Research Proposal Submitted to the National Science Foundation

by

Stanford University
School of Medicine
Stanford, California 94305

A Program in Genetics and Molecular Biology

(Renewal of GB 8739)

Principal Investigator:

Joshua Lederberg, Ph.D.
Professor and Chairman, Department of Genetics
Social Security N[redacted]

Proposed starting date: September 1, 1971 Amount requested: $79,107

Proposed duration: 36 months

Endorsements:

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Stanford University School of Medicine
Department of Genetics

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This is an application for the continued funding of certain aspects of the research program in Genetics and Molecular Biology in the Department of Genetics. As noted in our previous application, the initial award of this National Science Foundation grant enabled the department to make a smooth transition from its old quarters into the Clinical Sciences Research Building of the Medical School and to provide for several items of commonly used major equipment. The next phase was the support through the grant of certain essential departmental services and personnel used by all members of the department. These have included such diverse activities as providing partial support for the departmental research administrator, the technician in charge of the amino acid analyzer, the technician in charge of media preparation, and the supervisor of the departmental wash-up facility, as well as helping to cover maintenance costs on major equipment previously purchased on the grant and/or used jointly by the various projects, and minor renovation costs within the laboratories.

The present application is for continued funding of this type of activity. We have found that deliberate cooperative efforts in many of the above areas and potentially in at least one new one, that of a scientific computer programmer, allows much more efficient utilization of our facilities and resources and this, in turn, has had a significant impact on the momentum of our research effort.

The faculty group participating in this application consists of:

Professor Joshua Lederberg (Genetics of Bacteria; Instrumentation Research relevant to problems of molecular biology)

Professor L. L. Cavalli-Sforza (Population Genetics, Behavioral Genetics)

Professor L. A. Herzenberg (Immunogenetics and Somatic Cell Genetics)

Professor E. M. Shooter (Molecular Neurobiology)

Associate Professor A. T. Ganesan (DNA Synthesis and Genetic Recombination)

Professor Cavalli-Sforza has joined the faculty to fill the vacancy created by the departure of Professor W. F. Bodmer, who left to assume the Chair of Genetics at Oxford University during the past year.

In addition, a number of research associates of senior stature are connected with these and other programs;the details of these collaborative efforts are given under the description of the individual research activities. For example, the substantial engineering developmental program in automated instrumentation under NASA auspices has resulted in two significant advances in the main research areas of the department, namely the development of an automatic cell separator and of a cytoanalyzer for fluorochromatic cytotoxicity assay of human lymphocytes.

The Exchange Program in Genetics and Molecular Biology between Stanford University and the University of Pavia, Italy, funded in part by NSF, is also in operation and we anticipate that several Pavia faculty members and students will be visiting in our department for various periods of time over the next years.

A detailed budget for the first year of theproposed renewal follows, as well as budget outlines for the following two years.

Proposed Budget

Renewal of GB-8739

September 1, 1971 - August 31, 1972

	NSF Funded Calendar Year Man Months	Amount from NSF Grant
SALARIES AND WAGES		
1. Senior Personnel		
Principal Investigator:		
Prof. J. Lederberg	.5	none
Faculty Associates:		
Prof. L. L. Cavalli-Sforza		none
Prof. A. T. Ganesan		none
Prof. L. A. Herzenberg		none
Prof. E. M. Shooter		none
2. Other Personnel		
Scientific Programmer (J. Hwang)	3	4,000
Technical: Lab. Tech. (P. Evans)	6	4,000
Lab. Asst. (W. King)	3	2,000
		$10,000
Fringe benefits (15.2%)		1,520
Total Salaries and Benefits		$11,520
EXPENDABLE SUPPLIES AND EQUIPMENT		1,000
Materials for use in general departmental facilities: media room, wash and sterilization room, animal room; chemicals and glassware for department stores.		
OTHER COSTS		
Minor laboratory renovation		2,000
Maintenance of multi-use equipment		2,603
TOTAL DIRECT COSTS		$17,123
Indirect Costs (46% TDC)		7,877
TOTAL REQUESTED		$25,000

Projected Budget
(Renewal of GB-8739)

		1972-73		1973-74
Salaries		$10,500		$11,235
Staff Benefits	(Est. 16.7%)	1,754	(Est. 18.2%)	2,045
Total Personnel		12,254		13,280
Expendable Supplies		1,226		1,300
Other: Minor laboratory changes		1,500		1,500
Maintenance		3,000		3,000
Total Direct Costs		$17,980		$19,080
Indirect Costs (46% TDC)		8,270		8,777
Total		$26,250		$27,857

Budget Justification

The cooperative facilities established and funded in part by the previous grant have worked extremely well and we hope to continue to function in this manner. The central resource also makes possible trial ventures before the point that they can be incorporated into specific research projects.

Personnel:

In the present application we are requesting partial support for the scientific computer programmer. The scientific programmer acts as a general resource to all members of the department and because of her ready availability in the department has a profound effect on the way in which faculty, staff, students and fellows make use of our computer faciliities.

The technician in charge of media preparation services all of the biological laboratories in the department, providing media for research in bacterial genetics, tissue culture of mammalian cells and for bioassay of nerve growth factor.

The supervisor of the wash-up facilities has day-to-day charge of this function, specifying working schedules for the dishwashers and lab assistants assigned to the various laboratories, and rearranging work loads to maximize efficiency in an otherwise highly variable operation.

Expendable Supplies and Equipment:

These include materials for use in the common facilities, i.e., the media preparation room, the washing and sterilization facilities, the animal house, and for common departmental stocks of glassware, scintillation vials, chemicals and solvents.

Other Costs:

Minor laboratory alterations; relocation and rearrangement of facilities within laboratories to accommodate changing requirements of various projects.

Maintenance of shared-use equipment: the scintillation counters and ultracentrifuges are shared among all of the laboratories. Because these are in almost constant use there is a continuing requirement for service, and in the case of the ultracentrifuges, for drive replacements and rotors. Costs of maintenance are distributed among the various projects but the burden is heavy in view of the shrinking project funding and sharp increase in cost of basic maintenance contracts. Access to a central fund in the past years of this grant has considerably relieved the problem of deciding on a justifiable allocation of project funds.

Existing Facilities

The department covers some 11,000 square feet in the Clinical Sciences Research Building of the Stanford Medical Center complex, providing integrated areas for microbial, cell and human genetics, molecular neurobiology and computer work. Common facilities for wash-up and sterilization are available, as are hot and cold room facilities. The Instrumentation Research Laboratories are housed in 6,000 sq. ft. of appropriately designed areas on the ground floor beneath the Genetics Department. The department also makes large scale and effective use of the major computer facilities in the Medical School and University.

The department is equipped with all the usual apparatus for work in the fields outlined above. Major items of equipment include:

- Tricarb scintillation spectrometers
- Spinco analytical ultracentrifuge
- Spinco and IEC preparative centrifuges
- Spinco amino acid analyzer
- Zeiss spectrophotometer
- Bendix and Quadripole mass spectrometer with interfaces to the IBM 360/50 computer

Existing Funding

No other funding matches the purpose of this application; however, component elements of our investigations are supported as follows:

NIH

Grant No.	Title	Project Period	Recommended Level of Support
2 TO1 GM-295	Training Program in Genetics	1969-74	$687,177
AI-5160	Genetics of Bacteria	1968-73	$275,793
GM-14108	DNA Synthesis & Genetic Recombination in B. subtilis	1969-72	$ 89,211
GM-14650	Genetics of Human Tissue Antigens	1969-74	$125,194
NS-04270	Molecular Neurobiology	1970-75	$403,990
CA-04681	Genetic Studies with Mammalian Cells	1967-72	$272,352
AI-08917	Genetics of Immunoglobulins	1969-74	$243,198
GM-50199	Career Development - Dr. A.T. Ganesan	1971-75	$123,000
GM-17367	Automated Cell Sorting	1970-72	$225,330
69-2064	Cytotoxicity Assay Automation	1969-1971	$ 98,680
pending	Genetic Modifications of DNA Structure by Chlorine	1971-74	$166,194

Grant No.	Title	Project Period	Recommended Level of Support
NSF			
GB 29024	Exchange Program between Universities of Pavia and Stanford	1971-72	$ 41,096
GB 14458	Structure and Mechanism of Action of the Nerve Growth Factor	1969-71	$ 45,465
NASA			
NGR 05-020-004	Cytochemical Studies of Planetary Microorganisms (Instrumentation Research Laboratory)	1971-72	$240,000

L. L. Cavalli-Sforza - Population Genetics, Behavioral Genetics

1. Population genetics

a. General. In the past few years, Walter Bodmer and I have been writing a book on the "Genetics of Human Populations". I was stimulated to write such a book when I was invited to give a course on this topic at Stanford, 1960, and, on repetition in 1962, I shared that task with Walter. The book should be out in August 1971 (Freeman and Co., San Francisco). The manuscript incorporates a fair amount of research on population genetics which came about as a "side effect" of writing the book, such as an analysis of the distribution of unknown rates, factors which bias it, and how to evaluate this bias; molecular evolution and mean evolutionary times; factors of distribution of hemoglobin C and S in Africa, a study of the kinetics of a triallelic system; new methods of evaluating selection for a quantitative character, applied to birth weight as an example of stabilizing selection and to stature as an example of stabilizing and directional selection, and others. The size of the book has eventually grown beyond the originally intended size, and it is now hoped that we shall prepare a shorter version for undergraduates. Another commitment is that of preparing a review of Population Structure with C. Cannings, to appear in Rec. Adv. in Human Genetics.

b. Record linkage. A program of record linkage, based on parish books of baptisms, deaths, and marriages from the Parma Valley, was started several years ago. Only recently, thanks to the acquisition by the University of Pavia of an IBM 360/44, has it become possible to bring this program to near completion. It has thus become possible to build up genealogies to 14 generations, and the work is now extended to the entire material with a view toward obtaining demographic data of genetic interest. The computer work is being carried out by Mark Skolnick in Pavia, and, hopefully, it will be finished by June. It will then be possible to evaluate the results for usefulness in the solution of genetic and demographic problems. On the basis of this experience, we hope to apply these methods to the material existing in Salt Lake City, Utah. As this material covers almost all of Europe and some other parts of the world, it will be necessary, obviously, to choose one or more geographical areas of special interest.

c. Simulation of evolutionary trees. By simulating evolutionary trees, Ken Kidd and I have been estimating the error involved in reconstructing phylogenetic trees and are currently investigating which methods are best, and potential sources of error. Simulation data are still accumulating in Pavia where computing costs are especially low.

d. Evolutionary analysis. Ken Kidd and I, in collaboration with A. Boyce (Guildford University), are currently investigating a new method of reconstructing evolution in fossils, when some specimens are dated, although approximately. The work is carried out on data from the skulls of hominids and hominoids. It has become clear from the analysis of the data that the evolution of the human skull has constantly accelerated. This poses special methodological problems which are not solved to date.

e. Pygmy data. Data collected during the last five years are being analysed and will form the subject of an invited paper at the Paris Congress of Human Genetics, as well as of a monograph. We (Louise Wang) are currently investigating, using biological methods, the hypothesis that the tallest Pygmies may be XYY. We are also planning a campaign of yaws eradication (in which I will perhaps be unable to participate) coupled with a search for a correlation between this disease and the ABO blood groups. Preliminary data show an association in a direction opposite to that found for syphylis. It should be noted that the treponemata responsible for the two diseases are almost identical antigenically.

2. Behavioral Genetics
   a. Experiments with the transplantation of brain cells marked with H^3 thymidine into inbred mouse strains were carried out in 1968-69, and histological sections then prepared are being inspected and evaluated. If results prove satisfactory, trials will be made of transplantation to genetically defective mouse lines with neurological defects. Preliminary analysis indicates the transplantation technique may have to be modified to improve results (work done with Mike Durphy).
   b. If a polymorphism exists for given brain enzymes, and if it can be studied on cells other than the brain, it may be possible to show an association with mental disease or with peculiar behavioral attitudes. We are currently investigating (with S. Santachiara, and P. Pignatti) monoaminoxidase in brain cells, white cells, cultured cells, and other tissues, using electrophoretic techniques, and plan to extend this to other enzymes connected with the metabolism of transmitters.
   c. An analysis of substances excreted with apocrine and eccrine sweat, and with sebum, having connection with mental disease and/or peculiar behavioral states is being planned.

3. Interactions and similarities between biological and sociocultural evolution. Neolithic innovations permitting a considerable increase in population density, should have determined a population explosion, which may have been the cause of an expansion of neolithic people from the "nuclear area" in the Near East. This hypothesis might be further tested by an analysis of archaeological data at the demographic, anthropometric and other levels. Moreover, a reexamination of gene frequencies in the light of this idea shows that the genetic pattern of African populations may be explained by this theory, with very few exceptions, but a more elaborate analysis than that so far carried out will be necessary.

   An analysis of ethnographic data (mostly African) shows patterns that might be interpreted as "clones" of "mutants" (= innovations) spreading in the same way as an epidemic. This view needs further anthropological and statistical support.

VISITORS, 1971

POSTDOCTORAL:

C. Cannings
K. Kidd
P. Pignatti
S. Santachiara
N. Yasuda (1971-72, WHO Fellowship)

PREDOCTORAL:

M. Skolnick
M. Durphy (part-time, still to be decided)

RESEARCH ASSISTANTS:

A. M. Cavalli-Sforza
Louise Wang

A.T. GANESAN: DNA Synthesis and Genetic Recombination in *Bacillus Subtilis*

The aim of the project is to elucidate the basis of genetic control and biochemical mechanism of normal replication of chromosomes in *B. Subtilis* and the various steps the chromosomes go through in recombination during the process of transformation.

Chromosome replication in *B. Subtilis* was shown to occur in a particulate fraction containing "cell-wall-membrane" and enzymes capable of promoting semiconservative replication *in vitro*. The enzyme was shown to be different from *E. coli* DNA polymerase. The synthetic product, mainly short molecules, were biologically active. This system in principle demonstrated normal replication of DNA, yeilding a product free of template atoms that are genetically active. *In vivo* chromosome replicates, sequentially and semiconservatively from a fixed origin. In the above described system all small fragments of DNA acted as templates for synthesis.

To achieve a better understanding of the above process, we have devised a new approach, in which the chromosomes are kept intact along with the replicating unit, that can promote continued synthesis. Kornberg's polymerase, which interferes with the assay, has been eliminated from cells. This was accomplished by treating poisoned cells (cyanide, Azide) with nonionic detergent - Brij.58. The detergent eliminates most of the cellular proteins and DNA polymerase. The treated cells are permeable to pyrophosphates and enzymes. It is capable of continued DNA synthesis in the presence of added substrates. Normal DNA synthesis requires an additional energy requirement apart from the deoxynucleoside triphosphates supplied. This cofactor is a ribonucleoside triphosphate, in whose absence, one observes only repair process. Current studies are directed towards the nature of cofactor requirement and coupling of energy generation with chromosome replication. Here we are using several conditional lethal mutants for DNA synthesis.

We have mutants that lack Kornberg's polymerase similar to *E. coli* mutants. These can undergo recombination by transformation normally, suggesting that the known DNA polymerase may have only a very minor role, if any, in the process. The state of DNA molecules during recombination is analyzed by physical and genetic methods.

The above mentioned mutants are also used for studying phage SPO-1 infection to identify the induced enzymes that are responsible for phage DNA replication.

A.T. Ganesan:	Associate Prof. of Genetics
E. Elizur:	Visiting Prof. from Hebrew University, Israel
C.O. Yehle:	Postdoc. American Cancer Society
P. Cooper:	Postdoc. National Cancer Inst.
P.J. Laipis:	Grad-Student
J. Andersen:	Grad-Student

L. A. Herzenberg - Immunogenetics and somatic cell genetics.

Immunoglobulin loci. Four H-chain gene loci, Ig-1, Ig-2, Ig-3 and Ig-4 have been found in the mouse. corresponding to the H-chains in the classes γG_{2a}, γA, γG_{2b} and γG_L respectivly. The alleles at Ig-1 through Ig-3 were first defined serologically with alloantisera.

Detection of the reaction of alloantisera with immunoglobulin was by immunodiffusion in agar or, where more sensitive detection methods were necessary, by inhibition of precipitation of radioactive labeled antigens. The latter method was used for most of our analyses of allotypic differences.

Among some 70 inbred mouse strains, there are 8 alleles at the Ig-1 locus. These alleles display 11 distinct antigenic specificities of γG_{2a} immunoglobulins. Each allotype is cross reacting and is determined by various combinations of relatively few specificities.

Three other immunoglobulin loci closely linked to Ig-1 have been uncovered. Ig-2, Ig-3, and Ig-4 determine γA, γG_{2b} and γG_1 H-chains respectively. Ig-2 and Ig-3 were demonstrated by the same methods as Ig-1; i.e., cross reacting specificities found with alloantisera.

A fourth linked H-chain locus, Ig-4, has been defined, using allelic differences in electrophoretic mobility of γG_1 immunoglobulins and Fc fragments. Thus, 4 of the 5 H-chains in mice are controlled by closely linked genes (forming the H-chain chromosome region).

Allelic electrophoretic mobility differences have been found for the previously identified H-chain loci. Ig-1 through Ig-3 as well. One or more of the serologically defined allotypes at each locus is strictly associated with each electrophoretic mobility allotype. The use of electrophoretic mobility for allotype detection is new and of potentially great use for genetic studies of immunoglobulins.

Three heavy-chain allotypic specificities have been found on two of the mouse immunoglobulin classes, γG_{2a} and γG_{2b}. The other described immunoglobulin allotypic specificities in the mouse are restricted to one or another H-chain class. This work helps understand the possibly evolutionary origins of shared or common specificities and is of importance to theories of generation of diversity in immunoglobulins.

Use of Immunoglobulin Allotype as Functional Markers for Immunoglobulin Producing Cells and Their Precursors. The two pairs of congenic strains which we now have available after 14 backcross generations have turned out to be extremely valuable assets in the studies of cell interactions in the immune response. In both cases the strains are essentially identical except for the immunoglobulin chromosome region (and, of course, a small region of chromosome nearby). Thus cell transfer experiments can be made between mice of a pair of strains without any histoincompatibility reactions either of the graft vs. host or the host vs. graft variety. When mixtures of cells from the two strains of a pair are injected into an irradiated recipient, usually one of the congenic strains itself, the origin of the antibody producing cell precursors can be ascertained either by determining the allotype of serum antibody produced or by directly scoring the allotype of the antibody producing cell using the developed Jerne plaque assay.

In collaboration with Drs. Johanna L'age-Stehr, Ethel B. Jacobson and Graham Mitchell in this laboratory and Drs. Eva Chan and Robert Mishell in Berkeley, we have shown a number of important facts concerning the cellular basis for immunological memory and interaction of cells in the immune response. Using gradient and column fractionation methods, we have shown that antibody forming

cells are not themselves the carriers of memory but some cell not actively engaged in the process of antibody formation showed this function. These memory cells are derived from bone marow in all cases, but some have either passed through the mouse thymus or are dependent upon some function of the thymus. The two types of cells are called B cells and T cells. By depleting a suspension of spleen cells of T cells by a combination of treatment with anti θ=(θ is an alloantigen present on T cells only) and glass wool filtration, we have shown that although memory is carried by B cells, this memory cannot be expressed in the absence of some T cells. Thymus cells from an unimmunized animal will restore the capacity of a spleen cell population depleted of T cells fo give a secondary immune response both in vivo, on cell transfer into irradiated hosts, and in vitro in the Mishell-Dutton culture system. Additionally, we have shown that there is an increase in T activity for a specific antigen upon immunization. That is, thoracic duct lymphocytes or other sources of T cells (other than thymus) from immunized animals are numerically or qualitatively effective in restoring the immune response of these θ depleted spleen cell suspensions. For all this work, the congenic strains we have developed here plus the methodology of using the mouse allotypes as functional markers have been essential. This work continues.

Mouse Lysozyme and Production by a Reticulum Cell Sarcoma. One of the mineral oil induced tumors which we previously studied as sources of plasma cell tumors turned out to be a producer of lysozyme, not immunoglobulins, and morphologically is classified as a reticulum cell sarcoma. Large quantities of mouse lysozyme are produced by this tumor and structural, i.e. amino acid sequence comparisons with other lysozymes are now in progress. These will be correlated with serological comparisons between various mammalian lysozymes. --- work of Roy Riblet.

Is There a Transfer of Genetic Information in the Immune Response? Using allophenic or tetraparental chimeric mice in which two cell populations reside side by side in an animal from the 8-cell stage to maturity, Dale Hattis is investigating the title problem. The two types of cell differ by their immunoglobulin allotypes and H-2 surface alloantigens. Assays have been developed which allow scoring of individual antibody producing cells for both of these genetic markers simultaneously. Thus, any recombinant types of antibody producing cells can be detected. So far, no such recombinants have been found but statistical considerations allow us to state that genetic recombination or transfer of information is not an obligate part of the immune response but we cannot rule out that it occurs with frequencies of 10 or 20%. As more mice are tested the supper limit of such recombination can be set more precisely.

Fluorescence-activative and Volume-directed Electronic Cell Sorting. A major bioengineering effort aimed at sorting cells according to optical properties and/or volume has led to the successful completion of two separate machines for these purposes. With the fluorescence machine we are able to separate antigen reactive cells in low frequency from spleen or other lymphoid cell populations, θ positive cells and cell with immunoglobulins on their cell surfaces. Enrichments of up to several hundred-fold have already been achieved without loss of viability of the sorted cells. These cells are now being assayed for their immunological capabilities in a variety of cell transfer and in vitro cell transfer systems. The volume sorter is currently being used to separate tetraploid and binuclear cells from diploid human fibroblasts. This work, in collaboration with

Dr. Howard Cann and Dr. Jean-Jacques Cassiman in the Department of Pediatrics, should allow selection of viable cells of the desired diploid or tetraploid type for somatic cell genetic studies. The fluorescence sorter can possibly be used in this work to separate, for example, HL-A hybrids from each of the parental types of vice versa. This would be an extremely important tool in genetic analysis of the human genome in culture. Masuda, Hulett, et al.

Role of Carbohydrate Addition of Immunoglobulin Secretion. This work is being done by a visiting investigator, Dr. Fritz Melchers, in this laboratory because of the relevance of this work to other studies of myeloma proteins which have been done in this laboratory in the past. Dr. Melchers has shown that carbohydrate addition occurs in an orderly fashion in the usual course of immunoglobulin secretion by plasma cell tumor cells but that under conditions of lack of fucose or galactose, the last two sugars to be added, secretion can occur. Thus, at least the end of the carbohydrate chain is not required for secretion.

Chronic Allotype Suppression in Mice. Leonore Herzenberg has been working on allotype suppression in mice for several years and has found that suppression of synthesis of the paternal allotype is a rather short-lived consequence of exposure of fetal or newborn heterozygous mice to maternal anti paternal allotype antibody. More recently, she has found, however, that in the BALB/c x SJL strain combination suppression lasts in about half the animals for the life of the animals. Further, she has found very interestingly that suppression is caused by some induced active process. The maternal antibody apparently initiates or induces this active process which does not involve the continued production of anti paternal allotype antibody. Cells from chronically suppressed mice mixed with normal gentically identical cells producing the paternal allotype shut off production of the paternal allotype by the latter cells. We have not been able to dissociate the suppressing factor from the cells themselves, and one hypothesis to explain this phenomenon is that the animals own cells, not exposed to the paternal allotype, do not become tolerant to it but become synthesized or immunized to allotype on the surface of cells which produce it and kill all cells arising from stem cells which begin to produce this allotype. An alternative type of explanation is that some regulatory process is hyperactivated.

Leonard A. Herzenberg, Ph.D
Professor

Senior Research Associate

Leonore A. Herzenberg	-	suppression of immunoglobulin production, allotypic specificities, antiserum definition.

Postdoctoral Fellows

Ethel B. Jacobson	-	cell transfer studies with congenic mice.
Johanna L'age-Stehr	-	cell transfer studies
Graham F. Mitchell	-	cellular events in immune response as related to thymus-derived cells
Fritz Melchers	-	biosynthesis of immunoglobulin in lymphoid cells and possible regulatory phenomena involved in transport and secretion of these proteins
Tohru Masuda	-	gamma A allotype in mouse and techniques of antigen and antibody inducing cells.

Predoctoral Fellows

Timothy Coburn	-	cell separation studies
Roy Riblet	-	lysozyme-producing mouse tumor and lysozyme polymorphism
Dale Hattis	-	isopyknic centrifugation of immunoglobulins
Michel Julius	-	separation of antigen binding cells in normal and tolerant animals
Wesley Jan (Medical Student)		

Research Assistants

Marion Noble	-	thymus, bone marrow interaction experiments and suppression of immunoglobulin synthesis
Myrnice Ravitch	-	cell transfer assays and antiserum production
Derek Hewgill	-	primary assay development and antiserum production

Joshua Lederberg - Genetics of Bacteria

I. Grafting DNA Segments.

The DNA transfer system ("transformation") in Bacillus subtilis offers a particularly favorable opportunity to study the physical and chemical properties of DNA molecules in relation to their biological activity.

We are investigating the conditions needed to induce artificial insertions and inversions in B. subtilis with the aim of synthesizing new genotypes. In enteric bacteria, mapping studies have shown a remarkable congruence of gene sequences among bacterial species which have already diverged significantly in DNA homology as tested by hybridization-reanealing experiments. Conservation of gene order is also evident in the organization and persistence of operons, showing coordinate repression; these are frequent in bacteria but rare in higher forms. Most or all of the examples of inversions found experimentally in bacteria can be explained either as methodological artifacts or as consequences by crossing over of episome-bound segments with redundantly homologous segments of the whole DNA.

These observations were vague forerunners of the now accepted understanding that bacteria differ from eukaryons in 1) The simple organization of the chromosome as a single polynucleotide sequence, and 2) The absence of any common mechanism for "holo-ligation repair" of the chromosome. The clarification of the dark repair of single-strand breaks in DNA has pointed up mechanisms of template-directed repair (hemiligation) in bacteria. These are fundamental to recovery from UV-damage, to recombination mechanisms and doubtless to the post-editing of newly replicated DNA. There is, on the other hand, no good evidence for any normal mechanisms for joining or rejoining broken DNA strands except for hemi-ligation, i.e., when the broken strand is entwined with a complementary template strand that spans the broken ends.

We are then looking for holo-ligation, i.e., some way to join broken strands, or double helices, without the help of a homologous template. The potential extent and limitations of holo-ligation relate to many current problems in chemical genetics and cell biology. These include, for example:

1) Genetic variation in recovery from X-ray damage and the mechanism of recovery from double-strand scissions.

2) A sharper confrontation with chromosome structure of eukaryons in which broken chromosomes can indeed be rejoined (but is this a covalent nucleotide assembly?)

3) Transcription problems: framing, the reading of inverted sequences, and strand selection.

4) The barrier to promiscuous recombination of DNA from different sources.

Point 4 may be the most far-reaching for every level of genetic manipulation, both investigative and applied. The rejection of foreign DNA by competent Bacillus subtilis cells may illustrate one evolved mechanism whereby a cell has been able to protect itself against unlimited intrusion by foreign genes, arising by chemical scrambling or by virus infection. The research utility of freely moving genes from

another species into bacteria needs no elaboration, e.g. for the study of transcription-control, and to facilitate analyzing the genetic competence of DNA from differentiated tissues of higher organisms. The supplementation of defective human genes by graft-hybrid DNA would also lead to a therapy for genetic disease.

At present our experimental studies use bacterial DNA (a) as objects of cross-linking by chemical agents followed by enzymatic replication, and (b) as implants in frog and chick eggs to study the eukaryote's systems of DNA repair.

II. Mutagenesis by Chlorine. In association with Dr. Alan Duffield.

While investigating various ways of breaking and damaging DNA we became interested in chlorine as a possible mutagenic hazard.

Chlorine plays an indispensable role in the purification of our water supplies and urban settlement has depended upon chlorination or some equivalent means of removing polluting bacteria from water. Nevertheless we have limited information about the molecular mechanisms by which chlorine kills bacteria, and whether it might also damage mamalian cells. It has been assumed that the toxic effect of "active chlorine" is exerted through its reaction with cell membranes which are being destroyed in the process, but since hypochlorite will also inactivate certain viruses (1), other mechanisms may also be involved. The scanty published data suggests that the most likely route involves an attack of chlorine on the DNA of the microbe. This is supported by the isolation of appreciable quantities of chlorinated cytosine and uracil after hypochlorite treatment of Escherichia coli or tobacco mosaic virus and the loss of infectivity of the virus subsequent to such treatment (2,3). Additional evidence is also furnished by the mutagenic action of 5-halo-uracil, when it is incorporated into the DNA of bacteriophage T2 (3). Nevertheless chronic toxicity to man from chlorine has not been considered a hazard because the chlorinating agent is known to react rapidly with organics and the reagent is supposed to be destroyed in the body fluids. This conclusion however ignores the possibility that the chlorine may react with a variety of nitrogen containing compounds to form chloramines, which may themselves form potent chlorinating agents. Although the bactericidal potency of known chloramines is considerably less than hypochlorite itself, it is precisely the less reactive forms of chlorine that makes these agents potentially the most insidious Whilst there is an extensive literature on the stable end products of halogenation of the biologically important purine and pyrimidine bases and their nucleoside and nucleotide derivatives (4,5,6,7,8) no serious attempt has been made to look for, or characterize any of the labile chloramine intermediates which may be present in these reaction mixtures. This is somewhat surprising, as the chemistry of structurally related nitrogen heterocyclics suggests that fairly stable chloramine derivatives may be formed.

Preliminary Findings:

We have verified the formation of 5-chloropyrimidines in model experiments. The action of sodium hypochlorite (e.g. "Chlorox" solution at a dilution of 1:25; 10^{-3} molar) on the biologically interesting bases, at a physiological pH, leads to chlorine containing derivatives which behave as typical chloramines. These compounds give a positive color test with tolidine (9) and liberate iodine from potassium

iodide solution. The latter reaction can be used to quantitatively estimate the amount of labile chlorine present in the reaction mixtures. When cytosine was reacted in aqueous solution at a physiological pH with an equimolar equivalent of sodium hypochlorite 4-N-chlorocytosine was isolated in 80% yield. This compound was converted to 5-chlorocytosine which on addition of one mole of hypochlorite gave 4-N,5-dichlorocytosine. This latter compound produced the known (10) 5-chlorocytosine on mild hydrolysis. 5-Methylcytosine when reacted with aqueous hypochlorite afforded a 75% yield of 4-N-chloro-5-methylcytosine.

Specific Aims:

The elucidation of the reaction mechanisms involved in chlorination (by aqueous hypochlorite solution) of the biologically important purine and pyrimidine bases at a physiological pH will be investigated. Model compounds will be utilized where necessary and structure proofs of the products obtained will rely on classical chemical procedures supplemented by modern physical methods (e.g. NMR and mass spectral analysis). Oxidative side reactions (for instance rupture of the ribose ring of purine and pyrimidine nucleosides and nucleotides) may occur with aqueous hypochlorite and evidence for this and other side reactions will be sought.

Finally the chlorine-transfer reactions of chlorinated intermediates likely to be encountered in biological systems will be studied. Besides the N-chloro-pyrimidines already mentioned we will examine chlorinated amino acids, peptides and proteins in like fashion using simple amines and amides as model compounds where this would help elucidate reaction constants and mechanisms.

Methods of Procedure:

This research will primarily be associated with the reaction under physiological conditions of chlorine with purines and pyrimidines as free bases and in nucleotides and nucleic acids. Such chemical modifications have an obvious bearing on mutagenic hazards to genetic information within the cell. To facilitate the identification of the products obtained from these reactions a variety of modern chemical techniques will be used, combined gas chromatography-mass spectrometry (interfaced with the ACME computer system of the Stanford University Medical School) and other chromatographic techniques are available. (We have had considerable experience with such methods, see for instance publication numbers 1,2,3,4,16,18). In addition money is requested in the first year to purchase a Varian Aerograph High Pressure Liquid Chromatograph in view of the proven ability of this apparatus to separate a wide variety of purine and pyrimidine bases and of nucleotides themselves. Success has already been achieved in preliminary studies using this instrument at the Varian Aerograph Application Laboratory for the separation of 5-chlorocytosine from 4-N, 5-dichlorocytosine and 5-methylcytosine from its 4-N-chloro analog.

The High Pressure Liquid Chromatograph would be indispensible for the routine identification of the nucleotides obtained from chlorinated DNA by enzymatic hydrolysis. Chemical studies aimed at the identification of chlorinated bases from DNA would proceed concurrently with the basic chemistry involved in chlorination of single nucleosides and nucleotides.

On the biological side, we have tentative evidence that chlorine is mutagenic in microbial systems. The chemical and biological studies will be closely coordinated (a) to clarify basic issues in the mechanisms of action of chlorine in the modification of DNA and (b) to allow the formulation of critical tests for the possible transport of chloramines in body fluids to important tissue sites in animals (as models of possible human toxicity).

III. Metabolic and Environmental Monitoring. In association with Dr. Alan Duffield.

As an extension of NASA-funded work of the Instrumentation Research Laboratory we are also working on the development and application of automated methods of chemical analysis of body fluids and secretions, with a view to

(a) discovering individual differences in the metabolism of common nutrients (like tryptophan) that are already under suspicion as potential sources of mutations and of cancer, and

(b) discovering the intake of substances (food additives, drugs, and other environmental additives--including byproducts of smoking and air pollution) to which little attention has been directed so far, but which may behave like the metabolic deviations mentioned in (a). An excellent prototype of this would have been the discovery of cyclohexylamine and of its N-hydroxyl derivatives in the urine of an increasing part of the population as an index of their intake of cyclamates. There is little doubt that the procedures we are developing would have been capable of detecting such an output.

E. C. Levinthal and J. Lederberg - Relevance of Current Program of Instrumentation Research Laboratory to Problems of Molecular Biology

The activities of the Instrumentation Research Laboratory are largely supported by NASA in connection with their interest in an automated biological laboratory for the exploration of the planets. This has related to other biological interests in this department and elsewhere in the medical school in the areas of mass spectrometry, cell separation and classification, and the general question of computer managed instrumentation.

I. Mass Spectrometry. (MS)

That mass spectrometry should be a nexus of molecular and exo biology may be surprising. However, the same rationale that makes mass spectrometry a preferred tool for any studies qualifies it as a most sensitive and selective method for molecular analyses in molecular biology and medicine.

The problem of adapting MS to cytochemistry has several parts: First, there is the question of volatilizing the molecules of interest. This can be facilitated by chemical modification of particular classes of molecules. We have successfully applied this concept to problems of resolution and identification of optical isomers of aimno acids using the combination of a gas chromatograph and mass spectrometer. Molecules of biological interest are characterized by asymmetries at one or more of the carbon atoms incorporated in the molecule. From the viewpoint of the exobiologist this statement is the basis of the well-known significance of optical activity as a clue for the recognition of life. The preparation of volatile diastereoisomers, their separation by gas chromatograph and their further identification by a mass spectrometer provides a method important to both terrestrial and extraterrestrial biology. The general concept is elucidated in the papers enumerated in the bibliography. Initially the technique was applied to the high sensitivity scanning of amino acids for optical activity while other work has demonstrated the general applicability of the method. A second but much more difficult method, which has the advantage that it is more directly applicable to the goal of cytochemistry, could concievably utilize electron, heavy particle or photon beam energy. We have investigated the use of both heavy particles and laser photon beams with so far inconclusive results.

A second subdivision of this effort addresses itself to acquiring basic data on the mass spectra of a large number of monomers of biological interest. A report covering the work on amino acids has already been published. Work on nucleotides and related products is in progress.

Computer control of mass spectrometers described the third segment of the program. Full advantage of a mass spectrometer as a biological tool can only be achieved when the instrument is under computer control. The typical processes of calibration and optimization of oeprating parameters are sufficiently complex that they require automation if it is desired to analyze a large number of spectra in a short period of time. We have designed and built several effective systems for computer operation that provide a sophisticated level of control for quadrupole, time-of-flight, as well as high resolution mass spectrometers that use magnetic filters. These systems have been and are continuing to be used for biological research purposes. In the case of the quadrupole and time-of-flight systems the input to the mass spectrometer is a gas chromatograph also under computer control.

Fourthly, there is the question of data retrieval for subsequent computer analysis. The ultimate goal of a two dimensional micro-description of the distribution of molecules in a tissue by means of their mass spectra presents formidable problems of data handling. The bandwidth requirements are at least an order of magnitude greater than color video. High bandwidth data retrieval and buffer storage are required. We have not directly confronted this problem. General advances in the technology of high speed solid state switching devices and information storage methods lend some hope for the future. We have, however, made some modest steps. We have implemented an interface system for a direct data link from a high resolution mass spectrometer to an IBM 360/50 computer.

The fifth and last subdivision of the program really represents most clearly the ultimate goal for which the previously described efforts provide the technological tools. Ultimately the spectra acquired must be analyzed. This requires computer manipulation of chemical hypotheses. This poses a problem in both artificial intelligence and organic chemistry. A great deal of progress has been made in this direction. Most of this research is supported by the Advanced Research Projects Agency, Contract No. SD-183, and carried out in collaboration with Professor E. Feigenbaum of the Department of Computer Sciences.

While the high resolution mass spectrometer is perhaps the most capable single instrument for organic structural analysis, the sheer volume of its signal output poses formidable problems of data reduction and data analysis. These problems would be multiplied by the number of samples that would need to be processed by the micro-scanning mass spectrometer which is our ultimate goal. At one level, the problem is the identification of mass numbers with compositional formulas. However, no mass spectral signal is free of noise and great effort must then be spent to obtain an accurate determination of mass to ultimate resolution. Much of this effort is wasted when it does not answer a concrete question, i.e., which of a set of possible compositions is indicated by a given measurement. Even for all compositions, the corresponding mass numbers are not continuously distributed; they are rather the discrete set of numbers calculated from linear integral sums of nuclidic masses, and represented in the tables.

The tabulations and calculation programs (see bibliograph) are the first step in a control program for the mass spectrometer. As soon as the peak is identified within a given mass neighborhood, the competing possibilities should be computer, then weighted in accordance with any other available information. This allows the experimental problem to be restated as a choice among competing possibilities, and the signal information need be accumulated only long enough to lead to a meaningful choice among them.

In solving a structure, the chemist hypothesizes a series of trial structures, then matches them with the data (in this case a mass spectrum, but this can be generalized to any data set) and accepts or rejects his trial solutions, usually part by part, in a structure. Much of this tedious effort could be emulated or at least assisted by the computer in a program we call "mechanized induction".

For this purpose, a language has been devised for representing chemical structures in easily computer form: "DENDRAL 64". The development of this language required the filling of a surprising gap; the systematic application of simple topological principles to the field of chemical graphs - that is, a symbolic repre-

sentation of organic molecules. Existing notations were found to be quite defective,as the chemist already knows too well from his difficulties with nomenclature.(other organizations like Chemical Abstract Service also recognize the problem and are working on it, but tend to compromise topological rigor for the benefit of established traditions in notation). At any rate, with the help of some theorems on canonical forms of trees, and on Hamilton circuits of planar maps (for acyclic and cyclic structures respectively), a complete system has been worked out. This gives an algorithm by which the computer can generate an exact list of all isomers in a given composition.

By itself this is a futile approach to any but the simplest problems,since the number of possible isomers quickly exceeds the range of a fast computer. Heuristic and symbiotic methods are therefore called for whereby the computer emulates or cooperates in the use of human problem solving techniques in searching wisely selected parts of the space of possible solutions. Professor Feigenbaum and Dr. Richard Watson of the Computer Science Department participated in a cooperative effort to program efficient displays of structural ideas for conversational interaction with the computer. This was a step to evaluate the chemists problem solving heuristics,incorporating them in the machine program. The effort is limited at present by the existing computer facilities (a PDP-1 machine) with inadequate displays.

A complete description of the current status is given in an article by J. Lederberg and E. A. Feigenbaum. The following is the abstract of that paper.

"A computer program for formulating hypotheses in the area of organic chemistry is described from two standpoints: artificial intelligence and organic chemistry. The DENDRAL Algorithm for uniquely representing and ordering chemical structures defines the hypothesis-space; but heuristic search through the space is necessary because of its size. Both the algorithm and the heuristics are described explicitly but without reference to the LISP code in which these mechanisms are programmed. Within the program some use has been made of man-machine interaction, pattern recognition, learning, and tree-pruning heuristics as well as chemical heuristics which allow the program to focus its attention on a subproblem and to rank the hypotheses in order of plausibility. The current performance of the program is illustrated with selected examples of actual computer output showing both its algorithmic and heuristic aspects. In addition some of the more important planned modifications are discussed."

II. Cell Separation.

The high speed fluorescent cell separator has been developed into a working instrument usable with cells rendered fluorescent by a number of different methods. Much of the current work is concerned with separation of cells with distinctive immunological characteristics, using immunofluorescence techniques. A number of experimental programs in this area have been undertaken by graduate students and postdoctoral fellows associated with Professor Leonard Herzenberg of the Genetics Department. These programs include:

1. Studies on the role of theta antigen bearing cells from mouse lymphoid organs. In these studies, separated theta antigen bearing cells are implanted in irradiated mice to try to trace the role of these cells in the immune mechanism.

2. Similar studies on cells binding human serum albumin.

3. Studies on cells binding keyhole limpet hemocyanin. In these studies special attention is being paid to the phenomenon of tolerance, in an effort to see if this results from absence of antigen binding cells or from saturation of the binding sites on the cells.

In another area of interest a modification of the fluorescent cell separator has been developed for rapid automatic analysis of histocompatibility using the fluorochromatic assay. This instrument developed in connection with Professor Walter Bodmer (formerly of the Genetics Department, now at Oxford University) and Dr. Rose Payne of the Hematology Department uses cells incubated with fluorescein diacetate to develop internal fluorescence, then treated with test serum and complement. The extent of lysis is measured by the decrease in number of fluorescent cells.

Intensive effort is also under way in conjunction with Professor Paul L. Wolf of the Pathology Department, Director of the Clinical Laboratory of the Stanford University Hospital, on clinical application of the fluorescent separator. We are now investigating the possibility of developing a sensitive test for malaria, based on staining the nucleic acid of the malaria parasite with fluorescent dyes. Similar tests for other parasites also seem possible. These tests may use the instrument either as an independent assay or as a prescreening device, enriching the population of the parasite so as to simplify the job of a technician in infections with low parasite concentrations. A series of other possible clinical applications will be explored as time permits.

Engineering is proceeding on an advanced version of the separator, able to separate cells on the basis of information supplied from two optical channels simultaneously.

In cooperation with Professor Howard Cann of the Department of Pediatrics the volumetric separator is being applied to separation of tetraploid cells from diploid cells in human fibroblast culture. The tetraploid cells will then be cultured and diploids produced from these cultures studied to determine the somatic segregation characteristics of certain marker chromosomes.

E. C. Levinthal - Instrumentation Research Laboratory

A simplified version of the volumetric separator is being used as an analytical instrument to provide imporved volume distribution curves for various cell preparations. This instrument, like the others, uses a sheath flow system in which the cells are confined to a small central stream surrounded by a sheath of suspending fluid without cells. Restriction of the cell position to the center of the measuring orifice results in a much more exact relationship of signal to volume than has been the case in the instruments used previously.

Work is under way in the Applied Physics Department under Dr. Donald Winslow on the possible application of acoustic techniques, similar to those used in their acoustic microscope project, to detect differences between cell types. It is hoped that this parameter can also be used as the basis for useful cell separation.

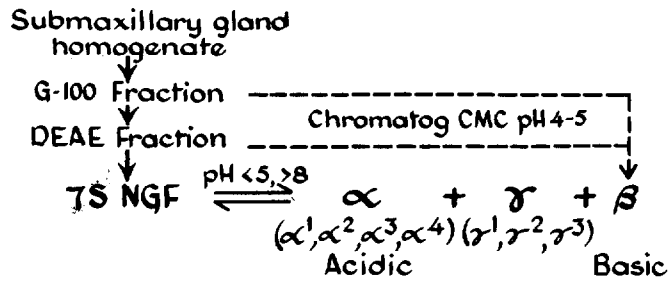
Some Aspects of Molecular Neurobiology

E. M. Shooter

The primary instructions for the development of the nervous system in terms of cell specialization, cell migration and the formation of intercellular connections are located in the genome and give rise to patterns of innate behavior. Throughout development internal and external environmental factors continue to modify gene expression in the nervous system and conversely variable gene expression may be one of the neural components which are the basis of learning. The Nerve Growth Factor protein is an agent which, at the least, accelerates fiber outgrowth from two specific types of neurons. Any understanding of how this protein achieves this result will help define some of the biochemical events in the development of the neuron and its axon. The work of Sperry and Jacobsen, among others, has illustrated the high degree of specificity with which certain synaptic connections are made. Part of this information may reside in the membranes of the nerve cell. In order to examine this hypothesis it is necessary to define both the composition of these membranes and how this composition changes with development. The second part of this program is, therefore, concerned with a study of the proteins and conjugated proteins of nervous system and other membranes.

The Nerve Growth Factor Proteins. The proteins which elicit nerve growth factor NGF activity have been the object of interest and investigation for more than a decade. The discovery by Levi-Montalcini of a factor which stimulates differentiation of certain neurons in embryonic sensory and sympathetic ganglia and its characterization as a protein were events of very considerable significance in neurobiology and their consequences are still being exploited. Soon after the protein character of NGF was established, it was found that very much more NGF activity could be obtained from the adult male mouse submaxillary gland than from the original tumor material and the gland became the preferred starting material for further investigation. The original method for isolating NGF from this gland was devised by Cohen. His preparation was optimally active at about 15 ng/ml in the in vitro plasma clot assay and sedimented at 4.3S. An alternative method was developed by Varon, Nomura and Shooter after the realization that the size of the NGF protein was critically dependent on pH. This new NGF protein was larger than that in the original preparation, was obtained in larger yield but had the same NGF potency. It was given the name 7S NGF, being defined by both its sedimentation properties and its biological activity. The continued characterization of 7S NGF and its constituent subunits revealed

that NGF activity was only one of several activities displayed by the complex. This emphasized that the 7S complex, as a major protein of the submaxillary gland, warranted study in its own right, as well as for its NGF activity. The isolation and properties of 7S NGF and its subunits are summarized below.



	7S NGF	α (Acidic)	γ (Acidic)	β (Basic)
Sp. biol. (NGF) act. ng/BU	10-20	—	—	10-20
Sp. enzymatic act. μmoles/min/mg	<3	—	180	—
Salvage factor μg/ml	0.04	3.0	—	—

The Biological Activities of 7S NGF. Because NGF activity was used as the assay to follow the isolation of 7S NGF, this was the first biological activity ascribed to the complex. The NGF potency of 10-20 ng/BU places the optimal *in vitro* response at a concentration of approximately 10^{-10}M. Besides producing the typical fiber halo around embryonic sensory of sympathetic ganglia, in the usual *in vitro* assay in a plasma drop, 7S NGF induces neurite growth in individual neuroblasts migrating on an acrylamide surface, and when injected into newborn mice, the 7S NGF complex results in a four- to six-fold increase in the size of the superior cervical ganglia. The 7S NGF, therefore, stimulates migration as well as differentiation of ganglionic neuroblasts. Higher than optimal concentrations of 7S NGF in the *in vitro* plasma clot assay produce less and less fiber outgrowth until finally no neurite halo is visible. Hoffman also noted that in the assay on acrylamide gel less response is observed at higher NGF concentrations. However, the effect at the highest concentrations was to cause cells to clump and disrupt. The nerve growth factor activity of 7S NGF is a property of the basic β subunit, both α and γ subunits being inactive in this regard.

The second activity displayed by 7S NGF is that of an esteropeptidase enzyme capable of hydrolyzing esters and to a less extent amides of substituted or free amino acids, in particular of arginine. Compared to trypsin, it shows

a much higher degree of specificity towards arginine rather than lysine containing substrates but unlike trypsin has a very low activity towards protein substrates such as casein. Only the γ subunit of the isolated three subunits of 7S NGF has this enzymatic activity. Although under typical conditions of assay 7S NGF displays a specific activity about one sixthat of the γ enzyme, it is now clear that this figure does not represent the true specific activity of the 7S species. The reasons for believing that the latter has a very low or even zero activity are discussed later. A number of enzymes with esteropeptidase activity have been reported in the mouse submaxillary gland. Gel filtration of extracts of the male gland resolves these esteropeptidases into three classes, the 7S complex containing the γ enzyme, enzymes with molecular weights in the range 60-70,000 and a third class with molecular weight of 25-30,000. Two distinct enzyme forms of the latter class have been purified and their synthesis shown to increase in female glands after testosterone treatment.

The third activity, which is a property of the acidic α subunits as well as of 7S NGF, has been termed the "salvage function". This is an ability to protect embryonic sensory ganglionic cells during dissociation and recovery from embryonic ganglia. In the absence of added 7S NGF, trypsin dissociation followed by washing and aspiration in trypsin-free media results in the recovery of a reproducible number of large neuronal and smaller non-neuronal cells from the ganglia. Addition of increasing amounts of 7S NGF, particularly to the wash solutions, allows the recovery of increasing numbers of both type of cells. The dose-recovery curve has a sigmoid shape but reaches a plateau at a particular concentration. Therefore, a quantitative measure of the salvage function can be made by estimating the concentration required for half maximal effect.

The β NGF Subunit. The β subunit elutes from G-50 Sephadex at a molecular weight approximating to that of trypsin, i.e. 24,300, and after brief exposure to sodium dodecylsulfate (SDS) shows a major migrating component on SDS-containing acrylamide gels corresponding to a molecular weight of 23,000 daltons. Continuing incubation in SDS with or without β-mercaptoethanol slowly converts this component into a second species of molecular weight 11-12,000. The conversion from the higher to the lower molecular weight form follows an apparent first order rate constant with a half life of about 2 hours at 37°. Dissociation of the native β subunit to components of approximately 11-12,000 daltons is also achieved by gel filtration on Sephadex in the presence of 6M guanidine hydrochloride, by succinylation or by reduction and carboxymethylation in the presence of denaturing agents. Since the reduction in molecular size can be achieved by denaturing agents in the absence of reducing agents it suggests that the β subunit comprises two similar chains which are not held together by disulfide bonds. Each of these individual polypeptide chains must, therefore, include three intra-chain disulfide links. That the chains are probably identical is shown by the fact that the β subunit still gives a single major band in isoelectric focusing gels or on ion exchange charomatography in the