

A PROGRAM IN GENETICS AND MOLECULAR BIOLOGY

Genetics Department

Stanford University School of Medicine

July 1, 1965 - June 30, 1968

Submitted by:

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This application is intended as a continuation of Grant No. G-6411
(expiring 6/30/65) entitled "Genetic Recombination in Bacteria".

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All of the members of this department share the need and intent to use the most advanced of the available tools of the physical sciences in furthering genetic research on various materials. These tools are both conceptual and instrumental. While each responsible investigator makes his own provision for essential funding of his research operations, the usual pattern of project funding sometimes impedes orderly planning and efficient development where our research aims and needs converge. This holds especially for analytical instruments whose use and justification must often be shared throughout the department, and also to some kinds of research assistance and other supporting personnel. Therefore, although each individual project might well benefit from additional support, these requirements are not the subject of the present application, lacking the expectation adequate funds would be available.

The faculty group participating in this application consists of:

Professor J. Lederberg	Genetic chemistry of bacteria
	Computer systems for biochemical analysis
Assoc. Professor L.A. Herzenberg	Immunogenetics and somatic cells
Assoc. Professor E. M. Shooter	Brain proteins - chemical ontogeny and polymorphism
Asst. Professor W. F. Bodmer	Genetic chemistry; leukocyte antigen polymorphism
E. Levinthal, Research Physicist	Instrumentation research

In addition, a number of research associates of senior stature play a key role in these and connected programs, including Drs. E. M. Lederberg and A. T. Ganesan (microbial genetics); S. Liebes (physics of mass spectrometry); S. Varon (neurobiology); B. Halpern and J. Westley (organic synthesis--reagents for ultramicroanalysis). A substantial engineering development program in automated instrumentation under NASA auspices is also converging with the main scientific aims of the department.

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Budget for a period of three years, beginning July 1, 1965:

<u>A. Personnel</u>	<u>Per annum</u>	<u>Over 3-year period</u>
Machinist	7,000	
Instrument designer (50% time)	4,500	
Storekeeper/expediter	<u>6,500</u>	
	18,000	
Payroll costs @ 8.5%	<u>1,530</u>	
Total Personnel	\$19,530	<u>\$ 58,590</u>
<u>B. Equipment</u>		
Tricarb Scintillation Counter		15,000
Chromatography column effluent scanner, plus basic Tricarb Scintillation Counter		10,000
Tricarb Automatic γ Counter		10,000
Spinco L2 or International P2 centrifuge		15,000
International BD2 medium centrifuge		14,000
Carey Spectropolarimeter		35,000
Virtis Freeze Drier		<u>5,000</u>
Total Equipment		<u>\$104,000</u>
<u>C. Consumable Supplies</u>		
Chemicals, glassware relating to above equipment	4,500	
Reference materials, library, etc.	<u>500</u>	
Total	\$ 5,000	<u>\$ 15,000</u>
Total Direct Costs		\$177,590
Indirect Costs at 20%		<u>35,518</u>
Total Amount Requested		<u>\$213,108</u>

Justification for Budget

Personnel:

From time to time the efficient progress of most of the research projects outlined depends on the modification of an existing piece of equipment or the manufacture of an entirely new custom made instrument. The matter may vary from fairly simple gadgets to more complicated original designs for new instruments. At the moment the services of a machinist are provided by the Instrument Research workshop on a very limited basis. The need for further help in this area on a Departmental basis is keenly felt, as are the services of a qualified designer. It is anticipated that such a person could give really effective help of a type that is not easily available at the moment. Since there is a reasonable limit to the amount of design time required, the designer is budgeted for on a 50% availability schedule.

It is also felt that the department would function more effectively if the common items of glassware, chemicals, media, etc. were held in a central storeroom, serviced and supervised by a departmental storekeeper. This individual would also be expected to be familiar with the quality and availability of all types of research equipment in our area, and to help in the processing and expediting of orders for such equipment as the need arose.

Equipment:

Many of the experimental approaches in the projects in genetic and neuro chemistry depend for their final data on scintillation counting. The rate of production of material for counting at the present time is beyond the capacity of the single Tricarb instrument in the department, and is expected to rise sharply. This is also true for the basic items of centrifuge equipment. Here the need for long equilibrium runs for pycnographic fractionation of DNA or the multistage procedures for cellular fractionation dictates the choice of

Justification for Budget (continued)

Spinco or International preparative ultracentrifuge to alleviate the continuous overloading of our present capacity. The medium International BD2 centrifuge listed would not only provide much needed large volume capacity at medium speeds, but also a horizontal rotor of very long path length for sensitive pycnographic fractionation.

The use of ^{131}I and ^{51}Cr in the immunoglobulin and leucocyte antigen work respectively calls for an automatic γ -scintillation spectrometer which is currently available. Furthermore, a unit which enabled chromatographic column effluents to be scanned for radioactivity would accelerate many procedures. Of necessity this unit has to include the basic scintillation counter.

A powerful tool for analyzing the conformation or changes in conformation of macromolecules is the measurement of optical rotary dispersion. Changes in optical rotation accompany the unwinding of the DNA bihelix or of the α -helix in proteins and may therefore be used as sensitive indicators of, for example, the amount of single-stranded DNA present in bihelical DNA at any given time, or the changes in protein conformation which accompany aggregation or dissociation of protein subunits or transition from active to inactive enzymatic forms. Of the commercial instruments on the market, the Carey model has the most desirable features.

Consumable Supplies:

It is our experience that we consistently underestimate the cost of scintillation counting as such. Low count vials plus the appropriate scintillation fluids cost approximately ten cents each. Assuming we use about 600 vials per scintillation counter per week - this is a low estimate reckoned at 100 vials for each of six individual research workers - this works out at approxi-

Justification for Budget (continued)

mately \$3,000 per year. The same applies to centrifuge tubes and to the chemicals, e.g, sucrose, buffer chemicals, which are routinely used in large amounts.

Existing Facilities:

The department at present covers some 5,000 square feet, excepting the Instrumentation Research laboratories and workshops, in eight laboratories, an office and library. In January 1966 it will move to the new Clinical Sciences Building of the Stanford Medical Center complex, to occupy an area of approximately 11,000 square feet, providing integrated areas for microbial, cell and human genetics, molecular neurobiology and computer work. Common facilities for wash up, sterilization and cold room will be shared with Pediatrics and Obstetrics. Additional areas in the new building will house the Instrumentation Research group. Animal room facilities specifically for Genetics will be on the floor above the main department. The department is equipped with all the usual apparatus for work in the fields outlined above. It has a number of specialized pieces of equipment for departmental use which this grant seeks to extend. At the moment these are:

Spinco Analytical Ultracentrifuge
Spinco Preparative Ultracentrifuge
International Preparative Ultracentrifuge
Tricarb Scintillation Spectrometer
Zeiss Spectrophotometer
Coulter bacterial counter
Bendix Mass Spectrometer
LINC Computer

No other funding quite matches the purpose of this application. However, component elements of our investigations are supported as follows:

From National Institutes of Health:

<u>Grant No.</u>	<u>Grant Title</u>	<u>Dates</u>	<u>Average Annual Direct Costs</u>
AI-5160	Genetics of Bacteria	9/1/64- 8/31/68	\$ 50,000
NB-04270	Molecular Neurobiology	12/1/64- 11/30/67	\$ 80,000
GM-12075	Genetics of Immunoglobulins	6/1/64- 5/31/69	\$ 30,000
CA-04681	Genetic Studies with Mammalian Cells	9/1/64- 8/31/67	\$ 35,000
Application Pending	Dissociation and Culture of Cells	9/1/65- 8/31/68	\$ 55,000

From National Aeronautics and Space Administration:

NsG 81-60	Cytochemical Studies of Planetary Microorganisms	4/1/64- 3/31/67	\$350,000
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J. Lederberg -

Integration of genetics, molecular biology and computer systems.

Our essential problems in the interface of molecular biology and genetics are essentially ones of ultrastructure, viz., what is the molecular sequence of amino acids or nucleotides in a biopolymer, and how is this organized in the cell. It seems obvious that the sheer complexity of the demanded information, not to mention the experimental difficulties of acquiring it, call for a fundamental enlargement of methodological power. It is equally obvious that the requisite analytical systems will be most efficiently developed around general-purpose digital computers used in real time. However, so far almost the only relevant applications of computers have been in chemical industry, and there has been negligible practical experience in applying them to versatile challenges of experimental laboratory practice. Probably the time is just now ripe to invest a substantial effort in such developments, even though computers are still relatively expensive devices (though perhaps not in proportion to their promised return). Fortunately, through other avenues, mainly NASA's interest in an automated biological laboratory, and the collaboration of a faculty group interested in artificial intelligence, in the Department of Computer Science, we have been able to make a small start in direct hookups of laboratory equipment to a small LINC computer, and we are in current negotiations with other agencies for the funding of a substantial computer to be devoted primarily to these kinds of study. It is our intention to push this as far as we can see tangible returns in the form of the efficient retrieval of experimental data. So far with one exception the applications have been relatively unexciting, though quite useful: e.g., the calculation and plotting of scintillation countings of column effluents and sedimentation fractions.

The most interesting exception is in mass spectrometry, which we are exploring as a tool for routine biochemical analysis, especially of amino acids

and nucleins, and possibly of their sequences. In principle this device offers a very high degree of ultimate sensitivity, measurable in molecules, and very high speeds of operation, measurable in thousands of repeated analyses per second. On these premises we are exploring the possibility of operating the mass spectrometer as a scanning microanalyzer, with a view to obtaining information on chemical composition of object points in the submicron range of size. The significance of such a device for the analysis of chromosomes and other cellular constituents should be obvious. While other funds support the principal costs of these studies, they connect quite closely at various points to exigent problems mentioned in this proposal. It is intended, as far as possible, to connect the instruments listed here to the data acquisition and computational facilities that we will be obtaining for developing such systems. At the present time, the LINC computer serves an indispensable function in controlling a Bendix TOF mass spectrometer and storing the data from it--which is emitted at over 20 megacycles, so we are not now able to use it efficiently.

As an aspect of this problem, we are also studying the computation of logical (contra numerical) problems of organic chemistry. The mere enumeration of possible structures is already a formidable problem, most of which has however been solved in the last few months. We hope ultimately to be able to formulate some of the more tractable issues of genetics and molecular biology in a form where they may be amenable to computer analysis. As an elementary example, a program has been written to extract the linear order, if any, from data on sets of overlapping deletions. When, say, 100 genotypes have been tested it might appear to be necessary to test 100! combinations; fortunately, this can be bypassed by another algorithmic approach and the problem made quite manageable.

J. Lederberg - Genetic chemistry of bacteria
(with Drs. E. M. Lederberg and A. T. Ganesan)

In addition to the related studies summarized by Dr. Bodmer we propose to continue the fractionation of B. subtilis DNA for differential genetic activity and to explore a membrane-complex with which nascent DNA seems to be associated for about ten seconds after synthesis, i.e. a possible physical site of DNA synthesis in the cell. This fraction also has high polymerase activity. In connection with the mechanics of transformation, both of these approaches are being combined in a test of sequential replication (following Sueoka, Cairns, et al). We hypothesize that DNA uptake consists of a matching of a proband molecule with the replicating region and that a molecule will be rejected (perhaps from the entire cell) if it does not match. These studies also include a detailed analysis of interspecific homology of B. globigii with B. subtilis.

References:

- Nester, E. W., Marion Schafer and J. Lederberg, 1963. Gene linkage in DNA transfer; a cluster of genes concerned with aromatic biosynthesis in *Bacillus subtilis*. *Genetics* 48:529-553.
- Ganesan, A. T. and J. Lederberg, 1964. Physical and biological studies on *Bacillus subtilis* transforming DNA. *J. Mol. Biol.* 9:683-695.
- Lederberg, J. Computation of Molecular Formulas for Mass Spectrometry. Holden-Day, Inc., San Francisco, 1964.
- Lederberg, J., 1965. Topological mapping of organic molecules. *Proc. Nat. Acad. Sci.* (in press).
- Ganesan, A. T. and J. Lederberg, 1965. A cell-membrane bound fraction of bacterial DNA. *Biochem. Biophys. Research Communications* (in press).

One of the long range aims of the projects currently in progress is the find structure mapping of mammalian genetic loci. This is a necessary step towards the extension of the detailed knowledge of microbial genetics to the mammal. Studies with mouse immunoglobulins offer the possibility of correlating genetic fine structure with structure of the proteins under the control of the gene locus.

It has been found that isoantigens on $7S\gamma_2$ -globulins of the mouse are controlled by alleles at the Ig-1 locus. Eight antigens have so far been identified in some 70 inbred strains examined. These antigens all cross react. By studying the cross reactions, eight antigenic specificities have been defined. Localization of these specificities to portions of the $7S\gamma_2$ molecules are now being attempted.

A second locus controlling β_{2A} immunoglobulin antigenic structure is closely linked to Ig-1. Both loci are concerned with the synthesis of one of the two polypeptide chains (H) of their respective immunoglobulins. The striking analogy with hemoglobins suggests that with immunoglobulins, also, a gene cluster may be uncovered.

The immunoglobulin antigens are also being used as cellular genetic markers. For example, both donor and host gamma globulin production continues for at least nine months in radiation chimeras. Also, production of donor type γ -globulin continues despite loss of tolerance to donor skin.

These and other related studies in progress with the immunoglobulins may also contribute to the understanding of the control of antibody structure and therefore, specificity.

L. A. Herzenberg - Immunoglobulins and Somatic Cells

Reviews:

Steinberg, A. G., 1962. Progress in Medical Genetics. II. Grune and Stratton, New York.

Klein, G., 1963. Somatic Cell Genetics, in Methodology in Mammalian Genetics, ed. J. J. Burdette. Holden-Day, Inc., San Francisco.

Publications:

Wunderlich, J. and L. A. Herzenberg, 1963. Genetics of a gamma globulin isoantigen (allotype) in the mouse. Proc. Nat. Acad. Sci. 49: 592-598.

Herzenberg, Leonard A., 1964. Study of the H-2 locus in murine cell cultures, p. 140-166. In Somatic Cell Genetics, (ed.) Robert S. Krooth. Univ. Michigan Press, Ann Arbor.

Herzenberg, Leonard A., 1965. A chromosome region for gamma_{2a} and beta_{2A} globulin H chain isoantigens in the mouse. Cold Spring Harbor Symp. Quant. Biol. (in press).

Herzenberg, Leonard A., Noel L. Warner and Leonore A. Herzenberg, 1965. Immunoglobulin isoantigens (allotypes) in the mouse. 1. Genetics and cross reactions of the 7Sγ_{2a} isoantigens controlled by alleles at the Ig-1 locus. J. Exp. Med. (in press).

Using as a hypothesis that the neural engram has a chemical entity, a possible candidate for the latter role, which meets such requirements as permanence during the lifetime of an animal, is the macromolecular constituent, protein. An extensive investigation is thus being undertaken of the proteins in brain. Analytical systems have been developed using acrylamide gel electrophoresis which resolve some twenty soluble proteins or groups of proteins in the sucrose homogenate of brain. Non-ionic detergents extract about an equal number of other protein or lipoprotein complexes from the insoluble cellular fraction of the homogenate. These primary analytical systems form the basis of a number of protein surveys noted below. They will be modified or extended to include chromatographic and gel filtration procedures as our knowledge of the physical properties of the brain proteins expands. In addition, the protein zones will be screened for enzyme activity with selected fluorescent and histochemical reagents. The cellular localization of the various proteins defined by the analytical techniques will be determined by subcellular fractionation. The comparison of the protein patterns from the brains of a wide range of inbred mouse strains should reveal whether there are genetic dimorphisms in brain protein analogous to the hemoglobin variants in man. Immunological assays will probably be required for the characterization and assay of any dimorphic protein. This would then provide the basis for the establishment of co-isogenic strains differing only in the gene controlling the production of this particular protein. Analysis of the protein patterns from certain neurological mutants may point to the presence or absence of specific proteins intimately concerned with brain function.

E. M. Shooter - Neurobiology (continued)

Since the most rapid development of brain structures occur during the early postnatal period, a survey will be made of the proteins in the developing brain of the mouse. Again, in order to test the hypothesis that specific protein structures are laid down in learning and then persist, labelled protein hydrolysate will be injected into young mice over an interval of several days to allow equilibration of the amino acid pools. These animals will then be reared to maturity on normal unlabelled diet and any residual label in the protein of the brain then sought by autoradiography and identified by the methods outlined above.

Publications:

- Boycott, B. and E. M. Shooter, 1965. Correlation of defined structural changes with the protein composition of lizard brain. (in prep.)
- Bucci, E., Fronticelli, C., Dance, N., and Shooter, E.M., 1965. Further studies on the haemoglobins modified by digestion with the carboxypeptidases. J. Mol. Biol. (in press).
- Huehns, E. R., N. Dance, E. M. Shooter and G. H. Beaven., 1962. The preparation and properties of haemoglobin α^A , a haemoglobin consisting of α^A -polypeptide chains. J. Mol. Biol. 5:511.
- Baldwin, R. L. and E. M. Shooter, 1963. The alkaline transition of BU-containing DNA and its bearing on the replication of DNA. J. Mol. Biol. 7:511.
- Raper, A.B., D. B. Gammack, E. R. Huehns and E. M. Shooter., 1960. Four haemoglobins in one individual. A study of the genetic interaction of haemoglobins G and C. Brit. Med. J. 2:1257.

Studies on recombination during transformation in collaboration with A. T. Ganesan have confirmed the physical incorporation of donor DNA into the recipient genome. Donor DNA labelled with ^2H and ^{15}N to give increased density and ^3H as a radioactive label is used with ^{32}P or ^{14}C labelled recipient cells and a system of biological markers which allows the selective identification of donor, recipient and recombinant transforming activities. The donor recipient complexes are isolated by pycnographic fractionation and further refractionated after shearing and denaturation by heat or alkali. The bond between donor and recipient DNA is resistant to shearing and denaturation. The relative shifts in density of donor versus recipient DNA suggest single stranded incorporation of the donor DNA and allow the estimation of the size of the incorporated region.

A thymine-requiring strain of B. subtilis has been used to study the involvement of DNA synthesis in the integration process. Measurement of ^{32}P uptake in the absence of thymine has shown that this strain is not leaky and that transformation can occur during a time when the overall amount of synthesis in the competent culture is less than 0.2% of the normal division cycle. Addition of 5-Bromouracil with donor DNA during transformation allows selective identification of that DNA which has been synthesized during the transformation process. Preliminary results have shown that the formation of recombinant biological activity is not dependent on any major amount of DNA synthesis, such as would be suggested by a copy choice model for recombination. Further results have also indicated that recently replicated molecules in the recipient cells do not incorporate donor DNA. Studies are currently under way to examine the fate of the incorporated donor DNA after further replication in the presence of 5BU or when recipient cells are grown in a deuterated ^{15}N medium. The aim of these

experiments is to obtain evidence concerning the specific involvement of DNA synthesis in the formation of donor-recipient DNA structures. It is also planned to examine in more detail the early fate of donor DNA using the systems described above.

At the present time there is still little or no indication concerning the mechanism of uptake DNA during transformation and the nature of the competent state of the recipient cells. It is proposed to investigate the pattern of DNA uptake during the acquisition of competence and the association of donor-recipient DNA complexes with different cellular fractions in the hope that this may clarify some of these unsolved problems.

A search for in vitro recombination using the B. subtilis transformation system has also been initiated. At present the intention is to use a biological assay to detect the formation of recombinants. This work is so far at the preliminary stage involving the screening of conditions for optimal detection of in vitro formation of recombinants.

Earlier studies have shown that pancreatic DNAase (DNAase₁) inactivates linkage at a much faster rate than the loss in molecular weight. It was suggested that this could be explained if incorporation of donor DNA occurs at the single stranded level and mild DNAase treatment, which does not affect molecular weight, also does not affect uptake of the donor DNA. In order to verify this, as has already been shown in pneumococcus by Lehrmann and Tolmach, it is proposed to do careful studies on the uptake of DNAase treated DNA.

Earlier work in collaboration with Dr. Kornberg's group in the Biochemistry Department had shown that newly synthesized DNA made using a native primer had no detectable biological activity in the B. subtilis transformation system. Since then an enzyme from phage T-4 has been purified to an extent where it no

W. F. Bodmer - Genetic chemistry of DNA mediated bacterial transformation.
(continued)

longer has any detectable nuclease activity and works only on single-stranded DNA as a primer. It is proposed to repeat some of the studies aimed at detecting biological activity of newly synthesized DNA using this enzyme system.

Publications:

Bodmer, W.F., 1962. Inactivation of linkage in *Bacillus subtilis* by deoxy-ribonucleases. *Genetics* 47:944.

Richardson, C.C., C. L. Schildkraut, H. V. Aposhian, A. Kornberg, W. Bodmer and J. Lederberg, 1963. Studies on the replication of DNA by *Escherichia coli* polymerase. Symposium on Informational Macromolecules. Academic Press.

Bodmer, W.F. and C. Schildkraut, 1964. Preparation and characterization of $^{15}\text{N}^{2,3}\text{H}$ labeled DNA from *Bacillus subtilis* and *Escherichia coli* Phage T2. *Anal. Biochem.* 8:229-243.

Bodmer, W.F. and A. T. Ganesan, 1964. Biochemical and genetic studies of integration and recombination in *B. subtilis* transformation. *Genetics* 50:717-738.

Bodmer, W.F. and A. T. Ganesan, 1964. The molecular basis for recombination in *B. subtilis* transformation. *Genetics* 50:236.

Bodmer, W.F. and S. Grether, 1965. The uptake and incorporation of thymine, uracil, uridine and 5-Fluorouracil into the nucleic acids of *Bacillus subtilis*. *J. Bact.* (in press).

Review:

Ravin, A.W. *Advanced in Genetics*. II. 1962.

W. F. Bodmer - Studies on Human Leukocyte Isoantigen Systems.

In collaboration with Dr. Rose Payne of the Department of Medicine, methods for the analysis of the reactions of leucocyte anti-sera containing mixtures of antibodies have been developed. An analysis was performed on the extensive data collected by Dr. Payne with the leucocyte isoagglutinins formed during pregnancy. This established the existence of a new three allele leucocyte isoantigen system in man. A major aim of the work at the present time is the establishment of further new isoantigen systems using these techniques with a view to tissue typing for transplantation. Development of sensitive, reliable and rapid procedures for testing sera is an essential part of such a program.

In collaboration with Dr. Terasaki of UCLA Medical School, the results of a cytotoxicity assay have been correlated with the agglutination assay. Recently a sensitive technique for such an assay using ^{51}Cr labelling has been developed for mouse antisera. In collaboration with Dr. Payne, it is proposed to develop this or similar assays for large scale studies in humans with the aim of establishing new leucocyte isoantigen systems.

Publications:

Payne, R., M. Tripp, J. Weigle, W. Bodmer, and J. Bodmer, 1964. A new leucocyte isoantigen system in man. Cold Spring Harbor Symposium on Quantitative Biology 29.

Sanderson, A.R., 1964. Nature 204:250.