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OFFICE OF NAVAL RESEARCH

ANNUAL PROGRESS REPORT

REPORT PREPARED BY:	Francis O. Schmitt
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CONTRACTOR:	Massachusetts Institute of Technology
PRINCIPAL INVESTIGATOR:	Francis O. Schmitt
TITLE OF PROJECT:	Investigations of the Chemical Composition and Molecular Organization of Nerve Axons

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V. PERSONNEL

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Technical Assitant

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II. OBJECTIVES

a. To investigate the molecular organization of neurons;

b. To isolate and characterize chemically and structurally constituents of axoplasm obtained by extrusion from squid giant fibers;

c. To attempt to relate such information to neuronal function and eventually to brain function.

III. ACCOMPLISHMENT OF RESULTS

A Kharasch laboratory at the Marine Biological Station at Montemar, Chile, has been outfitted with equipment needed to make physicochemical and analytical studies of the fibrous protein and other constituents of the axoplasm of the giant fibers of the large squid Dosidicus gigas. Dr. F. Huneeus-Cox, after a year's training in biophysical chemistry at M.I.T., has returned to Chile to conduct the experiments there. Axon material has been dissected from a large number of squid and stockpiled for future analysis. Initial experiments have been made in an investigation into the possible function of the fibrous protein which is ubiquitous to all neurons.

Consideration has been given to the possible role of neuronal macromolecules in encoding experiential information in long-term memory and learning.

IV. THE CHILEAN PROGRAM

As explained in last year's report, Dr. F. Huneeus-Cox was trained in this laboratory in the techniques of protein physical chemistry and in the nerve program of this laboratory. In July 1962 Dr. Huneeus returned to Chile and occupied the three laboratory rooms prepared for our Unit at the Marine Station in Montemar, adjacent to Valparaiso. He took with him a large variety of physical-chemical equipment and supplies because of the lack of such material in Chile. With the laboratory thus equipped and with the help of an assistant and a diener employed a few months after his arrival, Dr. Huneeus began the experimentation for which this rather elaborate planning was accumulated. This also constitutes the major effort of this laboratory in this program though the feedback with the home laboratory at M.I.T. was kept active (on a weekly basis) and nerve material was sent to M.I.T. for analysis and characterization.

Until November 1962, the development of the project was hampered by disappearance of the squid from the usual fishing grounds. Until squid could again be provided, Dr. Huneeus explored a variety of other local fauna, and in particular found that a local species of lamprey appeared to be suitable for studies on the nerve composition and structure. However, his investigation of these animals was postponed when squid again became plentiful. It is planned to keep the lamprey work for such periods during which no squid are available.

Since November 1962 nearly 1,500 large squid have been dissected and from most of these animals the axoplasm has been extruded, dialyzed, the dialyzable constituents frozen-dried for study at a later date, and the high molecular-weight constituents have been studied by viscometric, flow birefringence, and other techniques. These preparations have also been fractionated and further analytical studies have been performed on the fractions.

V. CHEMICAL INVESTIGATIONS OF AXOPLASMIC CONSTITUENTS

A. The Fibrous Protein (Neurofilaments)

1. **Stability.** The stability of the extruded axoplasm and, in particular, the maintenance of the structure of the highly asymmetric filamentous protein, the neurofilaments, has been studied as a function of ionic strength, pH, and temperature. It was found that at ionic strengths between 0.1 and 0.2 the protein was much more stable than at low or at higher ionic strengths, and the stability was maximal between pHs 7.2 and 7.5 with both the viscosity and flow birefringence of the preparations falling off rapidly as the pH was raised; the solutions also showed, on keeping, a progressive opalescence which ultimately resulted in the precipitation of the protein. This opalescence increased more rapidly in high ionic strength solutions. In low ionic strength solutions, the axoplasm showed a very high viscosity, but a surprisingly low birefringence, and the opalescence was also very small. Since it seems unlikely that all of this viscosity can be ascribed to electrostatic interactions between charged molecules and to the absence of counterions, it appears possible that in low ionic strength solutions a dissociation of the fibrous protein proceeds in a manner still unknown and previously unsuspected. Some further indication of a molecular change is given by the observation that electrophoresis of the axoplasm extruded and dialyzed at moderate ionic strength shows only two high molecular weight components present (in confirmation of earlier studies performed at M.I.T.), whereas after water dialysis four components are detectable. Earlier attempts to study the reversibility of the dissociation changes occurring in this filamentous protein as pH or ionic strength was raised and lowered have now to be re-interpreted in the light of the later findings of a dissociation at low ionic strength, and these experiments now need to be repeated.

2. X-ray Diffraction. In order to explore the structure of the filamentous protein, attempts have been renewed to obtain a fiber-type X-ray diffraction photograph of the extruded axoplasm. Attempts at M.I.T. to orient the fibers from centrifuged and purified axoplasm had previously failed, and more recently attempts have been directed to the recovery of the protein while preserving the orientation pre-existing in the axon. To this end the axoplasm has been extruded from the giant fibers and, maintaining the orientation of the plug, the salts have been dialyzed from the material through membranes or by immersion in aqueous acetone, and the stretched fiber or resulting protein plug has now been submitted for X-ray analysis. These plugs are strongly optically birefringent. Thus far no orientation in the X-ray diagram has been discerned, but an intensive effort will be maintained in this investigation.

3. Isolation of Subunits. Taking advantage of techniques explored by workers in the field of virus structure where the aggregation of subunits poses particular problems of analysis, the neurofilament protein from the axoplasm has been fractionated by previously described methods using ammonium sulphate, and the protein has been dissociated in 8 M urea, reduced by borohydride, and alkylated by iodo-acetic acid or iodo-acetamide. Such solutions have yielded a preparation which, although apparently monodisperse in the ultracentrifuge, in fact yields a boundary which, on analysis, appears non-Gaussian and therefore represents an interacting system or a system more complex than is immediately apparent. These preparations await further characterization. Electrophoretically these comprise only one component, whether in free boundary or zone electrophoresis, confirming the validity of the previously elaborated fractionation procedure. The amino-acid composition of the soluble alkylated subunits -- corresponding to F-1 in last year's report -- has been determined, and it agrees well with previous determinations.

1. Dialyzable Constituents

The low molecular weight constituents isolated from the axoplasm by dialysis have thus far been lyophilized and stored against a possibility that later in the year the squid will no longer be available for direct experiments. Further analysis of this material will be completed both in Chile and at M.I.T. Thus far the lyophilized dialyzable constituents of 360 squid axons have been accumulated. Meanwhile fully automated equipment for amino acid analysis has been assembled; it will be utilized in the analysis of the axon material shortly at M.I.T.

2. Physiological Experiments

In an attempt to understand the physiological role of the neurofilaments, experiments have been designed to inject both proteolytic enzymes and some of the dialyzable peptides of the axoplasm into the axon of a freshly dissected squid in which the neuromuscular junction has been retained intact.

It will be recalled that the axon filament protein is readily attacked by proteases such as trypsin. If the neurofilaments play a role in transfer of excitation to the innervated muscle, injection of protease into the axon should interfere with that transfer. With respect to the peptides the rationale was that if, in the peptides already demonstrated to exist in axoplasm, there were included a hormone active in ion transport, injection of this hormone into the fresh axon might alter the current flowing across the membrane (in the presence of the appropriate ion). The model for such a system is the toad bladder in which ion movements can be stimulated by as little as 10^{-11} M vasopressin (Leaf et al). The apparatus has been set up with the help of Dr. Luco of the Catholic University of Chile and at present the experiments are in an exploratory stage; there are no results to report.

V. LOBSTER NERVE PROTEIN

Last year's report listed some experiments by Mr. Whelchel on the characterization of proteins from lobster-claw nerve-axons. Some inconsistencies in these and earlier observations made by Maxfield in this laboratory led to a further investigation of this material. It was found that reproducible preparations were very difficult to obtain, but the over-all results did not convince us that the preparations isolated in this manner from lobster nerve contained any axon protein; rather they appear to derive from extracellular material, chiefly blood. Since this is not of immediate interest in this program, the detailed amino acid analyses projected were not performed.

VI. TECHNICAL DEVELOPMENTS

The resolution of the free-diffusion electrophoresis apparatus of Dr. K. Hannig for the separation of proteins, peptides and enzymes without alteration of biological properties has been demonstrated in experiments on collagen (Science, 139: 37, 1963). In these experiments the automated Technicon amino acid analyzer was proved adequate. To this equipment has recently been added an automatic ultraviolet analyzer (Model 1056, Vanguard Instrument Company) which provides monitoring function.

VII. THE BIOPHYSICAL AND BIOCHEMICAL BASIS OF MEMORY, LEARNING, AND COGNITIVE BEHAVIOR

The lectures by Dr. Leo De Maeyer, referred to in last year's report, reinforced the view that, if memory be coded in a specific type or types of macromolecules, the readout of the coded and stored information (memory) may involve a fast reaction such as might concern the fast transfer of elementary charged particles (protons or electrons) or of energy (excitation). Experiments to test such a process in a model system are being considered.

To facilitate advance in the whole area of the neurosciences relevant to the problem of the physical nature of the mind, a program has been initiated in collaboration with twenty-six other scientists. Known as the Neurosciences Research Program, this project has been formalized by the establishment of a Center in the House of the American Academy of Arts and Sciences in Brookline, Massachusetts, and a center staff including an Executive Officer (Dr. H. K. Gayer), an Information Specialist (Miss J. Morris), and secretarial staff. Four stated meetings of this group have already been held, and work sessions on two specific areas of the problem have been conducted. Plans are being made to catalyze the emergence and identification of a field which can best be described as molecular neurology, comparable to molecular genetics and molecular immunology. The larger systems aspects are also being studied by the group.

IX. PLANS FOR THE FUTURE

A. Immediate

Stockpiling of fibrous protein purified from dialyzed axoplasm and of lyophilized dialysate will be continued, so that in the Chilean winter when squid become scarce the analyses of amino acid composition and of structure by X-ray diffraction can be carried out. Meanwhile experiments on fresh material will be continued, particularly the attempts to isolate and characterize the monomer subunit of the filamentous protein.

Preliminary experiments indicate conditions important to success in injecting proteases into axoplasm to study the function of neurofilaments. Efforts will be made to gain headway in this problem which has been so intractable over the years.

B. Long-Range

The continuing twofold long-range aim is to advance the science of molecular neurology through investigation of the molecular organization, composition, and function of nerves and to seek evidence for the ability of macro-

molecules and their assemblies to function in the storage and retrieval of memory and in other aspects of learning and higher mental processes.

X. REPORTS AND PUBLICATIONS

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