

PROGRAM IN MOLECULAR NEUROBIOLOGY

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1. General theme: the neural engram as a chemical entity.

The underlying hypothesis of our program is that the engram has a material basis which it is profitable to pursue by the methods of macromolecular chemistry (=molecular biology).

This hypothesis may be incorrect, and one can hardly pretend that current knowledge permits a reliable judgement one way or the other ---many neuro-physiologists have despaired of finding a chemical engram and would focus their efforts on dynamic signal analysis rather than material circuit networks. If correct, the hypothesis may still take many forms. Thus, the engram may be more a problem of morphological rather than chemical analysis. Nevertheless, even if the results of such investigations were merely to define the chemical composition of synaptic terminals whose distribution rather than whose structure was the engram, this would still be important for further progress in neurobiology.

One approach to the hypothesis is to inquire whether any macromolecular constituents of the brain have some metabolic properties which parallel the learning process. For example, are any proteins synthesized during early life which have the same life-long persistence as memory, in contrast to the rapid turnover of most of the functional elements of the organism? Because proteins are the specific effectors for many cellular processes, we focus our attention on these in preference to other elements, which, however, cannot be ignored. In addition to the metabolic, cytochemical and developmental studies that are implied by these precepts, any genetic variations that could be discovered in brain proteins would furnish tools of immense value for further work. It should hardly be necessary to point out that a thorough knowledge of normal structure is absolutely essential for the understanding and thence prevention and mitigation of disease.

The hypothesis can also be stated in developmental terms: Learning is the development of the structure and chemistry of the brain under the influence of external stimuli. Thus, human intelligence is a corollary of the relative immaturity of the human brain at birth which allows for the extensive participation of environmental information in the actual development of the brain during its maturation. Another corollary of this conceptual approach is the importance of studying the metabolism of the brain during the early postnatal period when the most rapid development of brain structures and the most intense "learning" are fundamentally occurring. The question must also be posed, though it cannot now be answered, whether animals having more primitive intellectual functions can be suitable experimental material, in terms of the relative allocation of biosynthetic activity within their brains to learning processes, as compared to maintenance and consequent functions.

If some developmental control of protein structure is part of learning we may speculate on some specific mechanism that may be consistent with our general biochemical insight. We might just ask how a bit of information may be stored at a given switch point:

A. Choice among proteins, i.e., specific activation of one among a series of RNA messages from the DNA.

B. Choice among protein subunits to form diverse "isozymes" - analagous to the differentiation of adult hemoglobin from fetal hemoglobin, or cardiac from skeletal lactic dehydrogenase.

C. Episequential modification of the primary amino acid sequence - e.g., the selective removal of glutamine amide groups to give a series of variant proteins differing in ionic charge distribution from the basic fully amidated pattern.

It may be foolhardy to discuss these alternatives more deeply until we have accumulated much more information about the proteins of the brain, but these speculations may give some insight into the long range goals of neurochemical studies. Choice A may be substantiated through careful analytical work to separate the range of brain proteins into fractions which could then be localized by refined ultrastructural techniques. Choice B has had great encouragement from the realization that many mammalian proteins occur as series of "isozymes", different combinations of subunits offering another dimension in the strategy of developmental variation. Choice C has had new impetus since it was proposed in the original application: Zubay (Proc. Natl. Acad. Sci. 48: 894, May 1962) has found that glutamic acid is not bound directly to transfer RNA but only as glutamine, so that some secondary mechanism must be generally invoked to account for the choice between glutamic acid and glutamine in polypeptide sequences.

We can scarcely overestimate the technical problems that may hinder the resolution of these questions. Fortunately, Dr. Shooter's extensive experience with the analysis of hemoglobin is an ideal basis for an effort. Our general strategy is to enter the problem in a modest way to find the elements that give the most amenable opportunities to apply current technique, and to use this experience to make whatever technical innovations are within our capacity. We also plan to exploit, as far as possible, the opportunity for development of new analytical instrumentation which, fortunately, becomes available to us through the contiguity of the NASA supported programs in this department.

Having stated our general strategy I would then turn to the tactics, the specific problems chosen for study in the immediate future.

1. The genetic dimorphism of cerebral proteins in the mouse.
2. Identification of long lasting proteins in the brain.
3. Microscan mass spectrometer - new instrumentation for fine structure chemical analysis at the ultramicroscopic level.

1. The genetic dimorphism of cerebral proteins in the mouse

Bailey and Heald (J. Neurochem. 6: 342, 1961) have fractionated soluble brain proteins of the mouse by starch gel electrophoresis. They mention, in passing, the distinction of one component in two mouse strains. This suggests a genetic dimorphism, in a brain protein, analogous to the variation of hemoglobins in man, from the study of which we have learned so much of the molecular medicine of the blood. This study should be repeated, and, if confirmed, extended to other mouse strains. Then a choice should be made of any technically favorable opportunity to establish the genetic basis of the dimorphism and to characterize the protein as a basis for other work on its functional significance. To do this, it will be necessary to isolate the dimorphic protein and establish a convenient assay for it, perhaps an immunological one. This would then be the basis of a breeding program to establish co-isogenic strains differing only in the gene controlling this protein, and identical in every other respect. This breeding work could take several years unless we were fortunate to find the dimorphism in co-isogenic lines for other tissue antigens already established in this department or procurable from other centers.

The study would thus start out as a verification of existing claims to which the following elements would then be added: i) the examination of a wider range of inbred strains already available in the Stanford mouse colony, and being studied immunogenetically in respect to their tissue transplantation antigens by Dr. L.A. Herzenberg (Associate Professor of Genetics), ii) the comparison of the brain antigens with these differential tissue antigens, iii) the screening of the protein bands of the electrophoretograms for enzyme activity (esterase, phosphatase, glycosidase, nucleotidase, depolymerase) with sensitive fluorescent reagents being developed in the exobiology project in this department; other well established histochemical techniques for identifying enzyme activity are also in mind, iv) the use of further methods of protein fractionation, e.g., column and thin-layer electrophoresis and chromatography on a wide range of substrates, and pretreatment of the mixed extracts with selective reagents, v) the preliminary fractionation of the mixed extracts by sedimentation, thermal denaturation, disulfide reducing agents, and similar procedures in searching for improved methods of isolating the differential fractions, vi) isotopic labelling of the total cerebral protein followed by measures of the turnover rate of the respective fractions, vii) developmental studies of the fractions, viii) the development of immunological reagents for the characterization of the fractions, particularly important in searching for the distribution of these components in tissues other than the brain, ix) finally, the genetic program to obtain co-isogenic lines of mice to allow for testing the further impact of the protein dimorphism on the development of the animal's behavior.

Patently, it will not be possible to explore all of these lines of investigation within the scope of the present program. It is our intention to explore which aspects of the problem are more nearly within a realistic assessment of our reach.

2. Identification of long lasting proteins in the brain.

On one speculative hypothesis of the mechanism of memory, specific structures are laid down in learning and persist as material substances for the duration of memory, i.e., for a large fraction of the life span. As most proteins and other substances undergo rapid turnover, especially during the growth period, we can approach a test of the hypothesis by long term labelling experiments. For example, a group of very young mice (or perhaps monkeys, if preliminary experiments encourage it) would be injected with a C^{14} labelled protein hydrolysate for an interval of several days, to allow the equilibration of the amino acid pool throughout the body. These animals would then be reared to maturity on normal, non-isotopic diets for long periods, with intermittent monitoring of serum and catabolic C^{14} . During this interval, the dynamic elements of the brain (and other organs) should have turned over extensively and the label should have disappeared, but the label should be retained in any site which fulfills the conditions of the hypothesis. Whatever residual label can be found in this way by counting of bulk samples should then also be pursued: for its location by autoradiography, and its identity by chemical fractionation. Whatever the outcome of this experiment, it should be an important guide to our long-range program.

A.N. Davison (biochemical journal 78: 272, 1961) has now reported an experiment analogous to the one suggested here with findings quite encouraging to the hypothesis of the chemical engram. While most of the brain protein has a half life of only 22 days, a small fraction, characterized as lipoprotein and presumably originating from cell membranes, persists, having once been formed, without appreciable loss throughout the lifetime of the animal. The localization of these long lasting components, and their chemical characterization, are thus the next challenge in this analysis. We propose to repeat these experiments using labels other than C^{14} glycine, verifying the persistence of the C^{14} in the form of polypeptide, and attempting to localize these fraction by cytofractionation and microscopic studies (cf. Gray and Whittaker, The isolation of Nerve Endings from Brain, J. Anat. 96: 79, 1962; Cummins and Hyden, Adenosine triphosphate levels and adenosine triphosphatases in neurons, glia and neuronal membranes of the vestibular nucleus, Biophys. Acta 60: 271, 1962).

3. Microscan mass spectrometer - new instrumentation for fine structure chemical analysis at the ultramicroscopic level.

In many fields of biology we urgently need new methods of chemical analysis of structures that can now be seen under the light microscope at a resolution of 0.2 micron or under the electron microscope at .001 micron. For example, in planning instrumentation for exobiology, we found that many microscopic particles can be collected from the atmosphere or from soil that we could see but could not identify. A conclusion whether these were microorganisms or not would be very much helped if we could analyze such particles, e.g., for protein and nucleic acid. Further consideration of the instrumentation called for by this problem has led to the present project, the development of a microscan mass spectrometer, which may have the most exciting implications for neurobiology, as well as many other fields of biology and medicine.

Our point of departure is the electron probe microanalyzer (Castaing, Adv. Electronics and Electron Physics 13: 317, 1960) which in a few years has become a widely used analytical instrument, especially in industrial analysis of metals in alloys. An electron beam is focused on the specimen, and the X-rays emitted are then collected; their energy spectrum is characteristic of the excited atom and can be used to identify it. Cosslett (Ann. N.Y. Acad. Sci. 97: 329, 1962) had elaborated this instrument further by scanning the specimen with the electron beam, allowing an image of it to be created which shows the distribution of a given species of atom. For technical reason, the method is mainly effective for heavier atoms - such elements as iron or calcium can be readily mapped to a resolution of better than 1 μ . Useful, but much poorer performance is indicated for carbon, oxygen and nitrogen on account of their characteristics as X-ray emitters under electron excitation. It is characteristic of the method that it will give no information on the molecular, only on the atomic composition of the target.

We propose to construct a similar device for electron beam scanning, but to analyze the microtarget by the mass spectrum of the gas burned out of the specimen by the beam. High frequency mass spectrometers are commercially available that can analyze the molecular masses in a gas in an interval of 100 microseconds. Thus a 100 μ^2 specimen could be fully scanned in a period of one second at a resolution of 1 micron. Under ideal conditions the mass spectrum can give a wealth of information on the chemical composition of the target -- the structure of many new products and other organic compounds have been fully elucidated from their mass spectra alone (e.g. at Stanford university, Chemistry Department, Bjerassi et al., Proc. Nat. Acad. Sci. 48: 113, 1962 - Mass spectrometry in structural and stereochemical problems). Whether so much detail can be usefully obtained in a micro-scanning instrument remains to be seen. The large yield of information that can be generated (in principal, a million mass spectra per microgram of target material) will require careful attention to the simplification of the data by computer techniques. This problem is also shared by the exobiology program, and it must be thought of especially for the later stages of the instrument development.

The most immediate application of the microscan, requiring the least optimism as to its capabilities, is for the localization of isotope labels in the metabolic experiments. Although less suitable for some other purposes, tritium, the mass-3 isotope of hydrogen would be an ideal label for microscan localization, having such a large proportional mass shift compared to hydrogen. With such a label we could stress the spatial rather than mass spectral resolution, and hope to map the distribution of tritium to a fraction of a micron. After feeding tritium-

labelled amino acids we could then inquire whether long-lasting molecules synthesized from these amino acids are localized on the neuron membranes, or in synaptic knobs, or whether they are also represented in intracellular structures as well. With further elaboration, the instrument could also localize other isotopes, especially C^{14} and N^{15} with the special advantages of a) higher resolution, higher sensitivity and faster readout than autoradiography; b) availability for labels, like N^{15} that are not radioactive; c) the abundance of additional chemical information from the complete mass spectrum.

Even a crude microscan mass spectrometer could have a rewarding application in the direct determination of molecular weights of macromolecules. A solution of protein or DNA heavily labelled with tritium would be spread and dried on the target. The scan would give a number of bursts of tritium, each one representing a cluster source, a labelled macromolecule. Even without counting how many tritons occurred per burst, the number of bursts is the number of macromolecules which, divided into the total mass deposited, gives the average molecular weight of the particles. (These calculations are analogous to those in Rotman's work in this department on Microfluorometry for the detection of single enzyme molecules -- Proc. Nat. Acad. Sci. 47: 1981, 1962).

The chief technical problems we expect to encounter in this development have to do with the efficient gathering of the signal gas from the specimen, its ionization, and transport in the analyzer beam. Within the last few days, at a symposium on X-ray microscopy at Stanford University, Castaing has reported some new work of his own along related lines that gives the strongest encouragement to the practicability of our approach.