addiction drugs, pesticides, poisons, etc.

The analysis should be carried out initially on a sample of at least one hundred random individuals. Over 100 substances can be tested, but each test requires only 0.1 ml of serum and thus 20-30 ml of blood obtained from an individual will be enough for all tests. On the assumption that one or more proteins binding specifically a substance are found for most of these substances, this experiment should give a good sample of prospective polymorphisms from which the frequency of polymorphism and average heterozygosity for each can be computed and compared with that observed for enzymes (see Introduction).

Substances found to have polymorphic binding proteins can then be subject to the following series of observations:

1) Tests on families scored for other markers which have already been collected in other laboratories. Prospective collaborations are being considered. It is expected (but should be first tested) that in serum stored in freezers the specific binding activity is stable. The existence of a number of projects in which blood samples have been collected from families, examined and stored makes it easier and more efficient to test on such material inheritance of the protein differences (i.e. segregation analysis) and linkage of the corresponding genes to standard markers. Several such collections of samples are already available.

2) We plan to examine newborn infants born at Stanford Hospital of matings in which the mother is homozygous for a polymorphic protein of the type described, and the father heterozygous (or homozygous for another allele). The paternal protein would be searched in cord blood and if not present, the child would be followed further to establish the age of appearance of the paternal protein. This would give us a chance to seek regulatory genes for the developmental pattern of these proteins. For instance, we will seek variation among individuals of age of appearance of the protein and analyze the variation with family studies.

3) For every specific substance, patients with diseases that may be explained by a variation or absence of a binding protein, the specific substance should be examined.

D. SIGNIFICANCE

It is difficult to anticipate the total number of proteins that can be identified by this procedure, but existing information would suggest that it can be as high as several hundred. The method suggested then supplies a very economical procedure for testing a great number of potential polymorphisms. The frequency of polymorphic genes is one of the quantities which is of interest to estimate for comparison with the existing enzyme data. This result has obvious evolutionary significance in view of the present discussion on neutrality of polymorphic genes. If the proportion is the same as is known to be among enzymes, then this investigation may generate enough markers to more than double the existing genetic map of man, with all consequent advantages of increased precision in genetic counseling and research.

The interest offered by such new polymorphisms would be greatly enhanced by the possibility of detecting variation for regulatory genes in the manner explained before. This is one of the most difficult fields in human general genetics today, the development of which may be most fruitful.

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The interest offered by such new polymorphisms would be greatly enhanced by the possibility of detecting variation for regulatory genes in the manner explained before. This is one of the most difficult fields in human general genetics today, the development of which may be most fruitful.

Finally, each and every one of the proteins thus detected and identified may offer unique possibilities of further research and therapeutic developments. Taking again the model of transferrin, there is one well known case of congenital absence of this protein which was lethal (Heilmeyer et al., 1961). In similar cases, substitutional therapy by transfusion or plasma infusions may prove life saving. Several dangerous rare drug idiosyncrasies are known to exist, e.g. to chloramphenicol. Should they prove to be connected to the lack of a specific binding protein, transfusion or plasma infusions may again prove useful or at least these patients could be identified before becoming the victims of the administration of a drug potentially lethal for them. Cases of vitamin or hormone resistance might find similarly an unexpected explanation and therapeutic benefit.

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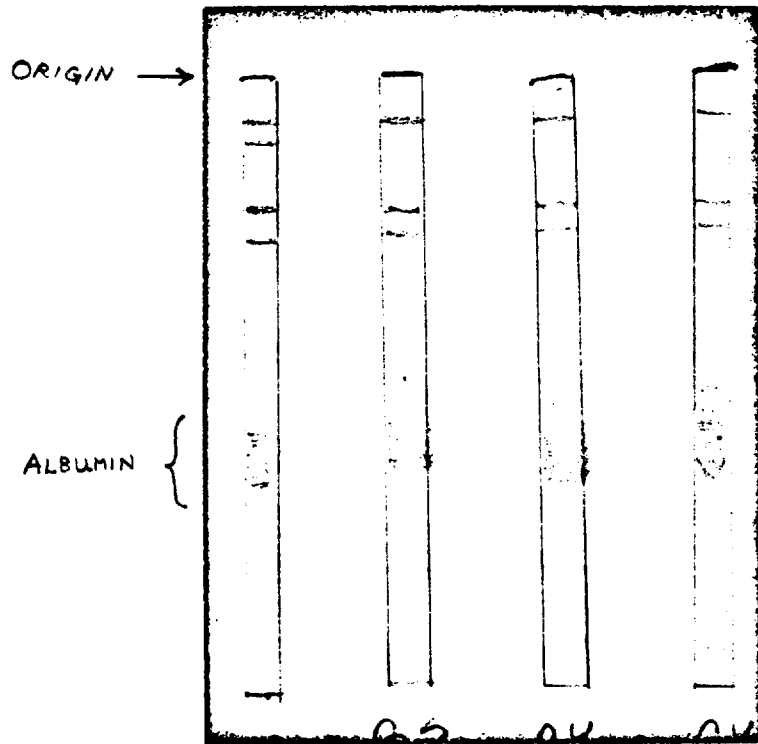


FIGURE 1

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM	THROUGH	
		1/1/74	12/31/74	
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT %/HRS.	AMOUNT REQUESTED <i>(Omit cents)</i>	
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION		TOTAL	
Cavalli, Sforza, L.	Principal Investigator or Program Director	10%	POLYMORPHISMS OF SPECIFIC BINDING PROTEINS	
Open - Genetics	Research Associate	100%		
Open - Genetics	Research Tech.	100%		
TOTAL →			\$ 30,489	
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$	
3. EQUIPMENT <i>(Itemize)</i>				
	Constant current power supply	\$1,000		
	Slab gel electrophoresis	300		
	Column acrylamide gel electrophor.	200		
	Destainer	200	\$ 1,700*	
4. SUPPLIES				
	Radioactive tracers	\$5,000		
	Chemicals, glassware, lab app.	1,000		
	Expendable lab supplies, (photographic supplies, etc.)	1,500	\$ 7,500	
5. STAFF TRAVEL	a. DOMESTIC	2 East coast meetings	\$ 1,000	
<i>(See Instructions)</i>	b. FOREIGN		\$	
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>				
	Venapuncture for blood samples		\$ 500	
7. ALTERATIONS AND RENOVATIONS			\$	
8. OTHER EXPENSES <i>(Itemize per Instructions)</i>				
	Office supplies, telephone, repro., postage, publications costs, etc.	1000		
	Central computer usage	1000	\$ 2,000	
9. Subtotal - Items 1 thru 8 →			\$ 43,189	
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
TOTAL STIPEND EXPENSES →			\$	
	b. TUITION AND FEES		\$	
	c. TRAINEE TRAVEL <i>(Describe)</i>		\$	
11. Subtotal - Trainee Expenses →			\$	
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>			\$ 43,189	

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE DIRECT COSTS ONLY (Omit Cents)							
DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED (<i>This application only</i>)					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	30,489	32,648	34,898	37,301	39,867		
CONSULTANT COSTS (<i>Include fees, travel, etc.</i>)							
EQUIPMENT	1,700*	500*	500*				
SUPPLIES	7,500	8,000	8,600	9,000	9,600		
TRAVEL	DOMESTIC	1,000	1,100	1,200	1,200	1,300	
	FOREIGN						
PATIENT COSTS	500	500	500	500	500		
ALTERATIONS AND RENOVATIONS							
OTHER EXPENSES	2,000	2,300	2,400	2,400	2,700		
TOTAL DIRECT COSTS	43,189	45,048	48,098	50,401	53,967		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (<i>Enter on Page 1, Item 4</i>) →					\$ 240,703		
<p>REMARKS: <i>Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)</i></p> <p>Budget explanation attached</p>							

BUDGET EXPLANATION

10% of Professor Cavalli-Sforza's time along with a full-time Research Associate and a full-time Research Technician are budgetted in support of this project. The Research Associate, a biochemical geneticist, will be responsible for the electrophoretic analysis of plasma proteins and will be assisted by the Research Technician.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The budget includes slab gel and column gel electrophoresis equipment, and associated power supply, etc., as well as supporting supplies. These supplies include radioactive tracers, chemicals and laboratory apparatus, glassware, and expendable supplies such as photographic plates, etc.

Travel funds are requested for attending two professional meetings on the east coast.

Patient costs covering venepuncture to obtain blood samples, are estimated at \$500 per year.

SECTION VI

The Impact of Genetic Counseling Practices on
Family Decisions and Behavior

Drs. Barnett, Cann, and Luzzatti

The Impact of Genetic Counseling Practices on Family Decisions and Behavior

Dr. C.R. Barnett, Principal Investigator
Drs. H. Cann and L. Luzzatti, Associate Investigators

A. INTRODUCTION

A.1 Objectives

The overall objective of this study is to provide systematic answers to some of the basic, unanswered questions in the practice of genetic counseling. (1) What is the impact of genetic counseling, that is, do families not receiving genetic counseling make decisions different from those who do? (2) What is the difference in counseling effectiveness between a physician trained in genetic counseling and a social worker trained in genetics? (3) What is the difference in effectiveness between a counselor who is directive in his counseling and one who maintains a neutral stance? (4) What is the relationship between the structure and content of a genetic counseling session and the pre-counseling training and attitude of the counselor? (5) What is the difference in effectiveness between a counselor who receives social and psychological information about the family before counseling and one who does not have such information? (6) What are the expectations of families seeking counseling and how do they use the information they obtain in making decisions?

A.2 Background

A recent review of the social aspects of human genetics (1) and an editorial on genetic counseling in the New England Journal of Medicine (2) have consisted largely of lists of questions regarding genetic counseling for which there are as yet no answers. While much has been learned over the years regarding the genetic basis for many diseases, their mode of inheritance and the probability of occurrence in a given population, little research attention has been paid to how this information is transmitted to patients and the use they make of it. Typical of the state of the art and the still prevailing emphasis on "genetic prognosis", rather than "genetic counseling", is a recent textbook on genetic counseling (3) which devotes only 3 of its 355 pages to the counselor-patient relationship.

The major issues in the field may be subsumed under three basic questions: 1) What is or should be the impact of counseling? 2) What should be the counselor's role? 3) Who should do the counseling?

The first question is most difficult to answer at this time since there are little data available on the impact of counseling. A few studies have looked at impact by measuring the number of children families have had post-counseling, or by learning of their post-counseling decision to practice or change

their methods of contraception. With regard to the findings from such studies, Hecht and Holmes (2) have noted: "What is the objective of genetic counseling? If it is to lessen the chance of subsequent affected sibs being born, the available data are discouraging." One of the major problems with studies which have reported somewhat favorable results (4) is that they have not utilized control groups. The only study that used a control group (made up of families with children affected with a non-genetic, chronic condition) reported that 50% of the control group decided to limit the size of their families, in the absence of any genetic counseling to do so (5). Studies to measure the effects of counseling have also used a number of other outcome criteria, such as knowledge of probability or risk and information about hereditary transmission of traits that was retained by the family. There are two major deficiencies in these studies. First, with one exception (6) none of the studies have made an assessment of knowledge before the counseling took place. Indeed, in some cases, the follow-up of knowledge retained by the families was as long as 4 to 10 years after counseling (4,7), with no control or assessment of the effect of other sources of information on the families.

A second deficiency with the studies which have tested post-counseling risk and genetic knowledge of families, is that there is no indication, from the point of view of families, that biological knowledge and information regarding risk is used by them in making decisions about reproduction. The actual decision-making process in the family has remained an unopened "black box".

The second question regarding the counselor's role involves sharp differences of opinion on two issues: the question of whether the counselor should be neutral or directive; and whether the counseling should be narrowly focused or broadly comprehensive. The traditional neutral stance is most often associated with the focused role prescribed for the counselor: "It can be argued that a counselor's job is simply to estimate....risk as well as possible and try to ensure that this is understood. This is, of course, true. It is entirely a matter for parents to decide whether to avoid having further children, or to seek sterilization or termination of pregnancy" (3). When a genetic counselor feels called upon to violate this principle of neutrality, he makes a point of explaining the deviation, as does Carter (4), in order to reassure low risk parents. There are two untested assumptions in the argument presented by experts on both sides of this controversy. The first assumption is that a neutral counselor will not communicate unconsciously by his tone of voice, mode of presentation or non-verbal cues, his true feelings about what decision a family should make. Secondly, it is assumed that presentation of his feelings of "what he would do if he were in their shoes" will have a marked effect on the family's decision. These assumptions can and should be tested, since findings may suggest that the argument for either position is irrelevant to the outcome of counseling.

The issue of whether genetic counseling should be narrowly focused or broadly comprehensive has been linked to the question of who should do the counseling. Thus those who have taken the position that genetic counseling should be considered part of family guidance, have argued that the family physician can best play this role (8). Those who argue that the primary purpose of genetic counseling is to answer questions the patient may have about risk, feel that counseling should be left to the clinical geneticist. Franz Kallman (9), who favors a comprehensive approach has phrased the question most realistically by suggesting the type of training needed by the counselor: "There can be no question....that the constructive management of genetic family problems requires either geneticists who are experienced in counseling techniques or family guidance workers who have adequate training in genetics".

The issue of whether to take a narrow or a broad approach to counseling could be settled if information were available regarding what problems they may acquire as a result of the counseling. At present, genetic counseling has been not subject to the same types of analysis that have been brought to bear on the physician-patient relationship in other situations (10). Thus, what happens during genetic counseling has been described only in anecdotal form (1).

The question of whether the geneticist, the family counselor, the family physician or any other type of professional or lay counselor can best meet the needs of the family seeking genetic counseling can be determined by systematic evaluation of what these people actually do in counseling and what impact they have on the families. When new roles have been established in other areas of care delivery, the behavior and effectiveness of people taking the new roles have been evaluated (11). There is no reason why the same approach cannot be taken with regard to this issue in genetic counseling.

A.3 Rationale

Genetic counseling involves at least two parties, the counselor and the family, and both parties to the event must be studied, as well as the event (counseling) itself to determine the effectiveness of genetic counseling under varying conditions.

The counselor will bring to counseling his expectations about the nature of the counseling situation, and a predetermined view of the risk and burden a defect may represent for the family. He may decide, beforehand, to communicate an optimistic, pessimistic or neutral point of view to the family. His "public" position may vary from his "privately" held view. He may, if he is supplied with additional information about the family, (their state of knowledge, their values regarding having children, differences between husband and wife on basic issues, other decisions they are in the process of making, and their expectations regarding counseling), tailor the information and counseling he provides to the specific needs of the family.

The family, as noted above, may have expectations regarding counseling that are at variance with those of the counselor. They also come to counseling with a state of knowledge about the risk of having a child with a defect, the burden it represents, the genetic and biological principles underlying the defect and the basis for computing risk. Further, in their own family decision-making experience they may make great or little use of probabilities in coming to decisions. The family also comes with a set of values or attitudes regarding what they want as individuals and as members of a family unit, and these values also determine the kind of information they seek and how they use the information. Families will vary even with regard to the number of sources of information they use, so that for some, the genetic counselor may be the principal source, while for others, the genetic counselor may be one among many.

The counseling session represents an interaction between these two parties and no matter what the prior expectations on either side, the event may differ from what the two parties believe will happen, and after the event, what they think happened. Thus, the event itself must be studied and compared both with prior expectations and with post-counseling recollections. Did the counselor consciously or unconsciously break his stance of neutrality, and was it noted by the family? Was the family so immersed in absorbing the information about burden and prognosis that they recollected little about the risk information given by the counselor?

The expectations of both parties in genetic counseling provides two measures of effectiveness of counseling, rather than the single measure (goals of the counselor) which has been used up to now. Information obtained prior to counseling about the values, knowledge, and decision states of the family, as well as their expectations may enable the counselor to satisfy both his and the family's expectations.

An ideal design for answering the basic questions regarding genetic counseling should satisfy the canons of experimental design even though the issues are basically behavioral and social. The study would be prospective in that it measures the status of the parties before the counseling takes place and then measures changes following counseling, against the pre-counseling base (6). It should randomly assign counselees to varying types of counselors (such as a physician or a social worker), and to counselors who have different types of information available to them about the family before counseling. Finally, control groups should be utilized to control for both the effects of the research contacts on the family, as well as a control group which does not receive genetic counseling, but may also make decisions about having children.

B. SPECIFIC AIMS

1. To test the hypothesis that genetic counseling can be done at least as effectively by a social worker with some

training in clinical genetics as by an M.D. trained in clinical genetics.

2. To test the hypothesis that genetic counselors, even when holding consciously to the principle of "neutrality," will divulge their "true" feelings to their counselees.

3. To test the hypothesis that counselors who are informed prior to counseling regarding the values, knowledge, decision status and counseling expectations of the counselees will be more effective than counselors who are not so informed.

4. To develop measures for determining the effectiveness of genetic counseling which utilize the goals of the counselees, as well as the objectives of the counselors.

5. To learn how families utilize information provided in genetic counseling (such as risk and burden) in reaching decisions about child bearing.

C. STUDY DESIGN

Four experimental groups and 3 control groups will be established in order to test the significance of the major variables in the study. All four of the experimental groups will be subject to the following procedures.

1. 48-72 hours, pre-counseling. Family receives pre-counseling interview by 2 members of research team and fills out inventory instruments to assess their values relating to child-bearing, family relationships and life expectations; their knowledge of probabilities, genetics and the disease or condition about which they are seeking counseling; the family decisions they have recently made or are in the process of making; and their expectations regarding the counseling they are to receive.

2. 24-48 hours pre-counseling. Genetic counselor writes a summary of his understanding of the case; his expectations regarding the session; the position he expects to take with the family (neutral, optimistic, pessimistic), and his personal feelings about the decision the family ought to make.

3. Family receives genetic counseling. The entire interview is audio-taped for analysis of the structure and content of the interaction.

4. 24-48 hours post-counseling. Summary and evaluation of the counseling session by the genetic counselor including his prediction about the decision the family will make and differences between his expectations recorded at point #2 and what actually occurred during the counseling at point #3.

5. 48-72 hours post-counseling. Interview and administration of instruments to the family, similar to point #1. Probes on: their view of the counseling session; what they learned; were

expectations met; what position did they feel the counselor took.

6. 1 month post-counseling interview with family. Information obtained as at #1; probes on other information obtained by family sources of information, new experiences which have led to value changes and decisions.

7. 6 month post-counseling interview with family. Information obtained as in #6.

8. 1 year post-counseling interview with family. Information obtained as in #6.

The 4 experimental groups will vary according to whether they receive counseling by an M.D. trained in medical genetics or by a social worker trained in genetic counseling. They will also vary according to whether the counselor receives or does not receive information about the family obtained from the pre-counseling contact (point #1, above). The families in all 4 of the experimental groups defined below will be subject to the procedures outlined above (#1-8).

Families seeking or referred for genetic counseling will be assigned randomly to one of the following treatment groups:

- Group E-1. Receives counseling from M.D. trained in medical genetics. Counselor receives no information obtained from pre-counseling interview.
- Group E-2. Receives counseling from M.D. trained in medical genetics. Counselor receives information about the family obtained in pre-counseling research interview.
- Group E-3. Receives counseling from social worker trained in genetic counseling. Counselor receives no information obtained from pre-counseling research interview.
- Group E-4. Receives counseling from social worker trained in genetic counseling. Counselor receives information about the family obtained in pre-counseling research interview.

It has been our experience with other longitudinal studies (12) that multiple interviews with families in order to obtain research data actually provide considerable psychological and social support for the family. In the case of the proposed study, it could even influence the decision made by the family by helping them to focus on the problems they face and to make more explicit the alternatives they may have. In order to control for the effects of the interviews and instruments on the decisions that may be made by the families, the following 2 control groups will be established by random assignment of families:

Group C-1. Family does not receive pre-counseling research interview (#1 above). Receives counseling from M.D. trained in medical genetics (as does Group E-1). Counselor completes pre- and post-counseling summary (points #2 and #4). Family does not receive post-counseling follow-up (points #5, #6, and #7) until 1 year post-counseling (point #8).

Group C-2. Family does not receive pre-counseling research interview (#1 above). Receives counseling from social worker trained in genetic counseling (as does Group E-3). Counselor completes pre- and post-counseling summary (points #2 and #4). Family does not receive post-counseling follow-up (points #5, #6, and #7) until 1 year post-counseling (point #8).

A third control group (C-3) will consist of parents who have a child with a chronic, non-genetic condition and who have not received genetic counseling. This group will provide an overall control on the effect of genetic counseling on family decisions, particularly with regard to knowledge and limitation of family size. Like control groups C-1 and C-2 they will be interviewed one year after receiving information from a physician (in this case, information about the diagnosis and prognosis for their child).

ENTRANCE CRITERIA FOR THE STUDY

For families in the 4 experimental groups and families in control groups 1 and 2:

1. Family must seek or be referred for and receive genetic counseling at Stanford University Medical Center.
2. Family must be intact, i.e. there must be a couple in an already-established marriage or common-law relationship.
3. Family must be willing to participate in the number of sessions involved for data collection. Counseling costs and transportation for research interviews will be borne by the project to encourage participation.

Families in control group 3 will meet the same criteria, except that they will have a child with a non-genetic, chronic condition diagnosed at Stanford University Medical Center or the Children's Hospital at Stanford.

The purpose of the entrance criteria is to control for some of the background variables which must be considered in data analysis. Patients receiving genetic counseling outside of the medical center must be presumed to be a population with somewhat different characteristics than the population seen at the medical center, and the counseling they receive must also be assumed to be somewhat different. A population outside of the medical center

could be studied only by increasing the size of the study population by 100%. Use of a medical center population, combined with the requirement that families be intact, will serve to provide a population with some homogeneity with regard to income, education, occupation and family situation (13). This requirement, for example, rules out from the study couples seeking genetic counseling before marriage, unmarried teen-age mothers, etc. While the impact of counseling on these groups is deserving of study, given the number of variables in the study, control of some of the population characteristics is necessary. These criteria will also allow for random assignment of families to treatment and control groups thus obviating the difficulties and possible bias of selective matching.

INSTRUMENTS AND SCHEDULING

The first year of the study will be devoted to the development and validation of the instruments to be utilized, the training of personnel to do the coding of the transcripts of the counseling and interview sessions, and a pilot test of the study design. Approximately 50 families will be utilized during the first year. During the second and third year of study, approximately 125 families will be taken in and followed each year. The 4th year will be devoted to continued one year post-counseling follow-up of the families and data analysis. The 5th year will be exclusively data analysis and write-up of the study.

Among the instruments to be developed are those to assess the attitudes, decision state, knowledge and expectations of families relevant to genetic counseling. These are the instruments to be utilized at point #1 in the study design and at future follow-up points. These instruments will be pre-tested with a variety of patients to determine their ability to distinguish significant differences among families, their ease of administration and numerical scoring. Face validity will be determined through use of standard pre-test procedures (14). Particular attention will be given to the development of instruments which will determine the ability of the families to apply probability figures to every-day life situations.

During the development period, genetic counseling sessions will be tape recorded and a scoring system developed for analysis of the sessions. Coders, who will have no knowledge of the pre-counseling data obtained from the families or the counselors, will apply the scoring system. Using an adaptation of the interaction methods developed by Bales (15), both the structure and the content of the sessions will be analyzed. These data will be tested against the pre-counseling data obtained from both the counselors and families and against the recall, post-counseling, of counselors and families.

Pre-coded and pre- and post-counseling forms to be used by the counselors will be developed. Counselors will record their understanding of the case, the stance they propose to take and

their own personal feelings about the decision the family should make. The standardized post-counseling report will include their evaluation of the session, any changes from the pre-counseling stance and their estimate of the decision the family might make as a result of the counseling. The expectations of the counselor regarding the session will be compared with the pre-counseling expectations elicited independently from the couple. Similarly, the post-counseling summary from the counselor will be compared with the post-counseling view of the session obtained from the counselees.

Post-counseling interviews will also be conducted with the families (points #5-8 in the study design). Some of the same pre-counseling instruments will be used along with a standard interview format combining general and specific probe questions similar in form to the type developed by the study director for a study of family response to the birth of premature infants (12, 13). Included in the post-counseling interviews will be questions to elicit family reactions to the counseling, their assessment of the point of view taken by the counselor, decisions they may have reached and the reasons for making the decisions they have arrived at. On the basis of our previous family studies, the husband and wife will be interviewed separately to prevent contamination of the decision-making process by forcing consensus or facilitating husband-wife communication. Since the pre-counseling assessment will also be obtained independently, one form of data analysis will be to see to what extent the values and information of the husband and wife coincide after counseling.

The timing of data collection for the post-counseling period, beyond the first post-counseling interviews, is not rigidly established. One purpose of the first year of developmental work is to determine the best timing that will take us closest to the point at which families do make decisions.

LIMITATIONS

There may be some loss of subjects to follow-up, but this will be minimized by paying transportation and counseling costs. The number of families who refuse to participate in the study will be kept to a minimum through the same devices, but background data will be obtained in any case to see whether refusing families differ in important respects from the study population.

The findings of the study will not apply, of course, to couples who seek pre-marital counseling, to individuals who do not constitute a family unit, and to those who do not seek or are referred for counseling. Further, it is anticipated that because of the nature of the entrance criteria, the population will have fairly homogeneous middle class characteristics (as defined by income, occupation and education).

A number of genetic counseling studies have attempted to determine the relationship between the decisions families make about reproduction and the risk and burden they face. As noted previously, the meaning of risk from the family's point of view has not been determined. Further, there appear to be significant differences among counselors regarding the nature of the burden for the same disease. Therefore, we have not chosen to classify families on the basis of risk and burden before assigning them to the experimental or control groups. Risk and burden will be analytic variables in the study and random assignment of families should provide an appropriate mix of these variables in each group.

SIGNIFICANCE

The study will provide the first systematic test of the significant questions relating to the practice and impact of genetic counseling. The study is unique in the experimental nature of the design. The instruments to be developed in the course of the study should be useful to counselors in guiding their practices and in evaluation of their effectiveness. Conceptually, the study places genetic counseling within the general framework of family decisions, so that the effect of variables other than counseling on decision-making can be assessed.

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SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM	THROUGH	
		1/1/74	12/31/74	
1. PERSONNEL <i>(List all personnel engaged on project)</i>			TIME OR EFFORT %/HRS.	AMOUNT REQUESTED <i>(Omit cents)</i>
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION			TOTAL
Barnett, C.	Principal Investigator or Program Director	20%	IMPACT OF GENETIC COUNSELING PRACTICES	
Cann, H.,	Assoc. Prof./Peds.	10%		
Luzzatti, L.	Professor of Peds.	10%		
Open	Research Assoc.-Stat	20%		
Open-Pediatrics	Res. Assoc.-Soc. wk.	50%		
Open-Pediatrics	Interviewer	50%		
Open-Pediatrics	Interviewer	25%		
Open-Pediatrics	Statistical Clerk	50%		
Open-Pediatrics	Data Coder	65%		
Open-Pediatrics	Typist	100%		
			TOTAL	\$ 47,377
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>				\$
3. EQUIPMENT <i>(Itemize)</i>				
Tape Recorder				\$ 500 *
4. SUPPLIES				\$
5. STAFF TRAVEL <i>(See Instructions)</i>	a. DOMESTIC	1 East coast meeting		\$ 500
	b. FOREIGN			\$
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>				
Genetic counseling				\$ 2,500
7. ALTERATIONS AND RENOVATIONS				\$
8. OTHER EXPENSES <i>(Itemize per instructions)</i>				
Office supplies, telephone, repro., postage, publication costs, etc. 1200				
Central computer usage 1200				\$ 2,400
			Subtotal - Items 1 thru 8	\$ 53,277
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
				TOTAL STIPEND EXPENSES
	b. TUITION AND FEES			\$
	c. TRAINEE TRAVEL <i>(Describe)</i>			\$
	11. Subtotal - Trainee Expenses			\$
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>				\$ 53,277

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION		1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					7TH YEAR
			2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	
PERSONNEL COSTS		47,377	50,732	54,229	57,964	61,950		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>								
EQUIPMENT		500*						
SUPPLIES								
TRAVEL	DOMESTIC	500	600	600	600	700		
	FOREIGN							
PATIENT COSTS		2,500	5,000	5,000				
ALTERATIONS AND RENOVATIONS								
OTHER EXPENSES		2,400	2,600	2,800	3,000	3,200		
TOTAL DIRECT COSTS		53,277	58,932	62,629	61,564	65,850		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →						\$ 302,252		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget explanation attached

BUDGET EXPLANATION

Dr. Barnett has a 9 month academic appointment supported jointly by the Department of Pediatrics and the Department of Anthropology. His project salary is computed on the basis of 10% time during the 9 month academic year and 70% time during July and August for an average of approximately 20% during each year.

The social worker (50% time), a research associate (20% time), two interviewers (50% and 25% time respectively), a statistical clerk (50% time), a data coder (65% time) and a typist (100% time) are required for the project. Two interviewers are required because husband and wife will be seen separately. The research associate is a biomathematician experienced in design of and data analysis for behavioral research projects. Computer time will be used for data analysis.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The tape recorder will be used by the typist to transcribe tape recordings of genetic counseling sessions and pre- and post-counseling interviews in the project on genetic counseling impacts on the family.

To insure that we obtain adequate patient material for the project on genetic counseling, we propose to waive the fee for this service to any participating family and therefore include these costs in our budget. The cost of genetic counseling to a family is \$50. This does not include laboratory tests and amniocentesis. We anticipate doubling the number of patients in the second and third years of the project. In years 4 and 5 no new patients will be studied although follow-up interviews will be carried out for those studied in year 3.

SECTION VII

Overall Budgets

DIRECTOR'S OFFICE BUDGET EXPLANATION

Salary support for the Program Director (Professor Lederberg) has been included entirely under the subproject budget for Screening and Characterization of Inborn Errors of Metabolism Using GC/MS. The 20% of his time budgetted there, includes support for his role in overall program direction as well as his direct involvement in that research project. This 20% allocation has not been subdivided between that budget and the present Program Director's Office budget. Such a suballocation would be difficult to make realistically since the apportionment of Professor Lederberg's time will vary from time to time, depending on program needs.

This budget does include support for 30% of the Program Director's secretary. She will support the Director in overall program management as well as in liaison work with the Visiting Committee and in implementing the planned annual symposium on aspects of genetic disease. An important responsibility of the Director is maintaining current awareness of the relevant literature which spans a number of fields. Ms. Redse will spend considerable time in assisting at this task with the help of modern information services and devices. She will also undertake to disseminate notices to the appropriate collaborating investigators.

Ms. Redse's salary is increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

Secretarial support for the individual Principal Investigators is provided in those respective subproject budgets.

The budget also covers estimated expenses for Visiting Committee honoraria and travel and expenses related to the planned annual symposia.

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM	THROUGH	
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT	AMOUNT REQUESTED <i>(Omit cents)</i>	
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION	%/HRS.	TOTAL	
Redse, R.	Principal Investigator or Program Director Program Director's Secretary	30	PROGRAM DIRECTOR'S OFFICE	
TOTAL →			\$ 3,414	
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$ -	
3. EQUIPMENT <i>(Itemize)</i>			\$ -	
4. SUPPLIES			\$ -	
5. STAFF TRAVEL <i>(See Instructions)</i>		\$ -		
a. DOMESTIC		\$ -		
b. FOREIGN		\$ -		
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>			\$ -	
7. ALTERATIONS AND RENOVATIONS			\$ -	
8. OTHER EXPENSES <i>(Itemize per instructions)</i>			\$ 5,000	
Visiting Committee honoraria and travel and expenses for annual symposium. Communications: information services (e.g., abstracts, ASCA; MEDLINE).				
9. Subtotal - Items 1 thru 8 →			\$ 8,414	
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			\$
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
	TOTAL STIPEND EXPENSES →			\$
b. TUITION AND FEES			\$	
c. TRAINEE TRAVEL <i>(Describe)</i>			\$	
11. Subtotal - Trainee Expenses →			\$	
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>			\$ 8,414	

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE DIRECT COSTS ONLY (Omit Cents)							
DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	3,414	3,656	3,908	4,177	4,464		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>	-	-	-	-	-		
EQUIPMENT	-	-	-	-	-		
SUPPLIES	-	-	-	-	-		
TRAVEL	DOMESTIC	-	-	-	-		
	FOREIGN	-	-	-	-		
PATIENT COSTS	-	-	-	-	-		
ALTERATIONS AND RENOVATIONS	-	-	-	-	-		
OTHER EXPENSES	5,000	5,300	5,700	6,000	6,400		
TOTAL DIRECT COSTS	8,414	8,956	9,608	10,177	10,864		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →					\$ 48,019		
<p>REMARKS: <i>Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)</i></p> <p style="text-align: center;">Budget explanation attached</p>							

AGGREGATE BUDGET EXPLANATION

The following budget is the aggregate of the five individual subprogram budgets and the Program Director's Office budget. Individual budget items such as personnel appearing in more than one budget and supply items like "chemicals, glassware, etc." have been combined to show only one such item each in this overall budget.

In considering the percentages of personnel time committed to this project, it should be noted that faculty personnel, as distinct from staff, also receive a portion of their salary from the institution. This institutional salary support assists in covering faculty efforts along research lines overlapping those in the present application. Therefore the faculty time percentages reflect contractual lower bounds on faculty commitment to this program.

Salaries are uniformly increased at a rate of 6% per year to cover expected merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; 22.3%, 9/78-8/79.

Budget items other than "Equipment" and "Patient Costs" are in general increased by 6% per year to cover inflation, except in specific instances noted in the individual budgets.

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM 1/1/74	THROUGH 12/31/74	
1. PERSONNEL (List all personnel engaged on project)			TIME OR EFFORT	AMOUNT REQUESTED (Omit cents)
NAME (Last, first, initial)	TITLE OF POSITION	%/HRS.	TOTAL	
Lederberg, J.	Principal Investigator or Program Director	20	AGGREGATE PROGRAM BUDGET	
Cann, H.	Assoc. Prof. of Peds.	50		
Kretchmer, N.	Prof. of Peds.	10		
Herzenberg, L.	Prof. of Genetics	10		
Cavalli-Sforza, L.	Prof. of Genetics	10		
Barnett, C.	Assoc. Prof. of Peds.	20		
Luzzatti, L.	Prof. of Peds.	10		
Tsuboi, K.	Senior Scientist	20		
Duffield, A.	Research Associate	100		
Pereira, W.	Research Associate	100		
Rindfleisch, T.	Research Associate	20		
Hulett, H.	Research Associate	20		
Open - Genetics	Research Associate	100		
Open - Pediatrics	Research Associate	100		
(continued)				
TOTAL →			\$ 345,600	
2. CONSULTANT COSTS (Include Fees and Travel)				\$
3. EQUIPMENT (Itemize)				
see attached				\$ 126,200*
4. SUPPLIES				
see attached				\$ 35,700
5. STAFF TRAVEL (See Instructions)	a. DOMESTIC 7 East Coast(3,500), 1 Mid-west (300), 1 West Coast(200)			\$ 4,000
	b. FOREIGN			\$
6. PATIENT COSTS (Separate Inpatient and Outpatient)				
Venepuncture (500) and genetic counseling (2,500)				\$ 3,000
7. ALTERATIONS AND RENOVATIONS				
Relocate equip., power, etc. for GC/MS data system				\$ 1,800
8. OTHER EXPENSES (Itemize per instructions)				
see attached				\$ 21,500
9. Subtotal - Items 1 thru 8			\$ 537,800	
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES (See Instructions)			
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER (Specify)	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
	TOTAL STIPEND EXPENSES →			\$
b. TUITION AND FEES				\$
c. TRAINEE TRAVEL (Describe)				\$
11. Subtotal - Trainee Expenses			\$	
12. TOTAL DIRECT COST (Add Subtotals, Items 9 and 11, and enter on Page 1)				\$ 537,800

continued

1. PERSONNEL	TITLE	% TIME
Open	Research Assoc.--Stat.	20
Open - Pediatrics	Research Assoc. - Soc. Worker	50
Veizades, N.	Research Engineer	33
Steed, E.	Research Engineer	33
Tucker, R.	Computer Programmer	75
Wegman, A.	Senior Res. Assist.	10
Waters, R.	Research Assist.	100
Sakaguchi, S.	Research Assist.	50
Van West, B.	Research Assist.	100
Makk, G.	Research Assist.	100
Open - Pediatrics	Research Assist.	50
Summons, R.	Post.Doc. Fellow	100
Open - Genetics	Grad. Res. Assist.	50
Open - Genetics	Grad. Res. Assist.	50
Pearson, D.	Electronics Tech.	40
Wyche, M.	Lab. Tech.	100
Boswell, M.	Lab. Tech.	100
Open - Genetics	Engin. Tech.	50
Open - Genetics	Research Tech.	100
Open - Pediatrics	Interviewer	50
Open - Pediatrics	Interviewer	25
Open - Pediatrics	Statist. Clerk	50
Open - Pediatrics	Data Coder	65
Jamtgaard, R.	Administrator	15
Redse, R.	Secretary-Prog.Dir.	30
Allen, M.	Secretary - IRL	25
Murray, R.	Secretary - Peds.	50
Meyering, P.	Secretary - Gen.	10
Open - Pediatrics	Typist	100
Harlow, W.	Machinist	10
Cusumano, M.	Laboratory Diener	50
Open - Genetics	Lab Glasswasher	25

continued

3. EQUIPMENT

4 Column Gas Chromatograph	\$14,400*
Mini-computer System & Inst. Interface	59,900*
Amino Acid Analyzer	32,500*
Event Counter	2,700*
Digital Voltmeter	900*
Carbon Dioxide Incubator	2,500*
Laminar Flow Hood	1,000*
Fluorescence Microscope	9,000*
Eppendorf Microfuge	500*
Buchler Power Supply	600*
Constant Current Power Supply	1,000*
Slab Gel Electrophoresis	300*
Column Acrylamide Gel Electrophoresis	200*
Destainer	200*
Tape Recorder	500*

Total Equipment \$126,200*

4. SUPPLIES

Chemicals, glassware & lab. apparatus	\$18,000
GC Supplies (Columns, Phases, etc.)	1,100
Dry ice & liquid nitrogen	500
Data recording media (GC/MS, Calcomp, etc.)	1,800
Mini-computer supplies (start-up & continuing)	1,600
Mass spectrometer repair parts & supplies	2,300
Electronic parts & supplies	1,900
Amino acid analyzer supplies	2,000
Radioactive tracers	5,000
Expendable supplies (photo. plates, etc.)	1,500

Total Supplies \$35,700

8. OTHER

Visiting committee honoraria & expenses for annual symposium	\$5,000
Office supplies, telephone, repor., postage, publication costs, etc.	6,200
Mini-computer maintenance	6,000
Freight on capital equipment	500
Central computer usage & terminal rental	3,800

Total Other \$21,500

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION		1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					7TH YEAR
			2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	
PERSONNEL COSTS		\$345,600	370,068	431,528	461,243	492,966		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>								
EQUIPMENT		126,200*	23,000*	18,100*	5,300*	6,000*		
SUPPLIES		35,700	37,700	42,400	44,800	47,700		
TRAVEL	DOMESTIC	4,000	4,700	4,900	4,900	5,400		
	FOREIGN							
PATIENT COSTS		3,000	5,500	5,500	500	500		
ALTERATIONS AND RENOVATIONS		1,800						
OTHER EXPENSES		21,500	24,900	26,400	27,800	29,700		
TOTAL DIRECT COSTS		537,800	465,868	528,828	544,543	582,266		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →						\$ 2,659,305		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

See individual subprojects for budget explanations

SECTION VIII

Concluding Remarks

Dr. Lederberg

CONCLUDING REMARKS

(Joshua Lederberg)

The overt justification for the proposed Center is, of course, the collective value of the well-defined research projects that will make up its day to day work. However, its most important utility in the long run may be a new institutional arrangement that will generate now unforeseen lines of investigation. Molecular biology as a basic science has leaped far beyond its practical application to human problems. The more intimate intellectual association of gifted scientists and clinicians envisaged here, the development of an active interface among workers who spend much of their time still either in basic laboratory work or in the clinic, is the only possible way of advancing these applications.

Speaking more personally, I can point to a number of anticipations from my own research career that might have been explored more aggressively had I then pressed them in an interdisciplinary context. My early work (with Tatum, Zinder and others) on genetic recombination and on viral transduction in bacteria was foreseen years ago as laying a groundwork for the development of a genetics of somatic cells and of means of importing reparative genetic information ("genetic engineering"). Subsequently, I tried to stress that the new molecular genetics would overtake other lines of concern about genetic impairment, and improvement, and that ethical issues would loom as large as technological opportunities in public policy debates. While public reactions to these potentialities are in some respect overdrawn, geneticists obviously must inculcate and exhibit great sensitivity to the ethical issues of genetic intervention and how they are perceived by the public (1).

Although much of my own laboratory research might go under the heading of genetic engineering of bacteria (2) I have been persuaded that prenatal diagnosis represents the most important practical avenue of genetic therapy or rather pre-emption of disease. For this reason, the application of automated technologies (growing in large measure out of NASA-supported work) is emphasized in this application.

What is more difficult to foresee is the intensive development of the whole repertoire of tools of molecular genetics. Somatic cell genetics is well launched; but we will surely also have to learn how to use specific DNA replication, hybridization, transcription, and translation, i.e. the full range of gene action in vitro, to solve pressing diagnostic problems.

Laboratory research shares a place with other interests. I can summarize these under the heading of a concern for the overall process of science, and its application for human benefit. These meta-scientific interests have been expressed in the public arena, as in efforts to enhance public understanding of science and provision for its support. More recently, I have been more involved in efforts to enhance the infrastructure of science, with work on new instrumentation coupled with feeble steps towards the use of computers for scientific "intelligence" (3).

The Genetics Research Center is a plan to bring all of these themes to a focus on an area most likely to advance genetic knowledge -- my fundamental scientific base -- and to generate tangible health benefits for the mutual advantage of both disciplines, and to satisfy the public's motives for continued investment in the health sciences.

Bibliography

1. Lederberg, J.: in Challenging Biological Problems, Oxford U. Press, N.Y. 1972, p. 7 (J.A. Behnke, ed.) "Biological Innovation and Genetic Intervention".
2. Sgaramella, V.: Proc. Nat. Acad. Sci. 69:3389, 1972.
"Enzymic Oligomerization of Bacteriophage P22 DNA and of Linear Simian Virus 40 DNA."
3. Lederberg, J.: in Biochemical Applications of Mass Spectrometry, John Wiley & Sons, N.Y. 1972, p. 193 (G.R. Waller, ed.) "Use of a Computer to Identify Unknown Compounds: The Automation of Scientific Inference."

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SECTION IX

Professional Personnel

KEY PERSONNEL AND BIOGRAPHICAL MATERIAL

Joshua Lederberg, Ph.D. - Principal Investigator, Professor and Chairman, Department of Genetics.

Professor Lederberg will serve as the Principal Investigator for the overall program, and for part 1, automated screening methods (GC/MS) for inborn errors of metabolism. In addition, Professor Lederberg maintains active research programs in genetic recombination in bacteria; mechanisms of DNA replication and reunion; interactions of environmental mutagens with DNA; and computer emulation of human cognitive processes.

Howard Cann, M.D. - Associate Program Director, Associate Professor of Pediatrics.

Dr. Cann will assume responsibility for coordinating laboratory research with clinical applications within the program. This will involve screening of patients for their suitability of participation in the relevant research projects, obtaining appropriate patients for projects, informing patients about the research projects and obtaining their consent for participation, insuring that proper evaluation, care and counseling are provided to patients and families involved in these projects and planning for the application of the research findings to patient care. Dr. Cann will be directly involved with research projects on genetic markers in amniotic fluid, separation of fetal cells from the maternal circulation, the impact of genetic counseling on family decisions and screening and detection of inborn errors of metabolism. Dr. Cann's research activities pertain to human polymorphisms and to somatic cell genetics and his clinical work involves him with hereditary illness and genetic counseling. He has set up and directs a laboratory for typing for various polymorphisms of erythrocyte antigens and enzymes and serum proteins. He has initiated an investigative program for applying somatic cell hybridization to prenatal diagnosis of hereditary disorders.

L.L. Cavalli-Sforza, M.D. - Professor of Genetics

The investigations of Professor Cavalli-Sforza have long contributed and continue to contribute important information on human population genetics. He has pioneered in applying demography to studies of population and genetic structure of man. Particularly pertinent to this program is his interest and work on variation among populations in the frequency of inherited disorders and their determining genes.

Professor Cavalli-Sforza also directs a laboratory program oriented toward the detection of specific binding proteins in

blood. The detection of genetic polymorphisms of these proteins will be carried out by Professor Cavalli-Sforza as a research activity of this Genetics Center.

Norman Kretchmer, M.D., Ph.D. - Professor of Pediatrics.

Professor Kretchmer will participate in the research project dealing with screening and detection of inborn errors of metabolism. He is well known for his research and clinical work in various inborn errors of metabolism. One of Professor Kretchmer's main research interests presently deals with lactase intolerance in man. His research program also involves the developmental biochemistry of the urea cycle and the pyrimidine biosynthetic pathway in eukaryote cells and tissues. He has recently relinquished the chairmanship of the faculty committee for teaching the human biology curriculum at Stanford University. From 1959-1969 Professor Kretchmer was Chairman of the Department of Pediatrics.

Leonard Herzenberg, Ph.D. - Professor of Genetics.

Professor Herzenberg directs an active research program in immunogenetics with special emphasis on genetic and structural studies of mouse immunoglobulins and on the mechanism and control of antibody synthesis. The development under his direction of instrumentation for separating cells by means of fluorescent sensing has provided potential methodology for separating fetal cells from the maternal bloodstream. Professor Herzenberg will participate in the project which will apply this methodology to antenatal diagnosis of genetic disorders.

Clifford Barnett, Ph.D. - Professor of Anthropology and Associate Professor of Pediatrics.

Professor Barnett has worked in medical anthropology for 10 years. His research activities have dealt with cultural and genetic aspects of congenital dislocation of the hips, fertility and birth spacing of partially acculturated Guatemalan Indians, and mother and infant interactions in reference to premature infants. He has designed and will direct the research project on the impact of genetic counseling on family decisions and behavior.

Alan Duffield, Ph.D. - Research Associate in Genetics.

Dr. Duffield is an organic chemist who has carried out fundamental studies in mass spectrometry of organic compounds. He is presently directing research involving the development of a multicomponent, automated system for analysis of biologic substances. He shall apply this system, which involves a gas liquid chromatograph, a mass spectrometer and computer

facilities to screening, detecting, and studying inborn metabolic errors in various body fluids and tissues.

Kenneth Tsuboi, Ph.D. - Senior Scientist, Department of Pediatrics.

Dr. Tsuboi's research interests have included the physiological chemistry and enzymology of erythrocyte intermediate metabolism and more recently, the biochemical genetics of various erythrocyte enzymes. He is also working on the enzymological characteristics of various primate cell culture lines. In this program he will participate in the project on linkage and prenatal diagnosis of inherited disorders. In particular, he will work in the detection of polymorphic enzyme markers in cultured amniotic fluid cells.

Luigi Luzzatti, M.D. - Professor of Pediatrics.

Professor Luzzatti is the director of the clinical cytogenetics laboratory and of the Birth Defects Clinics in the Department of Pediatrics. His clinical activities which are pertinent to this program, include comprehensive care for patients (and their families) with birth defects, genetic counseling and screening amniotic fluid samples (usually from women over 35 years of age) for chromosomal abnormalities. He will participate in the project which investigates the impact of genetic counseling on family decisions.

Herbert Schwartz, M.D. - Professor of Pediatrics.

Professor Schwartz's research interests in hemoglobin synthesis, structures and function in health and disease, and their application to the prenatal detection of hereditary disorders involving hemoglobin, e.g. sickle cell anemia and thalassemia, will be incorporated into the program on exploring the maternal bloodstream for fetal cells. Dr. Schwartz will collaborate with Drs. Herzenberg and Cann in preliminary studies of fetal erythrocytes separated from maternal blood. Dr. Schwartz directs the hematology service of the Department of Pediatrics.

Other members of the Department of Genetics who will not participate directly in this program but with whom we interact daily are Dr. Eric Shooter (Professor) and Dr. A.T. Ganesan (Associate Professor). Professor Shooter directs research into the structure and mechanism of action of the nerve growth factor protein. Dr. Ganesan is investigating the genetic control of chromosome replication in *B. subtilis*, the chemical basis of DNA replication, the mechanism of genetic recombination during DNA mediated transformation and the role of the nuclear membrane in chromosome replication in mammalian cells.

The clinical and research interests of Professor Irving Schulman, Chairman of the Department of Pediatrics, are devoted to various defects of coagulation, including inherited disorders. Other members of the Department of Pediatrics whose clinical and research activities relate to this program are Dr. Phillip Sunshine (Associate Professor of Pediatrics) and Dr. John Johnson (Assistant Professor of Pediatrics), Director and Associate Director of the Newborn and Premature Infant Nurseries, respectively. Dr. Sunshine and Dr. Johnson are together investigating the metabolic consequences of neonatal ornithine transcarbamylase deficiency and detection of heterozygotes for the gene which determines this disorder. The research activities of Dr. Merton Bernfield, Associate Professor of Pediatrics, are designed to assess the morphogenetic behavior and cell surface properties of human cells and to utilize these assessments in studies of cells derived from individuals with birth defects. Dr. John Gribble, Assistant Professor of Pediatrics, is a pediatric hematologist whose research activities have dealt with in vitro biosynthesis of hemoglobin and biochemical and physiological aspects of pinniped hemoglobins. Dr. Gribble's clinical activities involve evaluation and treatment of patients with hereditary disorders of blood coagulation, including genetic counseling of their families. The clinical and research activities of Dr. R.O. Christiansen (Assistant Professor of Pediatrics and Director of the Pediatric Metabolic and Endocrine Service) and Dr. Judith Koehler (Assistant Professor of Pediatrics and Director of Pediatric Neurology) are quite relevant to the Genetics Center program. Even though the research activities of all of these individuals will not be supported by this grant, we are including bibliographic sketches on each of them because of their relevance to the overall Genetics Center program.

Within one or two years after the activation of this program we anticipate the direct participation of one or more colleagues from the Department of Obstetrics and Gynecology. A search for chairman of this department is presently under way, and we expect that this individual will bring competence to Stanford in fetal physiology and fetal monitoring. We look forward to interacting in this program with our obstetrical colleagues in various projects pertaining to antenatal detection of genetic disorders and selective abortion.

Research Support Summary for Departments of Genetics and Pediatrics
 Relevant to Genetics Research Center

Program Director: J. Lederberg
 Associate Program Director: H. Cann

Principal Investigator: (unless otherwise noted)	Grant Title	Current Year	Total Award	Grant Term	Budgeted % time
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HOWARD CANN
 Associate Professor of Pediatrics
 Department of Pediatrics

1) NIH:HL-15008	Pre-Natal Detection of Sickle Cell Anemia	\$ 38,717	\$118,000	9/72-8/75	
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LUCA CAVALLI-SFORZA
 Professor of Genetics
 Department of Genetics

1) AEC:AT-04-3-326	Mutation Rates and Mutational Loads in Man	32,000	32,000	10/72-9/73 (Renewal pending)	10%
2) NIH:NS-10711	Genetic Study of Metabolism of Neural Transmitters	65,000	207,000	9/72-8/75	20%
3) NIH:GM-20467	Gene Diffusion, Natural Selection and Drift in Man	47,587	147,351	5/73-4/76	20%

ADAYAPALAM GANESAN
 Associate Professor of Genetics
 Department of Genetics

1) NIH:GM50,199	Development Award-Research Career Program Chromosome Replication, Recombination and Cell Division	21,433	Support recommended for 2 additional years ending 12/31/75, the amounts to be determined annually.		
2) NIH:GM14108	DNA Synthesis and Genetic Recombination	34,902	211,996	6/72-5/77	50%

LEONARD HERZENBERG
 Professor of Genetics
 Department of Genetics

1)	NIH:GM-17367	Automated Cell Sorting - Clinical and Biological Uses	160,802	585,977	1/73-12/75	15%
2)	NIH:AI-08917	Genetics of Immunoglobins	52,774	246,386	5/69-4/74	20%
3)	NIH:CA-04681	Genetic Studies of Mammalian Cells	80,994	424,981	9/72-8/77	30%
4)	NIH:HD-01287	Fetal-Maternal Immunological Interactions	38,198	222,897	5/73-4/78	10%

JOHN D. JOHNSON
 Assistant Professor
 Department of Pediatrics

1)	United Cerebral Palsy	Developmental Aspects of Heme Protein Catabolism	28,772	53,322	7/71-6/73	
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NORMAN KRETCHMER
 Professor of Pediatrics
 Department of Pediatrics

1)	NIH:RR-00081	Clinical Research Center for Premature Infants	418,532	1,974,008	10/69-9/74 (Renewal pending)	5%
2)	NIH:HD-02147	Biochemical Studies of Development	206,593	1,309,278	6/66-5/74 (Renewal pending)	15%
3)	National Found. CRBS-252	Growth and Differentiation of the Placenta	23,293	23,293	7/72-6/74	15%
4)	NIH:HD-00047	Human Development and Pediatrics Training Grant	90,588	448,936	7/70-6/74	10%
5)	NIH:HD-00391	Regulation of Enzyme Action During Development	42,289	183,822	9/68-8/73 (Renewal pending)	15%

NORMAN KRETCHMER (continued)

6)	NIH:CA14917	Pyrimidine Synthesis and Cellular Proliferation in Colon	42,689	130,737	6/73/5/76	20%
7)	Educational Found. of America	Epidemiology, Etiology, and Physiology of Diarrhea in the American Indian	45,156	92,915	7/73-6/75 (Renewal pending)	5%

JOSHUA LEDERBERG
Professor and Chairman
Department of Genetics

1)	NASA:NGR-05-020	Cytochemical Studies of Planetary Micro-organisms	180,000	3,800,000	9/60-8/73 (Future support dubious)	11%
2)	NIH:AI-05160	Genetics of Bacteria	60,000	280,000	9/68-8/73 (Renewal pending)	15%
3)	NIH:RR-00612	Resource Related Research - Computers and Chemistry (E. Feigenbaum, Principal Investigator; J. Lederberg and C. Djerassi, Co-Investigators)	194,408	675,000	5/71-4/74 (Renewal pending)	0%
4)	NIH:RR-00785	Stanford University Medical Experimental Computer Facility (SUMEX)	765,573	4,246,621	8/73-7/78 (pending)	20%
5)	NIH:GM0295	Training Grant in Genetics	143,964	756,650	7/69-6/74	15%

GILDA LOEW
Research Associate
Department of Genetics

1)	NSF:GB17980	Quantum Chemical Investigations of Heme Proteins and Ferredoxins	34,799	34,799	2/72-1/74 (Renewal pending)	50%
2)	NIH:DA00770	Quantum Chemical Studies of Opiate Narcotics	57,557	184,139	9/73-8/76 (Pending)	50%

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ERIC M. SHOOTER
Professor of Genetics
Department of Genetics

1) NIH:NS04270	Molecular Neurobiology - Proteins in the Nervous System	84,558	404,979	12/70-11/75	40%
2) NSF:GB31982	Structure and Mechanism of Action of the Nerve Growth Factor	60,000	60,000	1/72-12/73	20%

KENNETH TSUBOI
Senior Scientist
Department of Pediatrics

1) NIH: Contract- Div. of Biol. Standards	Biochemical Parameters of Primate Cell Cultures	23,598	47,196	6/72-5/74	
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BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Clifford R. Barnett	TITLE Associate Professor of Pediatrics Professor of Anthropology	BIRTHDATE (Mo., Day, Yr.) 8/17/29	
PLACE OF BIRTH (City, State, Country) New York, New York	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S. Citizen	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female	
EDUCATION (Begin with baccalaureate training and include postdoctoral)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
The City College, New York, New York	B.S.S.	1950	Anthropology-Psychology
Cornell University, Ithaca, New York	M.A.	1951	Anthropology
Cornell University, Ithaca, New York	Ph.D.	1960	Anthropology
HONORS Executive Board, Society for Applied Anthropology 1967-70, Vice-President, 1971-72, President 1972-73, Past President 1973-74; Executive Board, Society for Medical Anthropology 1971-73; Chairman, Committee on Ethics, American Anthropological Association, 1971-74; Fellowship recipient, Milbank Memorial Fund, 1971-72.			
MAJOR RESEARCH INTEREST Cultural Anthropology		ROLE IN PROPOSED PROJECT Investigator	

RESEARCH SUPPORT (See instructions)

Research Grant: Genetic Studies in the Lake Atitlan Basin, Guatemala, GM 15593; \$14,130 for the current year; \$300,000 for the six year period 1967 - 1973; National Institutes of Health. Howard M. Cann, M.D. - principal investigator.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

- 1964- Associate to Full Professor, Anthropology; Associate Professor, Pediatrics, Stan. Uni
- 1964-1970 Assistant Director to Acting Director, Program in Medicine and the Behavioral Sciences, Stanford University.
- 1962-1964 Research Associate to Associate Project Director, Navajo-Cornell Field Health Research Project, Dept. of Public Health, Cornell Univ. Medical College.
- 1961-1963 Resident Anthropologist, Navajo-Cornell Field Health Research Project at Many Farms, Arizona; Resident in Professional Practice, Russell Sage Foundation.
- 1955-1961 Senior Research Associate to Team Chairman, Foreign Areas Studies Division of Special Operations Research Office of the American University, Washington, D.C.

Publications (selected)

- "Untreated Congenital Hip Disease: A Study of the Epidemiology, Natural History and Social Aspects of the Disease in a Navajo Population". With D. Rabin, W. Arnold, R. Freiburger and G. Brooks. Supplement, American Journal of Public Health 55, 2. 1965.
- "Genetics of Diego Blood Groups in Guatemalan Indians: Use of Antiserums to Diego a and Diego b Antigens". With Howard M. Cann and Betty Van West. Science, 162:1391, 1968.
- "Neonatal Separation: The Maternal Side of Interactional Deprivation". With P.H. Leiderman, R. Grobstein, M. Klaus. Pediatrics 45:197, 1970.

(publications continued)

Publications (continued)

- "Child Spacing in a Highland Guatemala Community". With Jean Jackson and Howard M. Cann. In Culture and Population: A Collection of Current Studies, Polgar, (Ed.). Monograph 9, Carolina Population Center, Chapel Hill, 1971. pp. 139-148.
- "Health Care Experiment at Many Farms". With W. McDermott and K. Deuschle. Science 175:4017, January 7, 1972, pp. 23-31.
- "The Effects of Denial of Early Mother-Infant Interaction on Maternal Self-Confidence". With M. Seashore, A. Leifer and P. Leiderman. Child Development 43:1203, 1972.
- "Effects of Mother-Infant Separation on Maternal Attachment Behavior". With A. Leifer, P. Leiderman and J. Williams. In Press, Personality and Social Psychology.

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Merton R. Bernfield	TITLE Associate Professor	BIRTHDATE (Mo., Day, Yr.) April 9, 1938
PLACE OF BIRTH (City, State, Country) Chicago, Illinois	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.A.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Illinois, Urbana, Illinois	B.S.	1959	Medicine
Graduate Division, University of Illinois, Chicago, Illinois	M.S.	1961	Biochemistry
College of Medicine, Univ. of Illinois, Chi.	M.D.	1961	

HONORS
 Alpha Omega Alpha, 1959 Ross Award for Pediatric Research, 1971
 Borden Undergraduate Research Award in Medicine
 C.V. Mosby Company Research Award

MAJOR RESEARCH INTEREST Developmental Biochemistry	ROLE IN PROPOSED PROJECT Investigator
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RESEARCH SUPPORT (See instructions)
 NIH GM-15086-05 Ribonuclease and Oligoribonucleotide Synthesis, September 1, 1970 to August 30, 1975. Current year \$26,816. Total \$140,381.
 NIH HD 06763 Extracellular Materials and Embryonic Organ Formation, May 1, 1972 - April 30, 1977. Current year \$45,401. Total \$203,648.
 National Foundation-March of Dimes R-73-146 Morphogenetic Behavior of Human Cells July 1, 1973 - June 30, 1974. Total \$40,000.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List only most representative publications. Do not exceed 3 pages for each individual.)

1970 - Associate Professor, Department of Pediatrics, Stanford University School of Medicine; Stanford, California
 1970 - Co-Director, Medical Scientist Training Program, Stanford University School of Medicine; Stanford, California.
 1969-1970 Associate Director, Medical Scientist Training Program, Stanford University School of Medicine; Stanford, California
 1967 - Director, Birth Defects Research Center; Associate Director, Birth Defects Clinic, Department of Pediatrics, Stanford University School of Medicine; Stanford, California
 1967-1970 Assistant Professor, Department of Pediatrics, Stanford University School of Medicine; Stanford, California
 1967-1967 Chief Resident, Department of Pediatrics, Stanford Medical Center, Stanford University
 1965-1966 Research Investigator, National Institute of Child Health and Human Development with Dr. Clifford Grobstein, in the Department of Biology, University of California, San Diego; La Jolla, California
 1963-1965 Research Associate, National Heart Institute, with Dr. Marshall Nirenberg; Bethesda, Maryland
 1962-1963 Assistant Resident, Department of Pediatrics, New York Hospital-Cornell Medical Center; New York, New York
 1961-1962 Rotating Internship, Research and Education Hospitals, University of Illinois, Chicago, Illinois

Biographical Sketch of Dr. Merton Bernfield

Page Two, continuation of page one

Publications

- Bernfield, M.R. and Nirenberg, M.W.: RNA Codewords and Protein Synthesis. IV. The nucleotide sequences of multiple codewords for phenylalanine, serine, leucine, and proline. *Science* 147, 479-484, 1965.
- Bernfield, M.R. and Rottman, F.M.: Ribonuclease and Oligoribonucleotide Synthesis. III. Oligonucleotide synthesis with 5'-substituted uridine 2', 3'-cyclic phosphates. *J. Biol. Chem.* 242, 4134-4143, 1967.
- Maenpaa, P.H., and Bernfield, M.R.: Quantitative Variation in Serine Transfer Ribonucleic Acid during Estrogen-Induced Phosphoprotein Synthesis in Rooster Liver. *Biochemistry* 8, 4926-4935, 1969.
- Bernfield, M.R.: Collagen Synthesis during Epitheliomesenchymal Interactions. *Develop. Biol.* 22, 213-231, 1970.
- Bernfield, M.R., and Wessells, N.K.: Intra- and Extracellular Control of Epithelial Morphogenesis. *Develop. Biol. Supplement* 4, 195-249, 1970.
- Bernfield, M.R., Banerjee, S.D., and Cohn, R.H.: Dependence of Salivary Epithelial Morphology and Branching Morphogenesis upon Acid Mucopolysaccharide-Protein (Proteoglycan) at the Epithelial Surface. *J. Cell. Biol.*, 52, 674-689, 1972.

NAME		Associate Professor of Pediatrics March 31, 1929	
Howard M. Cann, M.D.			
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX	
Chicago, Illinois	U.S. Citizen	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female	

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Colorado, Colorado	B.A.	1950	Chemistry, Cum Laude (General Studies)
University of Colorado School of Medicine, Colorado.	M.D.	1954	

HONORS
 1971 - 1972 National Institutes of Health Special Research Fellowship, Genetics Laboratory, Department of Biochemistry, University of Oxford, England.
 1966 - 1971 Scholar in Academia Medicine, The John and Mary R. Markle Foundation.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT (Continued on Page --)
Human Somatic Cell Genetics	ASSOCIATE INVESTIGATOR

RESEARCH SUPPORT (See instructions)

Research Grant: Genetic Studies in the Lake Atitlan Basin, Guatemala, GM 15593; \$28,260. for the current year; \$300,000. for the six year period 1967 - 1973; 35% effort; National Institutes of Health.
 National Institutes of Health Special Research Fellowship for sabbatical leave at the University of Oxford (Professor Walter F. Bodmer), Genetic Control of Human Transplantation Antigens, 1-FO3, HD51401-01; \$12,371. for one year, September 1, 1971 - August 31, 1972, 100% effort.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

September 1, 1971 - August 31, 1972 National Institutes of Health Special Research Fellowship, Genetics Laboratory, Department of Biochemistry, University of Oxford, England.
 1970 - Associate Professor, Department of Pediatrics, Stanford University School of Medicine.
 1964 - 1970 Assistant Professor of Pediatrics, Stanford University School of Medicine
 1963 - 1964 Instructor, Department of Pediatrics, Stanford University School of Medicine.
 1962 - 1963 National Science Foundation Senior Postdoctoral Fellow, Institute of Genetics, University of Pavia, Pavia, Italy.
 1960 - 1962 Postdoctoral Fellow, Department of Genetics, Stanford University School of Medicine, Palo Alto, California.
 1957 - 1960 United States Public Health Service, Washington, D.C., Director, National Clearinghouse for Poison Control Centers. Draft Classification: 4A.
 1956 - 1957 Assistant Resident and Senior Resident in Pediatrics, Stanford University Hospital, San Francisco, California.
 1955 - 1956 Assistant Resident in Pediatrics, University of Colorado Medical Center, Denver, Colorado.
 1954 - 1955 Rotating Intern at San Francisco Hospital, University of California Service, San Francisco, California.

Committees and Consultant

- 1968 - Committee on Drugs, American Academy of Pediatrics.
- 1965 - 1968 Genetic Consultant, Hereditary Defects Unit, California State Department of Public Health.
- 1965 - Genetic Consultant, Congenital Malformations Branch, Epidemiology Section, Dental Health Center, U.S. Public Health Service.

Societies and Organizations

- American Academy of Pediatrics
American Association of Poison Control Centers
American Federation for Clinical Research
American Public Health Association, Epidemiology Section
American Society of Human Genetics
Western Society for Pediatric Research

Specialty Board Certification

- 1960 American Board of Pediatrics

State and National Certification

- Diplomate of National Board of Medical Examiners
Licensed to practice medicine in California and Colorado.

BIBLIOGRAPHY

1. Cann, H.M. and Herzenberg, L.A.: In Vitro Studies of Mammalian Somatic Cell Variation. I. Detection of H-2 Phenotype in Cultured Mouse Cell Lines, J. Exp. Med. 117:259, 1963.
2. Cann, H.M. and Herzenberg, L.A.: In Vitro Studies of Mammalian Somatic Cell Variation. II. Isoimmune Cytotoxicity With a Cultured Mouse Lymphoma and Selection of Resistant Variants. J. Exp. Med. 117:267, 1963.
3. Berrai, I. and Cann, H.M.: Segregation Analysis of Juvenile Diabetes Mellitus, J. Med. Genet. 2:8, 1965.
4. Cann, H.M.: Computer Analysis in Human Genetics: Segregation Analysis and Demographic Genetics, Ann. New York Acad. Sci. 126:728, 1965.
5. Berrai, I., Cann, H.M., Cavalli-Sforza, L.L. and DeNicola, P.: The Effect of Parental Age on Rates of Mutation for Hemophilia and Evidence for Differing Mutation Rates for Hemophilia A and B, American J. Hum. Genet. 20:175, 1968.
6. Cann, H.M. and Cavalli-Sforza, L.L.: Effects of Grandparental and Parental Age, Birth Order and Geographic Variation on the Sex Ratio of Liveborn and Stillborn Infants, American J. Hum. Genet., 20:381, 1968.
7. Cann, H.M.: Principles of Human Inheritance, in Human Genetics, Birth Defects, Original Article Series, The National Foundation, Vol. IV, November, 1968.

BIBLIOGRAPHY (Continued)

8. Cann, H.M., Van West, B., and Barnett, C.R.: Genetics of Diego Blood Groups in Guatemalan Indians: Use of Antiserums to Di^a and Di^b Antigens, Science, 162: 1391, 1968.
9. Greenstein, R.M., Harris, D.J., Luzzatti, L.L. and Cann, H.M.: Cytogenetic Analysis of a Boy with the XXXY Syndrome: Origin of the X-Chromosomes, Pediatrics, 45:677, 1970.
10. Dungy, C.I., Aptekar, R.G. and Cann, H.M.: Herediatry Hydrometrocolpos with Polydactyly in Infancy. Pediatrics, 47:138, 1971.
11. Barnett, C.R., Jackson, J. and Cann, H.M.: Childspacing in a Highland Guatemala Community, in Culture and Population: A Collection of Current Studies, Polgar, S. (ed.), Carolina Population Center, Univ. North Carolina, Chapel Hill, 1971.
12. Watson, W. and Cann, H.M.: Genetic Counseling in Dermatology. Pediat. Clin. N. America 18:757, 1971.
13. Watson, W., Cann, H.M., Farber, E. and Nall, L.: The Genetics of Psoriasis. Arch. Dermatol., In Press.
14. Bodmer, W.F., Bodmer, J.G., Coukell, A., Cann, H.M. and Van West, B.: Some Further Data on the Joint Segregation of HL-A and Haptoglobin. Ann. Hum. Genet. (London), In Press.

Book Review

Carter, C.O.: Human Heredity, Cytogenetics 2:55, 1963.

HONORS (Continued from Page 17)

1964 Society for Pediatric Research
1963 - 1968 Research Career Development Award, National Institutes of Health,
U.S. Public Health Service
1962 - 1963 National Science Foundation Senior Postdoctoral Fellowship.

DO NOT TYPE IN THIS SPACE-INDICATING MARGIN

CURRICULUM VITAE

NAME: L. L. Cavalli-Sforza

TITLE: Professor of Genetics

BIRTHDATE: January 25, 1922

PLACE OF BIRTH: Genoa, Italy

NATIONALITY: Italian

SEX: Male

Married, four children

SOCIAL SECURITY NUMBER:
[REDACTED]

ACADEMIC HISTORY:

University of Pavia, Italy

M.D., 1944

Cambridge University, U.K.

M.A., 1950

Universities of Pavia and Parma, Italy

Libera Docenza, 1952 (Microbiology)

1952 (Genetics)

1960 (Statistics)

EMPLOYMENT RECORD:

1943-44 Intern of Genetics Department, University of Pavia
(then located at Istituto Italiano di Idrobiologia, Verbania,
Italy)

1944-45 Intern and resident at Verbania Hospital, Italy

1945-47 Assistant in research, Istituto, Sieroterapico Milanese,
Milan

1948 Scholarship in genetic statistics, John Innes Horticultural
Institution, London (U.K.)

1948-50 Research Fellow and Assistant in Research, Genetics De-
partment, Cambridge University (U.K.)

1950-57 Director of Research in Microbiology, Istituto Siero-
terapico Milanese (Milan, Italy)

1951-54 Lecturer in Genetics and in Statistics, Science Faculty
(part-time), University of Parma and University of Pavia,
Italy

1958-62 Professor of Genetics, University of Parma, Italy

1962-70 Professor of Genetics, and Director of Istituto di Genetica,
University of Pavia (Italy)

1970- Present Professor of Genetics, Sranford University, Palo Alto,
California

Earlier Sojourns in the United States:

- | | |
|---------|--|
| 1954 | Rockefeller Fellow (six months) at Genetics Department, University of Wisconsin, Madison, Wisc. |
| 1958 | Research Associate at University of Wisconsin |
| 1960 | Visiting Professor at Genetics Department, Stanford University (three months) |
| 1962 | Visiting Professor at Genetics Department and Statistics Department, Stanford University (one month) |
| 1964-65 | Visiting Professor at Department of Biology, Harvard University (three months) |
| 1968-69 | Visiting Professor at Genetics Department, Stanford University (one year) |

Expeditions to Africa in the Fall-Winter periods of 1965-66, 1966-67, 1967-68, 1968-69, 1969-70, 1970-71, conducting research in the following countries: Central African Republic, Cameroon, Zaire and Ethiopia

ACADEMIC HONORS:

President, Biometric Society, 1967-68
Vice President, International Congress of Genetics, Tokyo, 1968
Foreign Honorary Member of American Academy of Arts and Sciences, 1969
Royal Anthropological Institute of Great Britain and Ireland, Huxley Award in Anthropology, 1972

MILITARY SERVICE: Medical Officer in the Italian Army, 1947-48

MAJOR RESEARCH INTERESTS: Bacterial genetics; presently, human population genetics and allied fields.

RESEARCH ACTIVITY:

Apart from early work in the fields of immunology and *Drosophila* population genetics, research activity during the first fifteen years was concentrated mostly on bacterial genetics. Among results of the analysis of sex and recombination in *E. coli* strains: the finding of the first *coli* mutant with a high recombination frequency (Hfr), of Hfr linkage with chromosome markers, and of the infective transmission of mating capacity (F episomes). Among results on the analysis of drug resistance in bacteria: a quantitative

Curriculum Vitae - 3
L. L. Cavalli-Sforza

technique for sib selection, demonstrating the spontaneous origin of drug resistant mutants; the analysis by crossing of polygenic inheritance for chloramphenicol resistance; streptomycin resistance as a genetic modifier. Some of this work was in collaboration with the Lederbergs.

From 1954, activity was concentrated increasingly on human population genetics -- the analysis of a population in the Parma Valley showed that microgeographic variation can largely be explained by drift alone. This required the development of special techniques, including the computer simulation of human populations, methods for the reconstruction of differentiation of racial groups, and the characterization of selective and random causes of variation. Several archives, from consanguinity records to parish books, have been the subject of study to extract genetic information. A full description of the history of consanguinity in Italy has resulted. Demographic analysis of parish book records by computer is still in progress.

An analysis, during six winters, of an African Pygmy population with the help of a large team of collaborators allowed to accumulate information on the population structure of hunters-gatherers, to detect new genetic types, to account for the physiology of low stature in Pygmies and other problems of this population. Most recent work has centered on the impact of technological developments and cultural change, essentially the domestication of plants and animals, on human biological evolution.

AUTHOR OF BOOKS:

"The Genetics of Human Populations" in collaboration with W. Bodmer (now Professor of Genetics at Oxford, U.K.), and a short handbook of biostatistics.

100. A. MORONI, 1960. Sources, reliability and usefulness of consanguinity data with special reference to Catholic records. International Symposium of Demography and Genetics, Geneva.
101. L.L. CAVALLI-SFORZA, 1960. Indagine speciale sulla consanguineità dei matrimoni. Istituto Centrale di Statistica, Roma.
102. A. MORONI, 1960. Analisi metodologica del rilievo della consanguineità. Parte Prima - Legislazione e materiale religioso e civile italiano per il rilievo della consanguineità. Folia Hereditaria et Pathologica, 9, fasc. 3: 199-247.
103. L.L. CAVALLI-SFORZA, 1961. Lezioni di metodologia statistica per ricercatori - Il numero di osservazioni necessarie in esperimenti semplici e l'efficienza dell'esperimento. Istituto di Calcolo della Probabilità, Istituto di Statistica, 4: Articolo Terzo.
104. L.L. CAVALLI-SFORZA, 1961. Un metodo per la stima della frequenza di mutazione dell'uomo; risultati preliminari. Atti A.G.I., VI: 151-162.
105. L.L. CAVALLI-SFORZA, J.A. ROPER, G. SERMONTI, S.I. ALIKHANIAN, D.A. HOPWOOD and J.F. STAUFFER, 1961. Panel discussion: microbial genetics and its application to fermentations. Sci. Repts., 1st Super. Sanita, 1: 441-483.
106. L.L. CAVALLI-SFORZA, 1961. Biologia e genetica. L'Ateneo Parmense, XXVII, fasc. 6: 1-20.
107. M. MAINARDI, I. BARRAI, L.L. CAVALLI-SFORZA, 1962. The distribution of the number of collateral relatives. Atti A.G.I., VII: 123-130.
108. L.L. CAVALLI-SFORZA, 1960. Metodi e problemi nella analisi degli effetti biologici delle radiazioni sull'uomo. VII Rassegna Internazionale Elettronica e Nucleare, Atti Ufficiali del Congresso Scientifico, Roma.
109. L.L. CAVALLI-SFORZA, 1962. Studying human evolution by census and computer. New Scientist, 13: 92-93.
110. I. BARRAI, L.L. CAVALLI-SFORZA and A. MORONI, 1962. Frequencies of pedigrees of consanguineous marriages and mating structure of the population. Ann. Hum. Genet., 25: 347.
111. L.L. CAVALLI-SFORZA, 1961. I fattori letali nell'Uomo. Primo Simposio di Statistica Medica, Roma, 11-12 giugno 1961.
112. L.L. CAVALLI-SFORZA, 1962. Risposta ad alcune critiche sul neodarwinismo. Rendiconti della Classe di Scienze Fisiche, Matematiche e Naturali; Serie VIII, Vol. XXXII, fasc.1.
113. L.L. CAVALLI-SFORZA, 1962. Indagine speciale su alcune caratteristiche genetiche della popolazione italiana. Note e Relazioni N. 17, Istituto Centrale di Statistica.

114. L.L. CAVALLI-SFORZA, 1963. Sir Ronald Fisher. Atti A.G.I., VIII: 1-8.
115. G. MODIANO, A.S. BENERECETTI-SANTACHIARA, F. GONANO, A. CAPALDO e L.L. CAVALLI-SFORZA, 1963. Analisis della popolazione leccese per gruppi sanguigni G6PD, aptoglobine, e transferrine. Atti A.G.I., 8: 64-79.
116. L.L. CAVALLI-SFORZA, 1963. Nuclear threat and human genetics. Exposure of man to radiation in nuclear warfare. Elsevier Publishing Company, Amsterdam.
117. L.L. CAVALLI-SFORZA, 1963. The distribution of migration distances, models and application to genetics. Entretiens de Monaco en Science Humaines, Premiere Session, 24-29 Mai, 1962. Human Displacements, 139-158.
118. E.M. LEDERBERG, L.L. CAVALLI-SFORZA and J. LEDERBERG, 1964. Interaction of streptomycin and a suppressor for galactose fermentation in E. coli K-12. P.N.A.S., 51: 678-682.
119. I. BARRAI, L.L. CAVALLI-SFORZA and M. MAINARDI, 1964. Testing a model of dominant inheritance for metric traits in man. Heredity, 19: 651-668.
120. A.W.F. EDWARDS and L.L. CAVALLI-SFORZA, 1964. Reconstruction of evolutionary trees. Systematic Association Publication N.6, Phenetic and Phylogenetic Classification, 67-76.
121. L.L. CAVALLI-SFORZA and A.W.F. EDWARDS, 1964. Analysis of human evolution. Genetics Today (Proceedings of the XI International Congress of Genetics, The Hague, Netherlands, September, 1963. Vol. 2: 932-952)
122. L.L. CAVALLI-SFORZA, I. BARRAI and A.W.F. EDWARDS, 1964. Analysis of human evolution under random genetic drift. Cold Spring Harbor Symposia on Quantitative Biology, 24: 9-20.
123. I. BARRAI, L.L. CAVALLI-SFORZA and A. MORONI, 1965. Record linkage from parish books. Mathematics and Computer Science in Biology and Medicine, Medical Research Council, 51-60.
124. A.W.F. EDWARDS and L.L. CAVALLI-SFORZA, 1965. A method for cluster analysis. Biometrics, 21: 362-375.
125. G. MODIANO, A.S. BENERECETTI-SANTACHIARA, F. GONANO, G. ZEI, A. CAPALDO and L.L. CAVALLI-SFORZA, 1965. An analysis of ABO, MN, Rh, Hp, Tf and G6PD types in a sample from the human population of the Lecce Province. Ann. Hum. Genet., London, 29: 19-31.
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133. CAVALLI-SFORZA, L.L., 1967. Some old and new data on the genetics of human populations. The Alabama Journal of Medical Sciences, 3: 376-381.
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139. BARRAI, I., H.M. Cann, L.L. CAVALLI-SFORZA, P. De Nicola, 1968. The effect of parental age on rates of mutation for hemophilia, and evidence for differing mutation rates for hemophilia A and B. Amer. J. Hum. Genet., 20: 175-196.

140. CAVALLI-SFORZA, L.L. L.A. Zonta, F. Nusso, L. Bernini, W.W.W. De Jong, P. Meera Khan, A.K. Ray, L.N. Went, M. Siniscalco, L.E. Nijenhuis, E. Van Loghem and G. Modinao, 1969. Studies on African Pygmies. I. A pilot investigation of Babinga Pygmies in the Central African Republic (with an analysis of genetic distances). *A.M. J. Hu. GEN.*, 21: 252-274.
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143. SANTACHIARA-BENERECETTI, A.S., G. Modiano e L.L. CAVALLI-SFORZA, 1968. Studio de Alcuni Polimorfismi Enzimatici dei Globuli Rossi nei Pigmei Babinga. *Atti A.G.E.*, 12: 99-101.
144. CAVALLI-SFORZA, L.L., 1968. Teaching of biometry in secondary schools. *Biometrics*, 24(3): 736-740.
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149. CAVALLI-SFORZA, L.L., 1969. Human diversity. *Proc. XII Intern. Congr. Genet., Tokyo, Japan, 1968*, 3: 405-416.
150. RIMOIN, D.L., T.J. Merimee, D. Rabinowitz, L.L. CAVALLI-SFORZA and V.A. McKusick, 1969. Peripheral subresponsiveness to human growth hormone in the African Pygmies. *New England Journal of Medicine*, 281: 1383-1388. (December, 1969).
151. CAVALLI-SFORZA, L.L., 1968. Studi sulla struttura genetica di una popolazione italiana. *Le Scienze*, 1(4): 7-19.

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153. MERIMEE, T.J., D.L. Rimoin, D. Rabinowitz, L.L. CAVALLI-SFORZA and V.A. McKusick, 1969. Metabolic studies in the African Pygmy. Unpublished.
154. PAOLUCCI, A.M., M.A. Spadoni, V. Pennetti and L.L. CAVALLI-SFORZA, 1969. Serum free amino acid pattern in a Babinga Pygmy adult population. Amer. J. Clin. Nutrition, 22(12): 1652-1659.
155. MALCOLM, L.A., P.B. Booth and L.L. CAVALLI-SFORZA, 1971. Inter-marriage patterns and blood group frequencies in the Bundi people of the New Guinea highlands. Human Biology, 43(2).
156. BARRAI, I., L.L. CAVALLI-SFORZA and A. Moroni, 1969. The prediction of consanguineous marriages. Japan Journal of Genetics, 44(1): 230-233.
157. CAVALLI-SFORZA, L.L., 1969. Genetic drift in an Italian population. Scientific American, 221(2): 30-37.
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159. BARRAI, I., L.L. CAVALLI-SFORZA and A. Moroni, 1969. Demography and genealogy. I. Family reconstitution by computer. World Conference on Records and Genealogical Seminar, Salt Lake City, Utah, 5-8 August, 1969.

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165. KIDD, K. K. and L. L. CAVALLI-SFORZA, 1971. Number of characters examined and error in reconstruction of evolutionary trees. In Mathematics in the Archaeological and Historical Sciences. Eds. F. R. Hodson, D. G. Kendall, and P. Tautu. Edinburgh University Press, Edinburgh. 335-346.
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167. CAVALLI-SFORZA, L. L., and W. BODMER. 1971. THE GENETICS OF HUMAN POPULATIONS. W. H. Freeman and Company, San Francisco.
168. AMMERMAN, A., and L. L. CAVALLI-SFORZA. 1971. Measuring the rate of spread of early farming in Europe. Man, ~~in press~~ 6:674-688
169. AMMERMAN, A. J., and L. L. CAVALLI-SFORZA, 1971. A population model for the diffusion of early farming in Europe. Proceedings of the Research Seminar in Archaeology and Related Subjects, Sheffield, Dec. 13-17, 1971. In press.
170. KIDD, K. K., P. ASTOLFI, L. L. CAVALLI-SFORZA, 1971. Error in the reconstruction of evolutionary trees. In GENETIC DISTANCE, Ed., J. F. Crow, in press.
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173. SKOLNICK, M. H., A. MORONI, and L. L. CAVALLI-SFORZA, 1971. The population structure of Parma Valley, Italy. IV Int. Congr. Human Genetics, Paris, Proceedings. ~~Abstract~~ ABSTRACT.
174. BODMER, W. F., and L. L. CAVALLI-SFORZA, 1971. Variation in fitness and molecular evolution. Proceedings of the Sixth Berkeley Symposium on Mathematical Statistics and Probability, Conference on Evolution, April, 1971. ~~In press.~~ June 1973
175. CAVALLI-SFORZA, L. L., 1971. Il carico genetico nell'uomo. Atti A. G. I., 16:
176. ZEI, G., A. MORONI, and L. L. CAVALLI-SFORZA. 1971. Age of consanguineous marriages. In "Genetique et Populations. Hommage a Jean Sutter." Institut National d'Etudes Demographiques. Travaux et Documents. Cahier n°60. Presses Universitaires de France. 147-153.
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Int. Congress Human Genet., Paris, Proceedings. In press.
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models of schizophrenia. Neurosciences Research Program Bulletin. ~~In~~
~~press~~. *NOVEMBER, 1972*
180. CAVALLI-SFORZA, L. L., 1972. Book review (David Rosenberg) in Psychiatry.
181. SGARAMELLA-ZONTA, L., and L. CAVALLI-SFORZA, 1972. A method of the
detection of a demic cline. Proceedings, Workshop on Population
Structure, Hawaii. In press.
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inheritance. I.: Group mean and within group variation. Theoretical
Population Biology. In press.
183. CAVALLI-SFORZA, L. L., and C. CANNINGS, 1972. Human Population Structure.
Advances in Human Genetics. Ed. K. Hirschhorn.
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Testing, Munksgaard, Copenhagen, 1972.
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Wesley Module, Addison-Wesley, Reading, Mass. In press.
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Ed. V. Cappelletti. In press.
187. CAVALLI-SFORZA, L. L., 1972. Some current problems of human population
genetics. Am. J. Hum. Gen. In press.
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submission to Theoretical Population Biology.

BIOGRAPHICAL SKETCH

FD-302 (Rev. 1-25-60)
Department of Pediatrics

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME CHRISTIANSEN, Robert O.	TITLE Assistant Professor of Pediatrics	BIRTHDATE (Mo., Day, Yr.) November 10, 1936
PLACE OF BIRTH (City, State, Country) San Francisco, California U.S.A.	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Stanford University Stanford University School of Medicine	B.A. M.D.	1958 1961	Basic Medical Sciences Medicine

HONORS

MAJOR RESEARCH INTEREST Endocrine Biochemistry	ROLE IN PROPOSED PROJECT
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RESEARCH SUPPORT *(See instructions)*

1. Support from September 1969 to September 1972: HD02147 (principal investigator: Norman Kretchmer, M.D., Ph.D.) Program Grant in Human Development, from which I was allocated \$25,000 per year, which supported ongoing projects herein described.
2. Current Research Support: HD06868-01, Biochemical Studies of Male Sexual Maturation, from 9/1/72 to 8/31/75. Budget for 9/1/72 to 8/31/73 is \$26,342.
3. Patient care costs for ongoing projects are derived from RR-70, General Clinical Research Centers Branch, and RR-00-81-11, Premature Infant Clinic Research Centers Branch, both of NIH.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List 2 or most representative publications. Do not exceed 3 pages for each individual.)*

Assistant Professor of Pediatrics, Division of Metabolism, Stanford Medical School, 1969-present

Post-doctoral Fellow, Department of Biochemistry & Molecular Biology, Cornell University, Ithaca, New York, 1967-1969

Post-doctoral Fellow in Metabolism, Department of Pediatrics, Stanford Medical School, 1967

Chief Resident in Pediatrics, Stanford Medical School, 1966

Post-doctoral Fellow in Metabolism, Department of Pediatrics, Stanford Medical School, 1965-1966

Resident in Pediatrics, Stanford Medical School, 1964-1965

Pediatrician, USAH, La Chapelle Saint Mesmin, France, 1962-1964

Intern, University of Utah Medical School, Salt Lake City, Utah, 1961-1962

Societies: American Association of University Professors
 American Association for the Advancement of Science
 Society for Pediatric Research
 Western Society for Pediatric Research
 The Endocrine Society
 Society for Developmental Biology

Licensure: National Board of Medical Examiners, 1962
 State of California, 1964
 Diplomate, American Board of Pediatrics, 1967

Awards: Special Fellowship, American Cancer Society, 1967-1969

1. Loyter, A., R.O. Christiansen, and E. Racker. Respiratory control in submitochondrial particles and Ca⁺⁺ transport. Biochem.Biophys.Res.Comm. 29:450, 1967.
2. Christiansen, R.O., L.A. Page, and R.E. Greenberg. Glycogen storage in a hepatoma: Dephosphophosphorylase kinase defect. Pediatrics 42:694, 1968.
3. Christiansen, R.O., A. Loyter, and E. Racker. Effect of anions on oxidative phosphorylation in submitochondrial particles. Biochem.Biophys.Acta 180:207, 1969.
4. Loyter, A., R.O. Christiansen, H. Steensland, J. Saltzgaber, and E. Racker. Energy-linked ion translocation in submitochondrial particles. I. Ca⁺⁺ accumulation in submitochondrial particles. J.Biol.Chem. 244:4422, 1969.
5. Christiansen, R.O., A. Loyter, H. Steensland, J. Saltzgaber, and E. Racker. Energy-linked ion translocation in submitochondrial particles. II. Properties of submitochondrial particles capable of Ca⁺⁺ translocation. J.Biol.Chem. 244:4428, 1969.
6. Loyter, A., C. Burstein, R.O. Christiansen, and E. Racker. The polarity of the mitochondrial membrane, Proceedings of the Bari Symposium, B.B.A. Library, Adriatica Editrice Bari, p. 235, 1970.
7. Greenberg, R.E., and R.O. Christiansen. The critically ill child. XV. Hypoglycemia. Pediatrics 46:915, 1970.
8. Loyter, A., R.O. Christiansen, and E. Racker. The two sides of the mitochondrial membrane in B. Chance (ed.), Johnson Foundation Symposia: "Probes of structure and function of macromolecules and membranes", p. 407, Academic Press, New York, 1971.
9. Monn, E. and R.O. Christiansen. Adenosine 3',5'-monophosphate phosphodiesterase: Multiple molecular forms. Science 173:540, 1971.
10. Monn, E. and R.O. Christiansen. "Guanylate kinase in man: Multiple molecular forms", Human Heredity 22:18-27, 1972.
11. Johnson, J.D., R.O. Christiansen, and N. Kretchmer. "Lactose synthetase in mammary gland of the California sea lion". Biochem.Biophys.Res.Comm., 47:393-397, 1972.
12. Monn, E., M. Desautel and R.O. Christiansen. Highly specific testicular adenosine 3',5'-monophosphate phosphodiesterase associated with sexual maturation, Endocrinology 91:716-720, 1972.
13. Johnson, J.D., Hansen, R.C., Albritton, W.L., Werthemann, V. and R.O. Christiansen. Hypoplasia of the anterior pituitary and neonatal hypoglycemia., J.Ped., in press.

Abstracts:

1. Christiansen, R.O., L.A. Page, and R.E. Greenberg. Analysis of glycogen storage in hepatoma: dephosphophosphorylase kinase defect. J.Pediat. 69:933, 1966.
2. Christiansen, R.O., and R.E. Greenberg. Hypoglycemia due to diminished hepatic gluconeogenesis. Proc.West.Soc.Pediat.Res. 14:79, 1967.
3. Christiansen, R.O., and R.E. Greenberg. Regulation of gluconeogenic enzymes in the newborn period. Soc.Pediat.Res.Abs. 37:158, 1967.
4. Greenberg, R.E., R.O. Christiansen, R.N. Remen, A.F. Kohrman, and G. Reaven. Physiologic mechanisms in infantile hypoglycemia. Pediat.Res. 1:292, 1967.
5. Christiansen, R.O., and A. Loyter. Respiratory control and calcium binding in submitochondrial particles. Fed.Proc. 27:527, 1968.
6. DiJeso, F., R.O. Christiansen, H. Steensland, and A. Loyter. Localization of inner mitochondrial membrane components. Fed.Proc. 28:663, 1969.
7. Christiansen, R.O., W.J.W. Fan, D.A. Belenky, and L.A. Page. Effect of

- chlorpropanide in central and nephrogenic diabetes insipidus. Amer. Pediat. Soc. & Soc. Pediat. Res. Abs., p. 192, 1970.
8. Christiansen, R.O., J.D. Johnson, J. Mellema, and G. Reaven. Severe prolonged neonatal hypoglycemia, nodular hyperplasia of the pancreas and hyperinsulinism. Amer. Pediat. Soc. & Soc. Pediat. Res. Abs., p. 193, 1971.
 9. Christiansen, R.O., E. Monn and M. Desautel. Highly specific isozyme of cyclic nucleotide phosphodiesterase associated with sexual maturation. Clin. Res., 20: 252, 1972.
 10. Hansen, R., J. Johnson, W. Albritton, R.O. Christiansen and V. Werthemann. Absent anterior pituitary and neonatal hypoglycemia, Clin. Res., 20:254, 1972.
 11. Christiansen, R.O. and M. Desautel. Highly specific testicular isozyme of cyclic nucleotide phosphodiesterase associated with sexual maturation., Ped. Res. 6:349, 1972.
 12. Christiansen, R.O., E. Monn and M. Desautel. Highly specific isozyme of phosphodiesterase associated with male sexual maturation. Abstracts, IV Int. Cong. Endo., 4:536, 1972.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

NAME Alan M. DUFFIELD		TITLE Research Associate	BIRTH-DATE (Month, Year, Day) December 16, 1936	
PLACE OF BIRTH (City, State, Country) Perth, Western Australia		PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) Australian, Permanent resident Temporary Visa	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female	
EDUCATION (Begin with baccalaureate training and include postdoctoral)				
INSTITUTION AND LOCATION		DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Western Australia		B. Sc (1st Class Hons)	1958	Organic Chemistry
University of Western Australia		Ph.D.	1962	Organic Chemistry
HONORS				
MAJOR RESEARCH INTEREST Applications of mass spectrometry to Biology and Biomedical Problems		ROLE IN PROPOSED PROJECT Organic Chemist/mass spectroscopist		
RESEARCH SUPPORT (See instructions) N/A				

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all of most representative publications. Do not exceed 3 pages for each individual.)

- 1970 - Research Associate, Department of Genetics, Stanford University School of Medicine
- 1969 - Head of the Mass Spectrometry Laboratory, Chemistry Department Stanford University
- 1965 - 69 Research Associate, Department of Chemistry, Stanford University
- 1963 - 65 Postdoctoral Fellow, Department of Chemistry, Stanford University
- 1962 - 63 Postdoctoral Fellow, Department of Biochemistry, Stanford University School of Medicine.

PUBLICATIONS SINCE 1971

1. An Application of Artificial Intelligence to the Interpretation of Mass Spectra. Mass Spectrometry, B.W.G. Milne, Ed., John Wiley and Sons, New York, 1971, pp. 121-178
By B. G. Buchanan, A. M. Duffield and A. V. Robertson

2. Mass Spectrometry in Structural and Stereochemical Problems. CCIV. Spectra of Hydantoins. II. Electron Impact Induced Fragmentation of some Substituted Hydantoins.
Org. Mass Spectr., 5, 551 (1971)
By R. A. Corral, O. O. Orazi, A. M. Duffield and C. Djerassi
3. Electron Impact Induced Hydrogen Scrambling in Cyclohexanol and Isomeric Methylcyclohexanols.
Org. Mass Spectr., 5, 383 (1971)
By R. H. Shapiro, S. P. Levine and A. M. Duffield
4. Derivatives of 2-Biphenylcarboxylic Acid.
Rev. Roumain. Chem., 16, 1095 (1971)
By A. T. Balaban and A. M. Duffield
5. Alkaloide aus *Evonymus europaea* L.
Helv. Chim. Acta, 54, 2144 (1971)
By A. Klásek, T. Reichstein, A. M. Duffield and F. Santavý
6. Studies on Indian Medicinal Plants. XXVIII. Sesquiterpene Lactones of Enhydra Fluctuans Lour. Structure of Enhydrin, Fluctuanin and Fluctuadin.
Tetrahedron, 28, 2285 (1972).
By E. Ali, P. P. Ghosh Dastidar, S. C. Pakrashi, L. J. Durham and A. M. Duffield
7. The Electron Impact Promoted Fragmentation of Aurone Epoxides.
Org. Mass Spectr., 5, 199 (1972)
By B. A. Brady, W. I. O'Sullivan and A. M. Duffield
8. The Determination of Cyclohexylamine in Aqueous Solutions of Sodium Cyclamate by Electron Capture Gas Chromatography.
Anal. Letters, 4, 301 (1971)
By M. D. Solomon, W. E. Pereira and A. M. Duffield
9. Computer Recognition of Metastable Ions. Nineteenth Annual Conference on Mass Spectrometry, Atlanta, 1971, p. 63
By A. M. Duffield, W. E. Reynolds, D. A. Anderson, R. A. Stillman, Jr. and C. E. Carroll
10. Spectrometrie de Masse. VI. Fragmentation de Dimethyl-2,2-dioxolanes-1,3-Insatures.
Org. Mass Spectr., 5, 1409 (1971)
By J. Kossanyi, J. Chuche and A. M. Duffield
11. Chlorpromazine Metabolism in Sheep. II. In vitro Metabolism and Preparation of 3H-7-Hydroxychlorpromazine.
Journées D'Agressologie, 12, 333 (1971)
By L. G. Brooks, M. A. Holmes, I. S. Forrest, V. A. Bacon, A. M. Duffield and M. D. Solomon
12. Mass Spectrometry in Structural and Stereochemical Problems. CCXVII. Electron Impact Promoted Fragmentation of O-Methyl Oximes of Some α,β -Unsaturated Ketones and Methyl Substituted Cyclohexanones.
Canadian J. Chem., 50, 2776 (1972)
By Y. M. Sheikh, R. J. Liedtke, A. M. Duffield and C. Djerassi

ADAYAPALAM T. GANESAN

Born: Madras State, India. May 15, 1932.

Citizenship: U. S. Citizen

Married Ann K. Cook, Ph.D. (Stanford University, Department of Genetics),
Research Associate, Department of Biology, Stanford University.

Education: 1947-1951 Annamalai University, Madras State, India. Physics,
Chemistry, English.
B. S. (1951) Botany.
1951-1953 Annamalai University, Madras State, India.
M. A. (1953) Plant Physiology and Genetics
1959-1963 Stanford University, Department of Genetics, Palo Alto,
California (National Institutes of Health Trainee under
Professor Joshua Lederberg).
Ph. D. (1963) Genetics. Thesis title: Physical and
Biological Studies on Transforming DNA from B. subtilis.

Professional Experience:

Research Fellow (1953-55), Department of Biochemistry, Indian Institute of
Science, Bangalore, India. Awarded Institute Fellowship.

Research Associate (1955-57), Botany Department, Indian Agricultural Research
Institute, New Delhi, India. Responsible for plant tissue culture,
teaching of genetics course (laboratory and lectures).

Fellowship (1957-59). Awarded by Rask Ørsted Foundation of Denmark for study at:

Department of Physiology, Carlsberg Laboratory, Copenhagen,
Denmark. Worked under Professor O. Winge, F.R.S. (fermentation
genetics and some aspects of cytology).

Department of Physiological Chemistry, Carlsberg Laboratory,
Copenhagen. Worked under Professor Holter (physiology of sporu-
lation in yeasts).

Department of Genetics, University of Copenhagen. Neurospora
genetics and methods.

Research Associate (1963-65), Department of Genetics, Stanford University, Palo
Alto, California. Assistant Professor (1965-70); Associate
Professor (1970-present).

Special Fields of Research Interest: DNA Replication and Recombination Mechanisms
in B. subtilis; in vitro Synthesis of Biologically Active DNA.
Repair of DNA Molecules and its genetics. Phage DNA Biosynthesis.
Cytogenetics of Eukaryotic Cells.

Recent Publications:

Studies on in vitro Replication of B. subtilis DNA. Cold Spring Harbor Symp.
Quant. Biol. 33:45 (1968).

ATP Dependent Synthesis of Biologically Active DNA by Azide Poisoned Bacteria.
Proc. Natl. Acad. Scie. 68:1296 (1971).

DNA Synthesis in Bacteriophage SPO-1 Infected Bacillus subtilis. J. Virology
9:263 (1972), with C. O. Yehle.

A Deoxyribonucleic Acid Polymerase I Deficient Mutant of B. subtilis. J. Biol.
Chem. 247:5867 (1972), with P. J. Laipis.

In vitro Repair of X-irradiated DNA Extracted from a Polymerase I Deficient
Bacillus subtilis. Proc. Natl. Acad. Sci. (November-1972), with P. J. Laipis.
69: 2211, 1972

In vitro DNA Synthesis and Function of DNA Polymerases in Bacillus subtilis
in "DNA Synthesis in vitro". Ed. R. D. Wells and R. B. Inman, University
Park Press, Baltimore, Maryland (1972), with C. O. Yehle and P. J. Laipis.

Source of Funds: NIH, General Medical Sciences
NIH Research Career Development Award (GM-50199)

Title of Project: DNA Replication and Recombination in Bacillus subtilis

Principle Investigator: A. T. Ganesan

Grant Number: GM 14108, GM-50199 (Award through November 1975)

Vol. 26, No. 5

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)	
Terence John Gribble	Assistant Professor	April 6, 1937	
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX	
Cardiff, Wales	U.S.A.	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female	
EDUCATION (Begin with baccalaureate training and include postdoctoral)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of the South, Sewanee, Tenn.	B.S.	1959	Chemistry
Stanford University, Stanford, California	M.D.	1964	
HONORS			
Sigma Pi Sigma, Physics Honor Society (1958); Phi Beta Kappa (1959); Alpha Omega Alpha; Faber Pediatric Award (1964); Borden Student Research Award (1964); Dernham Senior Fellow (1970). SOCIETIES: Western Soc. for Pediat. Research (1968); Amer. Soc. Hematolo			
MAJOR RESEARCH INTEREST		ROLE IN PROPOSED PROJECT	
Pediatric Hematology - Hemoglobin Synthesis		Investigator	
RESEARCH SUPPORT (See instructions)			

Dernham Senior Fellow, American Cancer Society, July 1, 1970 - June 30, 1973
Current year \$20,690. Total \$60,989.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List a or most representative publications. Do not exceed 3 pages for each individual.)

1969 to present - Assistant Professor of Pediatrics, Stanford University
1969 - Chief Resident in Pediatrics, Stanford University
1968 - Research Fellow in Pediatric Hematology, Stanford University
1966 - 1968 - U.S. Public Health Service (NIH) National Heart Institute, Bethesda, Maryland.

Publications (Selected)

Walters, T.R., Gribble, T.J., and Schwartz, H.C.: Heme Synthesis in Normal and Leukemic Leukocytes. *Nature* 197:1213, 1963.

Gribble, T.J. and Schwartz, H.C.: Effect of Protoporphyrin on Hemoglobin Synthesis. *Biochem. Biophys. Acta* 103:333, 1965.

Gribble, T.J., Comstock, T.J., and Udenfriend, S.: Collagen Chain Formation and Proline Hydroxylation in Monolayer Tissue Cultures of L-929 Fibroblasts. *Arch. Biochem. Biophys.* 129:308, 1969.

Comstock, J.P., Gribble, T.J., and Udenfriend, S.: Further Studies on the Activation of Collagen Proline Hydroxylation in Cultures of L-929 Fibroblasts. *Archives Biochem. and Biophys.* 137:115, 1970.

Lincoln, D.R., Edmunds, D.J., Gribble, T.J., and Schwartz, H.C.: Studies on the Hemoglobins of Pinniped. In press, January 1973, *Blood*.

Tune, B.M., Leavitt, T.J., and Gribble, T.J.: The Hemolytic-Uremic Syndrome in Children: Review of Twenty-Eight Non-Reparitized Cases With Long Term Follow-up. Accepted for publication in *J. of Pediatrics*

Use continuation pages and follow the same general format for each person.

NAME Leonard A. Herzenberg	TITLE Professor of Genetics	BIRTHDATE (Mo., Day, Yr) Nov. 5, 1931
PLACE OF BIRTH (City, State, Country) Brooklyn, New York	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Brooklyn College, Brooklyn, New York	A.B.	1952	Biology, Chemistry
California Institute of Technology Pasadena, Calif.	Ph.D.	1955	Biochemistry, Immunology
Pasteur Institute, Paris, Postdoctoral Fellow		1957	

HONORS

Phi Beta Kappa, Sigma Xi
Distinguished Alumnus Award, Brooklyn College, 1970
Genetics Study Section, National Institutes of Health

MAJOR RESEARCH INTEREST

Immunogenetics, somatic cell genetics

ROLE IN PROPOSED PROJECT

Principal Investigator

RESEARCH SUPPORT (See instructions)

N.I.H. GM-17367, Automated Cell Sorting-Clinical & Biological Uses, \$160,802 current year direct costs, total funds for project \$585,977 (1/1/73-12/31/75).
N.I.H/ AI-08917, Genetics of Immunoglobulins, \$52,774 current year direct costs, total funds for project \$246,386 (5/1/69-4/31/74).
N.I.H. CA 04681, Genetic Studies with Mammalian Cells, \$80,994, current year direct costs, total funds for project \$424,981 (9/1/72-8/31/77)
N.I.H. HD 01287, Fetal-maternal Immunological Interactions, \$35,000 current year direct costs, total funds for project \$101,572 (5/1/70-4/30/73)

RESEARCH AND OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1969-present Stanford University School of Medicine, Stanford, California, Professor of Genetics
1964-1969 Stanford University School of Medicine, Stanford, California, Asso. Professor of Genetics
1959-1964 Stanford University School of Medicine, Stanford, California, Assistant Professor of Genetics
1957-1959 National Institutes of Health, Bethesda, Maryland. Officer, USPHS (Dr. Harry Eagle)
1955-1957 Pasteur Institute, Paris, France (Prof. Jacques Monod, American Cancer Society Postdoctoral Fellow)
1952-1955 Ph.D. California Institute of Technology, Pasadena, California (Major: Biochemistry, Prof. H.K. Mitchell; Minor: Immunology, Prof. R.D. Owen)
1948-1952 A.B. Brooklyn College, New York

1. Mitchell, H. K. and Herzenberg, L.A.
Enzymatic degradation of cytochrome C
Colowick, S.P. and Kaplan, H. O. (eds.)
Methods of Enzymology 60:167-69, Academic Press, N.Y., 1955.
2. Mitchell, H. K. and Herzenberg, L.A.
Zone electrophoresis on sponge rubber
Analytical Chemistry 29:1229, 1957.
3. Herzenberg, L.A. and Herzenberg, L.A.
Adaptation to lactose
Nutrition Reviews 17:65-67, 1959.
4. Herzenberg, L.A.
Studies on the induction of beta-galactosidase in a cryptic strain
of escherichia coli
Biochimica et Biophysica Acta 31:525-38, 1959.
5. Herzenberg, L.A.
Discussion of genetics of somatic cells (Bar Harbor, Maine, Sept. 1960)
Burdette, W. J. (ed)
Methodology in Mammalian Genetics, p. 462-63, Holden-Day, Inc.,
San Francisco, 1963.
6. Herzenberg, L.A. and Roosa, R.A.
Nutritional requirements for growth of a mouse lymphoma in cell culture.
Experimental Cell Research 21:430-38, 1960.
7. Herzenberg, L.A.
Isolation and identification of derivatives formed in the course of
intracellular accumulation of thiogalactosides by escherichia coli.
Archives of Biochemistry and Biophysics 93:314-15, 1961.
8. Cann, H.M. and Herzenberg, L.A.
Detection and localization of H-2 antigen in cells grown in culture.
American Journal of Diseases of Children 102:477, 1961. (Abstract)
9. Herzenberg, L.A.
Chemical and serological characterization of purified H-2 antigens.
"Mechanisms of Immunological Tolerance" Proceedings of a Conference
of the Czechoslovak Academy of Science, Prague (Nov. 1961) p. 495-99,
1962.
10. Herzenberg, L.A. and Herzenberg, L.A.
Association of H-2 antigens with the cell membrane fraction of
mouse liver.
Proceedings of the National Academy of Sciences 47:762-67, 1961.

12. Herzenberg, L. A.
Histocompatibility antigens and tissue transplantation.
American College of Physicians, Annual Meeting, April 1962.
13. Herzenberg, L. A.
A genetic and immunologic approach to the purification of an histocompatibility antigen.
Leone, C. A. (ed.)
The Effects of Ionizing Radiations on Immune Processes, Gordon and Breach Science Publishers, N. Y., 1962.
15. Cann, H. M. and Herzenberg, L. A.
In vitro selection for isoantigenic variants of mammalian somatic cells.
American Journal of Diseases of Children, p. 533, 1962. (Abstract)
- 16 &
17. Herzenberg, L. A.
Part I. Steps toward a genetics of somatic cells in culture.
Part II. Maternal isoimmunization as a result of breeding in the mouse.
Journal of Cellular and Comparative Physiology, Suppl. 1, 60:145-57, 1962.
18. Roosa, R. A., Bradley, T. R., Law, L. W. and Herzenberg, L. A.
Characterization of resistance to amethopterin, 8-azaguanine and several fluorinated pyrimidines in the murine lymphocytic neoplasm, p. 388.
Journal of Cellular and Comparative Physiology, 60:109-26, 1962.
19. Wunderlich, J. R. and Herzenberg, L. A.
A second gamma globulin isoantigen (allotype) in the mouse.
Genetics 47:995, 1962 (Abstract).
20. Cann, H. M. and Herzenberg, L.A.
In vitro studies of mammalian somatic cell variation I. Detection of H-2 phenotype in cultured mouse cell lines.
Journal of Experimental Medicine, 117:259-65, 1963.
21. Cann, H. M. and Herzenberg, L. A.
In vitro studies of mammalian somatic cell variation. II. Isoimmune cytotoxicity with a cultured mouse lymphoma and selection of resistant variants.
Journal of Experimental Medicine , 117:267-84, 1963.
22. Mishell, R. I., Herzenberg, L. A. and Herzenberg, L.A.
Leukocyte agglutination in mice, Detection of H-2 and non H-2 isoantigens.
Journal of Immunology, 90:628-33, 1963.
23. Herzenberg, L. A. Tachibana, D. K., Herzenberg, L.A. and Rosenberg, L.T.
A gene locus concerned with hemolytic complement in mus musculus.
Genetics 48:711-15, 1963.

24. Wunderlich, J. and Herzenberg, L.A.
Genetics of a gamma globulin isoantigen (allotype) in the mouse.
Proceedings of the National Academy of Sciences 49:592-98, 1963.
25. Herzenberg, L. A., Rosenberg, P. and Herzenberg, L. A.
Gamma globulin isoantigens (allotypes) in the mouse.
Genetics 48:892, 1963. (Abstract).
26. Herzenberg, L.A., Mishell, R. I. and Herzenberg, L.A.
Gamma-globulin isoantigens (allotypes) in the house mouse.
Proc. of the XI International Congress of Genetics, The Hague,
The Netherlands, Sept. 1963. I:196. (Abstract).
27. Erickson, R. P. Herzenberg, L.A. and Goor, R.
Partial immune elimination of homologous red blood cells in mice.
Transplantation 2:175-82, 1964.
28. Herzenberg, L. A. and Cole, L. J.
Presence of donor specific gamma-globulins in sera of allogeneic
mouse radiation chimeras.
Nature 202:352-53, 1964.
29. Erickson, R. P., Tachibana, D.K. Herzenberg, L. A. and Rosenberg,
L. T.
A single gene controlling hemolytic complement and a serum antigen in
the mouse.
Journal of Immunology 92:611-15, 1964.
30. Papermaster, B. W. and Herzenberg, L.A.
In vitro selection of an isoantigenic variant from a cultured mouse
lymphoma heterozygous at the H-2 locus.
Genetics 50:274
31. Herzenberg, L.A.
Study of the H-2 locus in murine cell cultures
Krooth, R. S. (ed.)
Somatic Cell Genetics, p. 140-166, University of Michigan Press,
Ann Arbor, 1964.
32. Herzenberg, L. A.
A chromosome region for gamma_{2A} and beta_{2A} globulin h chain
isoantigens in the mouse.
Cold Spring Harbor Symp. Quant. Biol. 29:455-64, 1964.
33. Herzenberg, L. A., Warner, N. L., and Herzenberg, L.A.
Immunoglobulin isoantigens (allotypes) in the mouse.
I. Genetics and cross-reactions of the 7S gamma_{2A} isoantigens
controlled by alleles at the Ig-1 locus.
Journal of Experimental Medicine 121:415-38, 1965.
34. Warner, N. L. and Herzenberg, L. A., Cole, L.A. and Davis, W. E., Jr.
Dissociation of skin homograft tolerance and donor type gamma globulin
synthesis in allogeneic mouse radiation chimaeras.
Nature 205:1077-79, 1965.

35. Papermaster, B. W. and Herzenberg, Leonard A., 1966. Isolation and characterization of an isoantigenic variant from a heterozygous mouse lymphoma in culture. *Journal of Cell Physiology* 67:407-20, 1966.
36. Herzenberg, L. A., 1966, H-2 and immunoglobulin isoantigens (allotypes) in somatic cell genetics. (Antigenic variation of somatic cells and genetics cytogenetic aspects of cell antigenicity-introduction), p. 363-65. In Jan Klein, Marta Vojtiskova and Valdimire Zeleny (Genetic Variations in Somatic Cells, Proc. Symp. Mutational Process. Praha, August 9-11, 1965. Academia (Czechoslovakian publisher).
37. Herzenberg, L. A. and Herzenberg, L.A. 1966. Suppression of a γ G-Globulin allotype in mice by anti-allotype antibodies, pp. 227-32. In Jan Klein, Marta Vojtiskova and Vladimire Zeleny (ed.), Genetic Variation in Somatic Cells, Proc. Symp. Mutational Process, Praha, August 9-11, 1965. Academia (Czechoslovakian publisher)
38. Warner, Noel L., Herzenberg, L. A., and Goldstein, Gideon, 1966. Immunoglobulin isoantigens (allotypes) in the mouse. II Allotypic analysis of three γ G₂ myeloma proteins from (NZB X BALB/c)F hybrids and of normal γ G₂ globulins. *Journal of Experimental Medicine* 123:707-721, 1966.
39. Warner, Noel L., Herzenberg, Leonard A., 1966. Immunoglobulin isoantigens (allotypes) in the mouse. III. Detection of allotypic antigens with heterologous antisera. *Journal of Immunology* 97:525-31, 1966.
40. Herzenberg, Leonard A, and Warner, Noel L., 1967. Genetic control of mouse immunoglobulins, p. In B. Cinader (ed.), Regulation of the Antibody Response, Chapter XV. C. C. Thomas, Springfield, Illinois, 1967.
41. Tyan, Marvin L., Cole, Leonard J., and Herzenberg, Leonard A., 1967. Fetal liver cells: A source of thymus-dependent specific immunoglobulin production in radiation chimeras. *Proceedings of the Society of Experimental Biology and Medicine* 124:1161-63, 1967.
42. Klein, Jan, Martinkova, Jitka, and Herzenberg, Leonard A., 1967. Analysis of the histo-compatibility-2 (H-2) locus of NZB mice. *Transplantation* 5:1335-37, 1967.
43. Klein, Jan and Herzenberg, Leonard A., 1967. Congenic mouse strains with different immunoglobulin allotypes. I. Breeding scheme, histocompatibility tests and kinetics of γ G_{2A} globulin production by transferred cells for C3H. S. W. and its congenic partner CWB/5. *Transplantation* 5:1484-1495, 1967.
44. Welton, John, Walker, Samuel R., Sharp, Gordon C, Herzenberg, Leonard A., Wistar, Richard, and Creger, William P., 1968. Macroglobulinemia with bone destruction. Difficulty of distinguishing between macroglobulinemia and myeloma. *American Journal of Medicine* 44:280-88, 1968.

45. Warner, Noel L., and Herzenberg, Leonard A., 1967
Immunoglobulin isoantigens (allotypes) in the mouse. IV.
Allotypic specificities common to two distinct immunoglobulin
classes.
Journal of Immunology 99: 675-678, 1967.
46. Minna, John D., Iverson, G. Michael, and Herzenberg, Leonard A., 1967.
Identification of a gene locus for γG_1 immunoglobulin H chains and its
linkage to the H chain chromosome region in the mouse.
Proceedings of the National Academy of Sciences 58: 188-194, 1967.
47. Tyan M. L., Cole, L. J., and Herzenberg, Leonard A., 1967.
Fetal liver cells: A source of specific immunoglobulin production
in radiation chimeras, pp. 87-89. In J. Dausset, J. Hambrugger,
G. Mathe(Eds.) *Advance in transplantation*, Proceedings First Inter-
national Congress Transplantation Society, Paris, June 27-30, 1967.
(Munksgaard, Publisher).
48. Herzenberg, Leonore A., Herzenberg, Leonard A., Goodlin, Robert C.,
and Rivera, Edna C., 1967. Immunoglobulin synthesis in mice:
Suppression by anti-allotype antibody.
Journal of Experimental Medicine 126: 701, 1967.
49. Herzenberg, Leonard A., Minna, John D., and Herzenberg, Leonore A.,
1967.
The chromosome region for immunoglobulin heavy chains in the mouse:
Allelic electrophoretic mobility differences and allotype suppression.
Cold Spring Harbor Symposia on Quantitative Biology 32: 181-86, 1967.
50. Herzenberg, Leonard A., McDevitt, Hugh O., Herzenberg, Leonore A.,
1968.
Genetics of antibodies.
Annual Review of Genetics, 1968.
51. Tyan, M.L. and Herzenberg, L.A., 1968.
Ontogeny of the Mouse Immune System. II. Immunoglobulin-producing
Cells.
Journal of Immunology, 101: 446-450, 1968.
52. Tyan, Marvin L., McDevitt, Hugh O., and Herzenberg, Leonard A., 1968.
Genetic control of the antibody response to a synthetic polypeptide:
transfer of response with spleen cells or lymphoid precursors.
Abstract and paper submitted to 2nd International Transplantation
Congress, New York, Sept. 1968.
53. Herzenberg, Leonard A., and Tyan, Marvin, 1968.
Genetics of antibody formation: role of the thymus in the evolution
of the immune response. 12th International Congress of Genetics,
Tokyo, Japan, August 19-28, 1968.
54. Tyan, Marvin L. and Herzenberg, Leonard A., 1968.
Immunoglobulin production by embryonic tissues: thymus independent.
Proceeding of Society for Experimental Biology and Medicine, 128:
952-954, 1968.

55. Woods, Roy and Herzenberg, Leonard A.
Specificities common to mouse IgG and IgA molecules.
(In preparation).
56. Lanzerotti, Richard M. and Herzenberg, Leonard A.
Population of antibodies recognizing distinct allotypic specificities
in mouse immunoglobulin. V.
(In preparation).
57. Herzenberg, Leonard A., Pernis, B., and Kelus, A.S.
A second locus controlling rabbit heavy chain allotypes on the
Fd fragment of a second-class of immunoglobulin.
(In preparation).
58. Herzenberg, Leonard A., Carbonara, A., Tosi, R., and Pernis, B.
Comparison of a locus allotypic specificities in IgG, IgA, and
IgM in the rabbit.

NAME H. Russell Hulett	TITLE Research Associate	BIRTHDATE (Mo, Day, Year) May 5, 1920
PLACE OF BIRTH (City, State, Country) Nespelem, Washington	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Oregon State College, Corvallis, Ore.	B.S.	1941	Chemistry
Oregon State College, Corvallis, Ore.	M.S.	1942	
Stanford University, Stanford, Cal.	Ph.D.	1964	

HONORS Membership in:
 Phi Lambda Upsilon
 Pi Mu Upsilon
 Sigma Xi

MAJOR RESEARCH INTEREST Biomedical instrumentation, origin of life.	ROLE IN PROPOSED PROJECT Instrumentation development, investigation of specific cell separations, evaluation
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RESEARCH SUPPORT (See instructions)
 N/A

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List of most representative publications. Do not exceed 3 pages for each individual.)

- 1966 - present: Research Associate, Stanford University
- 1964 - 1968: Associate Professor, Head, Department of Chemistry, College of Notre Dam
- 1965 - 1966: Department Head, Itek Corporation
- 1959 - 1964: Research Director, Advanced Technology Labs
- 1957 - 1959: Subsystem Manager, Lockheed
- 1952 - 1957: Electronics Department Head, Detroit Controls
- 1951 - 1952: Electronics Department Head, Santa Barbara Research
- 1946 - 1951: Electronic Engineer, Hughes Aircraft
- 1942 - 1946: U. S. Army

Publications: (selected -- since 1965)

Hulett, H. R. and H. S. Loring. "Reactions of metals with tobacco mosaic virus." Federation Proceedings. 23:1943 (1965)

Hulett, H. R. and H. S. Loring. "Effect of particle length distribution on infectivity of tobacco mosaic virus." Virology 25:418 (1965)

Hulett, H. R. "Turbulence limitations in photographic resolution of planet surfaces." J. Opt. Soc. Am. 57:1335 (1967)

Ehrlich, P. and H. R. Hulett. "Living on capital." New Scientist 38:426 (1968)

Hulett, H. R. "Limitations on prebiological synthesis." J. Theor. Biol. 21:58-72 (1968)

Hulett, H. R., Bonner, W. A., Barrett, J. and L. A. Herzenberg. "Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence." Science 166: 747-749 (1969)

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

- Hulett, H. R. "Optimum world population." *Bioscience*. 20:160 (1970)
- Hulett, H. R. "Non-enzymatic hydrolysis of adenosine phosphates." *Nature* 225:1248 (1970)
- Hulett, H. R., Coukell, A., and W. Bodmer. "Tissue typing instrumentation using the fluorochromatic cytotoxicity assay." *Transplantation* 10:135 (1970).
- Hulett, H. R. and L. A. Herzenberg. "Approaches to prescreening with special consideration of flow systems." *Acta Cytologica* (in press)
- Hulett, H. R. and L. A. Herzenberg, W. Bonner and R. L. Wolf. "Rapid cell sorter -- a new tool for cell study with clinical applications." *Laboratory Investigations* 22:501 (1970).
- Hulett, H. R. "Shock synthesis of amino acids in simulated primitive atmosphere." *Science* 170=1000 (1970). Letter to the Editor.

Seven patents on electro-optical devices and instrumentation

Publications after 1970:

- Merrill, J. T., N. Veizades, H. R. Hulett, P. L. Wolf and L. A. Herzenberg. "An Improved Cell Volume Analyzer" *Rev. Sci. Instr.* 42, No. 8, 1157-1163 (1971).
- Bonner, W. A., H. R. Hulett, R. G. Sweet, and L. A. Herzenberg. "Fluorescent Cell Sorting" *Rev. Sci. Instr.* 43, 404 (1972).
- Hulett, H. R., R. G. Sweet, L. A. Herzenberg. "Development and Application of Rapid Cell Sorter," to be presented at O.R.N.L. symposium on Advanced Analytical Methods for the Clinical Laboratory, Mar. 15, 1973 (and later published in *Clinical Chemistry*.)
- Hulett, H. R. "Formaldehyde and Ammonia as Precursors to Prebiotic Amino Acids." *Science* 174, 1038, 1971.

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

Department of ...
Principal Investigator, ...

NAME John D. Johnson	TITLE Assistant Professor	BIRTHDATE (Mo., Day, Yr.) September 14, 1938
PLACE OF BIRTH (City, State, Country) Palo Alto, California	PRESENT NATIONALITY (if non-U.S. citizen, indicate kind of visa and expiration date) U.S.A.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Wabash College, Crawfordsville, Ind.	B.A.	1960	Zoology
Stanford Medical School, Stanford, Calif.	M.D.	1965	Medicine
Johns Hopkins Hospital, Baltimore, Md.		1965-67	Pediatrics, 2 yea
Stanford Medical School, Stanford, Calif.		1970	Post-Doctoral Fe Developmental Bi

HONORS
Phi Beta Kappa, Alpha Omega Alpha, Borden Award for Undergraduate Research (Stanford Medical School)

MAJOR RESEARCH INTEREST Developmental Biochemistry, bilirubin metabolism	ROLE IN PROPOSED PROJECT Investigator
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RESEARCH SUPPORT (See instructions)
United Cerebral Palsy Associations, Inc. Grant R-245-71,
Developmental Aspects of Heme Protein Catabolism
Current \$28,772. July 1, 1971 to June 30, 1973 Total \$53,322.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List 6 or most representative publications. Do not exceed 3 pages for each individual.)

- 1971 to present - Assistant Professor of Pediatrics, Stanford University
- 1971 to present - Assistant Director of Newborn Service, Stanford University
- 1970 - Post-Doctoral Fellow, Department of Pediatrics, Stanford University
- 1967-- 1969 - Research Associate, NICHD
- 1960 - 1963 - Pre-Doctoral Fellow, Department of Pediatrics, Stanford University

Publications

Johnson, J.D., Hurwitz, R., and Kretchmer, N.: Utilization of fat and glycerol for glycogenesis by the neonatal rat. *J. Nutrition*, 101, 299, 1971.

Johnson, J.D., Jant, B.A., Kaufman, S., and Sokoloff, L.: Effect of ionic strength on the RNA polymerase activities of isolated nuclei and nucleoli of rat liver. *Arch. Biochem. Biophys.*, 142, 489, 1971.

Johnson, J.D., Christiansen, R.O., and Kretchmer, N.: Lactose synthetase in mammary gland of the California Sea Lion. *Biochem. Biophys. Res. Comm.* 47, 393, 1972.

Johnson, J.D., Albritton, W.L., and Sunshine, P.: Hyperammonemia accompanying parenteral nutrition in newborn infants. *J. Pediatrics* 81, 154, 1972.

Johnson, J.D.: Neutral hetero- β -galactosidase from the small intestine of the rabbit. Submitted for publication to *Biochim. Biophys. Acta*.

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Judith P. Koehler, M.D.	TITLE Assistant Professor of Neurology and Pediatrics	BIRTHDATE (Mo., Day, Yr.) April 27, 1939	
PLACE OF BIRTH (City, State, Country) New York, New York	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S. Citizen	SEX <input type="checkbox"/> Male <input checked="" type="checkbox"/> Female	
EDUCATION (Begin with baccalaureate training and include postdoctoral)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Mount Holyoke College	A.B.	1960	
Dartmouth Medical School	B.M.S.	1962	
University of Pennsylvania	M.D.	1966	
HONORS American Academy of Neurology (Junior Member)			
MAJOR RESEARCH INTEREST Pediatric Neurology	ROLE IN PROPOSED PROJECT		
RESEARCH SUPPORT (See instructions)			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

- 1966-1967 Intern, Mixed Pediatrics, Montefiore Hospital and Medical Center, Bronx, N.Y.
 1967-1968 Junior Resident, Neurology, Albert Einstein College Hospital and Bronx Municipal Hospital
 1968-1970 Senior Resident, Neurology, Albert Einstein College Hosp. and Bronx Municipal Hospital Center, New York.
 1969-1971 Postdoctoral Fellow, Anatomy, College of Physicians and Surgeons, Columbia Univ., N.Y.
 1971-1972 Fellow in Pediatric Neurology, Columbia-Presbyterian Medical Center, N.Y.
 1972- Assistant Professor of Neurology and Pediatrics, Stanford Medical Center.

Publications (selected)

- Koehler, J.P., Lovelace, R.E., Spiro, A.J.: Basement membrane and capillary endothelial alterations in hypokalemic periodic paralysis: an electron microscopic study. (Abstract) Presented at the American Association of Neuropathologists, 47th Annual Meeting, June 1971.
 Koehler, J.P., Spiro, A.J., Lovelace, R.E.: I. Thyrotoxic periodic paralysis: light and electron microscopic observations of nerve and muscle. (In preparation).
 Koehler, J.P. and Chuzorian, A.: Tissue culture of medulloblastoma cells from spinal fluid, a useful diagnostic technique. (In preparation).
 Spiro, A.J., Koehler, J.P., Taylor, J.M.: Oculopharyngeal dystrophy: ultrastructural and histochemical observations of skeletal muscle. (Abstract) Presented at the American Association of Neuropathologists, June 1972.
 Koehler, J.P., Duffy, P.E., Carter, S. (1973) Selective type I fiber atrophy in childhood neuropathy: light and electron microscopic observations of peripheral nerve, Schwann cells, neuromuscular junction, and muscle. (In preparation).

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator.
Use continuation pages and follow the same general format for each person.)

NAME Norman Kretchmer	TITLE Harold K. Faber Professor of Pediatrics	BIRTHDATE (Mo., Day, Yr.) January 20, 1923
PLACE OF BIRTH (City, State, Country) New York, N.Y.	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.A.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Cornell University	B.S.	1944	Animal Physiology
University of Minnesota	M.S.	1945	Physiological Chem.
University of Minnesota	Ph.D.	1947	Physiological Chem.
Downstate Medical Center, State Univ. of	M.D.	1952	

HONORS Commonwealth Fund Fellow, 1952, 57, 65 President, Int. Organ. Study Human Development, 1969; President, Soc. Ped. Res., 1968;
Mead Johnson Award, 1958; Council, Amer. Soc. Clin. Invest., 1964-65;
Borden Award, 1969 Alumni Medallion SUNY, 1969.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT Investigator
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RESEARCH SUPPORT (See instructions)

RR 81 - Clinical Research Center for Premature Infants, 10/1/69-9/30/74
Amount current year: 418,532 - Total amount: 2,021,300- (5%) renewal pending

HD 02147 - Biochemical Studies of Development, 6/1/66-5/30/73,
Amount current year: 206,593 - Total amount: 1,309,278- (15%) renewal pending.

CRBS 252 - National Foundation - Cellular and Molecular Determinants of Morphogenesis
Amount current year: \$27,273 - Total amount: 54,546 (7/1/71-6/30/73)- (15%)

HD 00049 - Human Development and Pediatrics Training Grant, 7/1/70-6/30/74,
Amount current year: \$90,588 - Total amount: 448,936.- (10%)renewal pending

(Please see continuation on attached sheet)

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List 2 or most representative publications. Do not exceed 3 pages for each individual.)

1971 to present - Harold K. Faber Professor of Pediatrics

1969 - 1972 - Chairman, Program in Human Biology (Baccalaureate Prog., Stanford Univ)

1959 - 1969 - Professor and Executive Head, Pediatrics

1953 - 1959 - Assistant then Associate Professor, Dept. of Peds, Cornell Univ.
New York Hospital

1950 - 1955 - Lecturer, Department of Biology, Brooklyn College

1948 - 1952 - Research Associate, Dept. of Pathol., State Univ. of New York

1947 - 1948 - Assistant Professor, Departments of Pathology and Biochemistry, Univ. of Vermont.

From 1952 - Various clinical appointments from Intern to Pediatrician-in-Chief

Publications (Six relevant publications are listed from a total of 101)

Kretchmer, N.: Lactose and lactase: A historical perspective. *Gastroenterology* 61:805, 1971.

Levine, R.L., Hoogenraad, N.J., and Kretchmer, N.: Regulation of activity of carb amyl-phosphate synthetase from mouse spleen. *Biochemistry* 10:3694, 1971.

Lebenthal, E., Sunshine, P., and Kretchmer, N.: Effect of carbohydrate and corticosteroids on activity of α -glucosidases in intestine of the infant rat. *J. Clin. Invest.* 51:1244, 1972.

Kretchmer, N., Ransome-Kuti, O., Hurwitz, R., Dungy, C., and Alakija, W.: Intestinal absorption of lactose in Nigerian ethnic groups. *The Lancet* 2:392, 1971.

Biographical Sketch of Dr. Norman Kretchmer

Page Two

Research Support, continued

- HD 00391 - Regulation of enzyme action during development,
9/1/68-8/31/73
Amount current year: 42,289 - total amount: 183,822 (15%)

Publications, continued

- Weichsel, M.E., Jr., Hoogenraad, N.J., Levine, R.L., and Kretchmer, N.
Pyrimidine biosynthesis during development of rat cerebellum
Pediatric Res. 6:682, 1972.
- Johnson, J.D., Christiansen, R.O., and Kretchmer, N. Lactose synthetase
in mammary gland of the California sea lion. Biochem. Biophys.
Res. Commun. 47:393, 1972.

Note: Renewal of HD-02147 is pending. This present renewal application,
HD 00391, in part overlaps with some material included in HD-02147.

BIOGRAPHICAL SKETCH (Give the following information for each KEY professional staff member, whether or not salary is requested. Begin with the Program Director.)		APPLICATION NUMBER (Leave blank)	
NAME (Last, first, initial) Luigi Luzzatti, M.D.	TITLE Professor of Pediatrics and Community & Preventive Medicine	BIRTHDATE (Mo., Day, Yr.) 9/6/14	
PLACE OF BIRTH (City, State, Country) Rome, Italy	PRESENT NATIONALITY (If non-U.S. citizen, indicate visa symbol) U.S. Citizen	SEX <input checked="" type="checkbox"/> MALE <input type="checkbox"/> FEMALE	SOC. SEC. NO. 570-42-7539
RELATIONSHIP TO PROPOSED PROGRAM Investigator			
EDUCATION (Begin with baccalaureate training and include postdoctoral)			
ORGANIZATION AND LOCATION	DEGREE	YEAR CONFERRED	DISCIPLINE
University of Minnesota, Mpls, Minn.	M.S.	1942	
University of Minnesota, Mpls, Minn.	M.D.	1943	
HONORS			
MAJOR RESEARCH OR PROFESSIONAL INTEREST (If applicable) Congenital Defects and Cytogenetics			
LIST RECENT RELEVANT PUBLICATIONS Miles, C.P., Luzzatti, L., Storey, S. and Peterson, C.D.: A male pseudo-hermaphrodite with a probable XO/XY mosaicism. Lancet, Sept. 1, 1962, 455. Luzzatti, L.: Failure to thrive - a diagnostic approach. Postgrad. Med. 35:270-8, March 1964. Peterson, C.D. and Luzzatti, L.: The role of chromosome translocation in the recurrence risk of Down's syndrome. Pediat. 35:463-9, March 1965. Weichsel, M.E., Jr., and Luzzatti, L.: Trisomy 17-18 syndrome with congenital extrahepatic biliary atresia and congenital amputation of the left foot. J. Ped. 67:324-7, Aug. '65			
PROFESSIONAL AND/OR RESEARCH EXPERIENCE (Start with present position and list recent significant experience relevant to program) (publications cont.) 1972- Professor of Pediatrics & Community and Preventive Medicine 1967- Director, Birth Defects Center, Stanford Univ. School of Medicine 1963- Director, Cytogenetic Lab., Stanford Univ. School of Medicine 1957-72 Associate Prof. of Pediatrics and Preventive Medicine, Stanford Univ. Sch. of Me 1955-67 Director, Pediatric Outpatient Dept., Stanford Univ. School of Medicine 1954-57 Assistant Prof. of Pediatrics and Preventive Medicine, Stanford Univ. Sch. of Me 1953-54 Director of Cerebral Palsy Training Program, Children's Hospital of San Francisco			

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Publications (continued)

- Craig, A., and Luzzatti, L.: Translocation in De Lange's syndrome? Lancet 2:445-6, August 1965.
- Craig, A., and Luzzatti L.: Translocation in trisomy D syndrome. A case of probable D/18 translocation. J. Pediat. 70:264-9, Feb. 1967.
- Greenstein, R.M., Harris, D.J., Luzzatti, L. and Cann, H.M.: Cytogenetic analysis of a boy with the XXXY syndrome: origin of the X-chromosomes. Pediatrics, 45:677-686, April 1970.
- Knight, L., Sakaguchi, S., and Luzzatti, L.: Unusual mechanism of transmission of a chromosome translocation from mother to offspring. American J. Dis. of Children 121:162-167. Feb. 1971.
- Knight, L., and Luzzatti, L.: Replication patterns of X & Y chromosomes in partially synchronized human lymphocyte cultures. Chromosoma, Vol. 40, 153-166, 1973.

Use continuation pages and follow the same general format for each person.

NAME Wilfred E. PEREIRA	TITLE Research Associate	BIRTHDATE (Mo., Day, Yr.) June 23 1936
PLACE OF BIRTH (City, State, Country) Madras, S. India	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) Indian, Permanent Resident Immigrant Visa	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Madras Medical College, Madras, India	B. Pharm	1960	Pharmaceutical Chemistry
Saugar Univ, Madhya Pradesh, India	M. Pharm.	1962	Pharm. Chem & Chem of Nat
U.C. Med. Center, San Francisco, Calif	Ph.D.	1968	Pharm. Chem & Pharmacolog

HONORS

MAJOR RESEARCH INTEREST Identification of Metabolites & drug metabolites in Biological fluids	ROLE IN PROPOSED PROJECT Organic chemist
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RESEARCH SUPPORT (See instructions)

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1968 - 1970 Post Doctoral Fellow, Dept. of Genetics Stanford University Med. School
 1970 - present Research Associate same institution
 During these four years I have been involved with peptide synthesis, amino acid analysis and synthetic organic chemistry. I helped develop methods for the separation of diastereoisomers by gas chromatography and have been involved with the routine use of gas chromatography mass spectrometry for the identification of urinary metabolites in normal and pathological urine and serum samples. My applications of mass spectrometry have included the development of mass fragmentography for the determination of the amino acid contents of soil and ~~xxxxxx~~ serum. My present project involves the screening of urine from leukemic patients for abnormal metabolites and to investigate the metabolic fate of anti-leukemic chemotherapeutic agents in the body.

PUBLICATIONS

1. Transesterification with an Anion-exchange Resin;
W. Pereira, V. Close, W. Patton and B. Halpern,
J. Org. Chem. 34:2032 (1969).
2. Alcoholysis of the Merrifield-type Peptide-polymer Bond with an Anion Exchange Resin;
W. Pereira, V. A. Close, E. Jellum, W. Patton and B. Halpern,
Australian J. of Chem. 22:1337 (1969).

3. The Action of Nitrosyl Chloride on Phenylalanine Peptides;
W. Patton, E. Jellum, D. Nitecki, W. Pereira and B. Halpern,
Australian J. of Chem. 22:2709 (1969).
4. Abnormal Circular Dichroism of α -Amino Acid Esters;
J. Cymerman Craig and W. E. Pereira,
Tet. Let. 18:1563 (1970).
5. The Use of (+)-2,2,2-Trifluoro-1-Phenylethylhydrazine in the Optical
Analysis of Asymmetric Ketones by Gas Chromatography;
W. E. Pereira, M. Solomon and B. Halpern,
Australian J. of Chem. 24:1103 (1971).
6. The Microsomal Oxygenation of Ethyl Benzene. Isotopic, Stereochemical,
and Induction Studies;
R. E. McMahon, H. R. Sullivan, J. Cymerman Craig and W. E. Pereira,
Arch. Biochem. Biophys. 132:575 (1969).
7. The Steric Analysis of Aliphatic Amines with Two Asymmetric Centers
by Gas-liquid Chromatography of Diastereoisomeric Amides.
W. E. Pereira and B. Halpern,
Australian J. Chem. 25:667 (1972).
8. Optical Rotatory Dispersion and Absolute Configuration -XVII.
 α -Alkylphenylacetic Acids;
J. Cymerman Craig, W. E. Pereira, B. Halpern and J. W. Westley,
Tetrahedron 27:1173 (1971).
9. The Optical Rotary Dispersion and Circular Dichroism of α -Amino and
 α -Hydroxy Acids;
J. Cymerman Craig and W. E. Pereira
Tetrahedron 26:3457 (1970).
10. The Determination of Cyclohexylamine in Aqueous Solutions of Sodium
Cyclamate by Electron-capture Gas Chromatography;
M. D. Solomon, W. E. Pereira and A. M. Duffield,
Anal. Let. 4:301 (1971).

Publications continued-

11. Chlorination Studies. I. The Reaction of Aqueous Hypochlorous Acid with Cytosine; *accn*
W. Patton, V. Brown, A. M. Duffield, B. Halpern, Y. Hoyano, W. Pereira and J. Lederberg,
Biochem. Biophys. Res. Commun. 48:880 (1972).
12. The Use of R-(+)-1-Phenylethylisocyanate in the Optical Analysis of Asymmetric Secondary Alcohols by Gas Chromatography;
W. Pereira, V. A. Bacon, W. Patton, B. Halpern, and G. E. Pollock,
Anal. Let. 3:23 (1970).
13. A Rapid and Quantitative Gas Chromatographic Analysis for Phenylalanine in Serum;
B. Halpern, W. E. Pereira, M. D. Solomon and E. Steed,
Anal. Biochem. 39:156 (1971).
14. Electron-impact Promoted Fragmentation of Alkyl-N-(1-Phenylethyl)-Carbamates of Primary, Secondary and Tertiary Alcohols;
W. E. Pereira, B. Halpern, M. D. Solomon and A. M. Duffield,
Org. Mass Spectrometry 5:157 (1971).
15. Peptide Sequencing by Low Resolution Mass Spectrometry;
V. Bacon, E. Jellum, W. Patton, W. Pereira and B. Halpern,
Biochem. Biophys. Res. Commun. 37:873 (1969).
16. A Gas Liquid Chromatographic Method for the Determination of Phenylalanine in Serum;
E. Jellum, V. A. Close, W. Patton, W. Pereira and B. Halpern,
Anal. Biochem. 31:227 (1969).
17. Quantitative Determination of Biologically Important Thiols and Disulfides by Gas Liquid Chromatography;
E. Jellum, W. Patton, V. A. Bacon, W. E. Pereira and B. Halpern,
Anal. Biochem. 31:339 (1969).
18. A Study of the Electron Impact-promoted Fragmentation of Promazine Sulfoxide and Promazine Using Specifically Deuterated Analogues;
M. D. Solomon, R. Summons, W. Pereira and A. M. Duffield,
Australian J. Chem. (1973, in press).
19. The Determination of Phenylalanine in Serum by Mass Fragmentography;
W. Pereira, V. A. Bacon, Y. Hoyano, R. Summons and A. M. Duffield,
Clin. Biochem. (In press).
20. Chlorination Studies II. The Reaction of Aqueous Hypochlorous Acid with α -Amino Acids and Dipeptides;
W. E. Pereira, Y. Hoyano, R. Summons, V. A. Bacon and A. M. Duffield,
Biochem. et Biophys. Acta (In press).

SECTION II - PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Thomas C. Rindfleisch		TITLE Research Associate		BIRTHDATE (Mo., Day, Yr.) 12-10-41
PLACE OF BIRTH (City, State, Country) Oshkosh, Wisconsin, USA		PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) USA		SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female
EDUCATION (Begin with baccalaureate training and include postdoctoral)				
INSTITUTION AND LOCATION		DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Purdue University, Lafayette, Ind. California Institute of Technology, Pasadena, CA		B.S M.S Ph.D	1962 1965 Thesis to be completed. All course work completed.	Physics Physics and examinations
HONORS Purdue University, Graduated with Highest Honors, Sigma Xi.				
MAJOR RESEARCH INTEREST Space sciences, computer science and image processing			ROLE IN PROPOSED PROJECT Technical Support	
RESEARCH SUPPORT (See instructions)				

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1971-Present Stanford University Medical School, Department of Genetics, Stanford, CA.
Research Associate - Mass Spectrometry, Instrumentation research.

1962-1971 Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.

Relevant Experience:

1969-1971: Supervisor of Image Processing Development and Applications Group.

1968-1969: Mariner Mars 1969 Cognizant Engineer for Image Processing

1962-1968: Engineer - design and implement image processing computer software.

- Rindfleisch, T. and Willingham, D., "A Figure of Merit Measuring Picture Resolution," JPL Technical Report 32-666, September 1, 1965.
- Rindfleisch, T. and Willingham, D., "A Figure of Merit Measuring Picture Resolution," Advances in Electronics and Electron Physics, Volume 22A, Photo-Electronic Image Devices, Academic Press, 1966.

Thomas C. Rindfleisch
PUBLICATIONS (cont'd)

3. Rindfleisch, T., "A Photometric Method for Deriving Lunar Topographic Information," JPL Technical Report 32-786, September 15, 1965.
4. Rindfleisch, T., "Photometric Method for Lunar Topography," Photogrammetric Engineering, March 1966.
5. Rindfleisch, T., "Generalizations and Limitations of Photoclinometry," JPL Space Science Summary Volume III, 1967.
6. Rindfleisch, T., "The Digital Removal of Noise from Imagery," JPL Space Science Summary 37-62 Volume III, 1970.
7. Rindfleisch, T., "Digital Image Processing for the Rectification of Television Camera Distortions," Astronomical Use of Television-Type Image Sensors, NASA Special Publication SP-256, 1971.
8. Rindfleisch, T., Dunne, J., Frieden, H., Stromberg, W., and Ruiz, R., "Digital Processing of the Mariner 6 and 7 Pictures," Journal of Geophysical Research, Volume 76, Number 2, January 1971.
9. Rindfleisch, T., "Digital Image Processing," To be published, IEEE Special Issue, July 1972.

SECTION II - PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Schulman, Irving		TITLE Professor and Chairman Department of Pediatrics		BIRTHDATE (Mo., Day, Yr.) 2/17/22
PLACE OF BIRTH (City, State, Country) New York City, New York		PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.A.		SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female
EDUCATION (Begin with baccalaureate training and include postdoctoral)				
INSTITUTION AND LOCATION		DEGREE	YEAR CONFERRD	SCIENTIFIC FIELD
New York University, New York New York University College of Medicine, New York		B.A. M.D.	1942 1945	
HONORS Phi Beta Kappa, Alpha Omega Alpha Mead Johnson Award for Pediatric Research, 1960 President, Society for Pediatric Research, 1966				
MAJOR RESEARCH INTEREST Coagulation physiology, Hemorrhagic diseases		ROLE IN PROPOSED PROJECT		

RESEARCH SUPPORT (See instructions)

See next page

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

Professor and Chairman, Dept. of Pediatrics, Stanford University- 1972
 Professor and Head, Dept. of Pediatrics, University of Illinois - 1961 - 1972
 Professor of Pediatrics, Northwestern University - 1958-1961
 Associate Professor of Pediatrics, Cornell University - 1956-1958
 Assistant Professor of Pediatrics, Cornell University - 1952-1956
 USPHS Post-Doctoral Fellow in Pediatrics, Cornell University - 1950-1952
 Resident in Pediatrics, Bellevue Hospital -1948-1950
 Military Service - 1946-1948
 Intern, Queens General Hospital, New York City - 1945-1946

Biographical Sketch (continued)

Research Support

HD-00568-12 Hemostasis and Hemorrhagic Disease in Children

Principal Investigator 20% time
Project period 1/1/70 - 12/31/72
Total Funds (Direct) \$122,993
Current Year (Direct) \$ 15,814 1/1/72 - 5/31/72
University of Illinois

HD-07300-01 ----- \$27,426 6/1/72 - 8/31/72
Stanford University

TI-AM-05344 Training Grant in Pediatric Hematology

Program Director 20% time
Period 7/1/67 - 6/30/72
Total Funds \$263,465
Current Year \$ 54,458

*Training grant renewed at University of Illinois for five years effective 7/1/72 (\$332,600)- application for new training grant in Pediatric Hematology has been submitted from Stanford University.

Representative Bibliography

Irving Schulman, M.D.

(Past 5 Years)

1. Abildgaard, C.F., Corrigan, J.J., Seeler, R.A., Simone, J.V., and Schulman, I.: Meningococemia associated with intravascular coagulation. *Pediatrics* 40:78, 1968
2. Johnson, C.A., Abildgaard, C.F., and Schulman, I.: Coagulation studies in children with cyanotic congenital heart disease. *Lancet* 2:660, 1968
3. Seeler, R.A., Forman, E.N., Bolger, J.F., Abildgaard, C.F., and Schulman, I.: Induction of intravascular coagulation and renal cortical necrosis in rabbits by simultaneous injection of thorotrast and endotoxin. *Brit. J. Haemat.* 16:501, 1969.
4. Forman, E.N., Abildgaard, C.F., Bolger, J.F., Johnson, C.A., and Schulman, I.: Generalized Shwartzman reaction: Role of the granulocyte in intravascular coagulation and renal cortical necrosis. *Brit. J. Haemat.* 10:507, 1969.
5. Honig, G.R., Forman, E.N., Johnson, C.A., Seeler, R.A., Abildgaard, C.F., and Schulman, I.: Administration of single doses of AHF (Factor VIII) concentrates in the treatment of hemophilic hemarthroses. *Pediatrics* 43:26, 1969.
6. Honig, G.R., Abildgaard, C. F., Forman, E.N., Gotoff, S.P., Lindley, A., and Schulman, I.: Some properties of the anticoagulant factor of aged pooled plasma. *Thrombosis et Diathesis Haemorrhagica* 22:151, 1969.
7. Schulman, I., Abildgaard, C. F., Johnson, C. A. and Lindley, A.: Immuno-suppressive therapy in the management of acquired inhibitors of Factor VIII in hemophilia. *Hemophilia and New Hemorrhagic States* (Brinkhous, editor), New York, The University of North Carolina Press, 1970, p.164.
8. Johnson, C.A., Abildgaard, C.F., and Schulman, I.: Functional studies of young versus old platelets in a patient with chronic thrombocytopenia. *Blood* 37:163, 1971.
9. Corby, D.G., Zirbel, C.L., and Schulman, I.: Thrombasthenia. *Am. J. Dis. Child.* 121:40, 1971.
10. Corby, D.G., Zirbel, C.L., Gibson, M.S., and Schulman, I.: Effects of antenatal drug administration on platelet function of newborn infants. *J. Ped.* 79:307, 1971.
11. Corby, D.G., and Schulman, I.: Effect of bilirubin on unwashed platelets in experimental hyperbilirubinemia. *J.Ped.* (in press).

BIOGRAPHICAL SKETCH

DEPARTMENT OF

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Year)
Herbert C. Schwartz	Professor of Pediatrics	May 8, 1926
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
New Haven, Connecticut	U.S.A.	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Alma College, Alma, Michigan			
Illinois Institute of Technology, Chicago			
Yale University, New Haven, Connecticut	A.B.	1948	Psychology-Chemistry
State University of New York, Brooklyn	M.D.	1952	

HONORS

John and Mary Markle Scholar in Academic Medicine (1962); Visiting Prof. State Univ. Netherlands (1967); SOCIETIES: Society for Pediatric Research (1961); American Soc. Clinical Investigation (1962); American Pediatric Society (1967), etc.

MAJOR RESEARCH INTEREST

Hemoglobin Structure and Synthesis

ROLE IN PROPOSED PROJECT

Investigator

RESEARCH SUPPORT (See instructions)

U.S.P.H.S. Grant RO1 AM 12467-10, Formation of Hemoglobin and Other Hemoproteins June 1, 1968, to May 31, 1973. Current year \$28,866. Total (Years 6-10) \$145,295.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project, or most representative publications. Do not exceed 3 pages for each individual.)

1968 - 1971 Professor of Pediatrics, Stanford University
 1969 - 1971 Chairman, Department of Pediatrics, Stanford University
 1963 - 1968 Associate Professor of Pediatrics, Stanford University
 1960 - 1963 Assistant Professor of Pediatrics, Stanford University
 1958 - 1960 Research Instructor in Medicine, University of Utah
 1957 - 1958 Research Fellow in Biochemistry, University of Utah
 1955 - 1957 Research Fellow in Medicine (Hematology), University of Utah

Publications (Selected from a total of 19.)

Schwartz, H.C., Cartwright, G.E., Smith, E.L., and Wintrobe, M.M.: Studies on the synthesis of Heme from Iron and Protoporphyrin, *Blood* 14:486, 1959.

Hill, R.L., Swenson, R.T., and Schwartz, H.C.: Characterization of a Chemical Abnormality in Hemoglobin G. *J. Biol. Chem.* 235:3182, 1960.

Gribble, T.J., and Schwartz, H.C.: Effect of Protoporphyrin on Hemoglobin Synthesis. *chem. Biophys. Acta.* 103:333, 1965.

Dallman, P.R. and Schwartz, H.C.: Myoglobin and Cytochrome Response during Regenerative deficiency in the Rat. *J. Clin. Invest.* 44:1631, 1965.

Walters, T.R., Welland, F.H., Gribble, T.J., and Schwartz, H.C.: Biosynthesis of Leukemic Leukocytes. *Cancer* 20:117, 1967.

Lincoln, D., Edmunds, D.J., Gribble, T.J., and Schwartz, H.C.: Pinniped Hemoglobin. *Blood* (in press, January, 1973).

C U R R I C U L U M V I T A E

Eric M. Shooter

- Born:** April 18, 1924 Mansfield, Nottingham, England.
- Married:** Elaine Arnold (Born Dec. 22, 1924) Newhall, Burton-on-Trent.
- Children:** Annette (Born Nov. 18, 1956) Redhill, Surrey, England.
- Permanent Address:** Department of Genetics
Stanford University School of Medicine
Stanford, California 94305
- 1942-45 Natural Sciences Tripos (Part II in Chemistry)
University of Cambridge
- 1942 Exhibitioner of Gonville & Caius College, Cambridge
- 1943 Minor Scholar of same.
- 1945 B.A. (Cantab.)
- 1945-46 Research under Professor Sir Eric Rideal in the Department of
nd 1946-49 Colloid Science, Cambridge and the Davy Faraday Laboratory of
the Royal Institution, London (Proteins of the ground nut).
- 1949 M.A. (Cantab.)
- 1950 Ph.D. (Cantab.)
- 1949-50 Postdoctoral Fellowship with Dr. J. W. Williams, Department of
Chemistry, University of Wisconsin, Madison, and partly with
Dr. D. E. Green, Enzyme Institute, University of Wisconsin.
(Enzymes of the electron transport system).
- 1950-53 Senior Scientist in charge of Biochemistry, Brewing Industry
Research Foundation, Nutfield (Proteins and enzymes of barley
and other brewing materials).
- 1953-63 Lecturer in Biochemistry, Department of Biochemistry, University
College, London with Professor Ernest Baldwin. (Molecular
biology of normal and abnormal haemoglobins; protein-ion inter-
actions of ribonuclease).
- 1961-62 U.S.P.H.S. International Fellow, Department of Biochemistry,
Stanford University School of Medicine, with Professor R. L.
Baldwin (Replication of DNA).
- 1963-68 Associate Professor of Genetics, Stanford University School of
Medicine (Molecular Neurobiology). Head of Neurobiology Group,
Lt. Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine.
- 1964 D.Sc. University of London (awarded for distinguished work in
the field of Biochemistry).
- 1968 Professor of Genetics, Stanford University School of Medicine.
- 1968-present Professor of Genetics and Biochemistry, Stanford University
School of Medicine.

69. Varon, S., Nomura, J. and E. M. Shooter, 1968. Reversible dissociation of the mouse nerve growth factor protein into different subunits. *Biochemistry* 7, 1296-1303.
70. Shooter, E. M., Smith, A. P., and S. Varon, 1968. Heterogeneity of the nerve growth factor protein and its subunits. *Fed. Proc.* 27, 464.
71. Herschkowitz, N., McKhann, G. M., Saxena, S. and E. M. Shooter, 1968. Characterization of sulfatide-containing lipoproteins in rat brain. *J. Neurochem.* 15, 1181-1183.
72. Smith, A. P., Varon, S. and E. M. Shooter, 1968. Multiple forms of the nerve growth factor protein and its subunits. *Biochemistry* 7, 3259-3268.
73. Greene, L. A., Shooter, E. M. and S. Varon, 1968. Enzymatic activities of mouse nerve growth factor and its subunits. *P.N.A.S.* 60, 1383-1388.
74. McKhann, G. M. and E. M. Shooter, 1969. Genetics of Seizure Susceptibility in Basic Mechanisms of the Epilepsies, H. H. Jasper, A. A. Ward, Jr., and A. Pope, Ed., Boston, Little, Brown and Company, Chapter 24.
75. Herschkowitz, N., McKhann, G. M., Saxena, S., Shooter, E. M. and R. Herndon 1969. Synthesis of sulfatide-containing lipoproteins in rat brain. *J. Neurochem.* 16, 1049-1057.
76. Goodall, P. T. and E. M. Shooter, 1969. Changes in Heme Environment due to subunit interaction in hemoglobin. *J. Mol. Biol.* 39, 675-678.
77. Smith, A. P., Varon, S. and E. M. Shooter, 1969. Equilibria of the Nerve Growth Factor proteins and their subunits. *Fed. Proc.* 28, 897.
78. Greene, L. A., Shooter, E. M. and Silvio Varon, 1969. Subunit interaction and enzymatic activity of mouse 7S Nerve Growth Factor. *Biochemistry* 8, 3735-3741.
79. Shooter, E. M., Smith, A. P., Greene, L. A. and S. Varon, 1969. Aspects of the dissociation equilibria between 7S NGF and its subunits. Abstracts of Second International Meeting of the International Society for Neurochemistry, p. 366.
80. Waehneltd, T. V., Grossfeld, R. M. and E. M. Shooter, 1969. The solubilization and electrophoretic analysis of membrane proteins from mouse brain. Abstracts of Second International Meeting of the International Society for Neurochemistry, p. 411.

81. Smith, A. P., Greene, L. A., Fisk, H. R., Varon, S. and E. M. Shooter, 1969. Subunit equilibria of the 7S Nerve Growth Factor protein. *Biochemistry* 8, 4918-4926.
82. Shooter, E. M. and S. Varon, 1970. Macromolecular aspects of the Nerve Growth Factor proteins in *Protein Metabolism of the Nervous System*. A. Lajtha, Ed., New York, N.Y., Plenum Press, 419-438.
83. Varon, S. and E. M. Shooter, 1970. The Nerve Growth Factor proteins of the mouse submaxillary gland in *Biochemistry of Brain and Behavior*, R. E. Bowman and S. P. Datta, Ed., New York, N.Y., Plenum Press, 41-64.
84. Shooter, E. M., 1970. Some aspects of gene expression in the nervous system in *The Neurosciences; Second Study Program*, F. O. Schmitt, Ed., New York, N.Y., The Rockefeller University Press, 812-827.
85. Shooter, E. M. and S. Varon, 1971. Biological activities of the subunits of the 7S Nerve Growth Factor protein in *Cellular Aspects of Neural Growth and Differentiation*, D. C. Pease, Ed., *UCLA Forum in the Medical Sciences*, No. 14, 269-272.
86. Shooter, E. M. and Elizabeth R. Einstein, 1971. Proteins of the Nervous System in *Annual Review of Biochemistry: Vol. 40*, E. E. Snell, Ed., Annual Reviews, Inc., Palo Alto, Calif. 635-652.
87. Perez-Polo, J. R., Bamburg, J. R. and E. M. Shooter, 1971. 7S Nerve Growth Factor: a subunit containing protein. *Fed. Proc.* 30, 1194.
88. Greene, Lloyd A., Varon, S., Pilch, A. and E. M. Shooter, 1971. Sub-structure of the β subunit of the mouse 7S Nerve Growth Factor. *Neurobiology* 1, 37-48.
89. Morris, S. J., Louis, C. F. and E. M. Shooter, 1971. Separation of myelin protein on two different polyacrylamide gel systems. *Neurobiology* 1, 64-67.
90. Perez-Polo, J. R. and E. M. Shooter, 1971. Comparison of the properties of native and modified β subunits of 7S NGF. Abstracts of the Third International Meeting of the International Society for Neurochemistry, Budapest, Hungary, p. 212.
91. Shooter, E. M., Bamburg, J. R., Perez-Polo, J. R., Pilch, A. and D. Straus, 1971. Structural studies on the 7S Nerve Growth Factor protein. Abstracts of Third International Meeting of the International Society for Neurochemistry, Budapest, Hungary, p. 428.

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Philip Sunshine	TITLE Associate Professor	BIRTHDATE (Mo., Day, Yr.) June 16, 1930
PLACE OF BIRTH (City, State, Country) Denver, Colorado	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) American	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Colorado, Boulder, Colo.	B.A.	1952	Pre-Medicine
University of Colorado, Denver, Colo.	M.D.	1955	Medicine

HONORS

Alpha Omega Alpha
Ross Award for Pediatric Research 1970

Council Western Society for Pediatrics
Research, 1972

MAJOR RESEARCH INTEREST Developmental Gastroenterology and Nutrition	ROLE IN PROPOSED PROJECT Investigator
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RESEARCH SUPPORT (See instructions)

RR 81 - Clinical Research Center for Premature Infants, 10/1/69 - 9/30/74
Amt. Current Year: \$385,882. Total Amount: - \$2,380,594 (Norman Kretchmer - Principal Investigator)

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List or most representative publications. Do not exceed 3 pages for each individual.)

Associate Professor of Pediatrics	1968-present	Stanford Univ. School of Medicine
Director, Center for Premature Infants	1968-present	Stanford Univ. School of Medicine
Instructor through Assist. Professor	1963-1968	Stanford Univ. School of Medicine
Fellow in Pediatrics	1961-1963	Stanford Univ. School of Medicine
Resident in Pediatrics	1959-1961	Stanford Univ. School of Medicine

Publications (Selected)

- Sunshine, P. and Kretchmer, N.: Studies of small intestine during development. III. Infantile diarrhea associated with intolerance to disaccharides. *Pediatrics* 34: 38, 1964.
- Sunshine, P. and Kretchmer, N.: Absence of intestinal disaccharidases in two species of sea lions. *Science* 144:850, 1964.
- Herbst, J.J., Hurwitz, R., Sunshine, P. and Kretchmer, N.: Effect of colchicine on intestinal disaccharidases: Correlation with biochemical aspects of cellular renewal. *J. Clin. Invest.* 49:530, 1970.
- Sunshine, P., Herbst, J.J., Koldovsky, O. and Kretchmer, N.: Adaptation of the gastrointestinal tract to extrauterine life. *Ann. N.Y. Acad. Sci.* 176:16-29, 1971.
- Sunshine, P., et al.: Hyperammonemia due to a defect in hepatic ornithine transcarbamoylase. *Pediatrics* 50:100, 1972.

BIOGRAPHICAL SKETCHES

Prof. J. Lederberg

Department of Genetics

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Tsuboi, Kenneth K.	Senior Scientist	February 7, 1922
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Okayama, Japan	U.S.A.	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
St. Thomas College, St. Paul, Minnesota	B.S.	1944	Chem.
University of Minnesota, Minn., Minn.	M.S.	1946	Biochem.
University of Minnesota, Minn., Minn.	Ph.D.	1948	Biochem.

HONORS

Established Investigator, American Heart Association, 1960 - 1964.

MAJOR RESEARCH INTEREST Biochemistry, Enzymology, Nucleotides, Muscle Proteins	ROLE IN PROPOSED PROJECT Investigator
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RESEARCH SUPPORT (See instructions)

Contract Award: Division of Biologic Standards, N.I.H., Biochemical Parameters of Primate Cell Cultures, June 1, 1972 to May 31, 1974. Current year \$23,598. Total \$47,196.

AM 03978 Metabolism of Human Erythrocyte, June 1, 1967 - May 31, 1972. Current \$12,500. Total \$102,880.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. (Do not exceed 3 pages for each individual.)

- 1966 to present - Senior Scientist, Biochemistry in Pediatrics
- 1960 - 1966 - Associate Professor, Biochemistry in Pediatrics, Stanford Medical School
- 1957 - 1960 - Assistant Professor, Biochemistry in Pediatrics, Cornell Medical School
- 1951 - 1957 - Research Associate, Biochemistry, Columbia University Medical School
- 1948 - 1951 - Research Associate, Oncology, University of Kansas Medical School

Publications (Five relevant publications are listed from a total of 48).

- Tsuboi, K.K., Greenberg, R.E., and Kretchmer, N.: Carbohydrate Metabolism, in Biological Basis of Pediatric Practice, R.E. Cooke, ed., McGraw-Hill (1968).
- Tsuboi, K.K., Fukunaga, K., and Petriccioni, J.C.: Purification and Specific Kinetic Properties of Erythrocyte Uridine Diphosphate Glucose Pyrophosphorylase. J. Biol. Chem. **244**, 1008 (1969).
- Tsuboi, K.K., Fukunaga, K.: Relationship of solute permeability to erythrocyte glycolysis. Biochem. Biophys. Acta, **196**, 215 (1970).
- Uchino, J. and Tsuboi, K.K.: Actin Accumulation in Developing Rat Muscle. Amer. J. Physiology, **219**, 154 (1970).
- Tsuboi, K.K., Fukunaga, K., and Chervenka, C.H.: Phosphoglucose Isomerase from Human Erythrocyte Preparation and Properties. J. Biol. Chem., **246**, 7586 (1971).