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ONR REPORT DR-145

PROGRESS REPORT

ABSTRACTS

BIOCHEMISTRY PROGRAM

Office of Naval Research

1969

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Department of the Navy

Washington, D.C.

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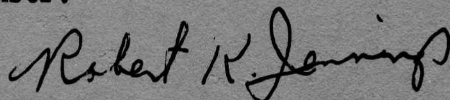
FOREWORD

Progress Report Abstracts of the Biochemistry Program is presented to a select and limited distribution list in the interest of coordination and communication among investigators being sponsored by this Program. It is hoped that, by this means, exchange of scientific information will be stimulated among those having related research interests.

These reports are preliminary in nature and do not constitute publication in the conventional sense. The material referred to herein will ordinarily be published in open scientific literature at a later date. These Abstracts are, therefore, to be considered as PRIVILEGED PERSONAL COMMUNICATIONS and must not be referred to without the written consent of the researcher and then only as a personal communication.

Credit for this volume belongs to the investigators who conducted the research and supplied the abstracts. We appreciate their cooperation and take pride in the high quality of the research that the Office of Naval Research has the privilege to sponsor.



ROBERT K. JENNINGS, Ph.D.
Biochemistry Program Director
Office of Naval Research

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INTRODUCTION

The series of annual abstract reports, viewed sequentially gives at least a partial view of the changes, and hopefully the progress, of the Biochemistry Program of the Office of Naval Research. Not all the changes reflect the influence of factors under the control of the Program Director. Changes in naval needs, in the overall distribution of responsibility for encouragement of basic research among the many government agencies, and changes in the methods, means and interests of the scientific community itself perhaps outweigh the plans for a particular balance among the several elements which constitute Biochemistry.

Over the past five years, the trend has been away from the type of organic chemistry of biological materials which only after assimilation into biology and medicine will provide the Navy with a return on its investment. We have been seeking areas in which truly basic research, not based on foregone conclusions as to what information would be generated, may nevertheless be expected to reveal new scientific principles, new processes and new products which the Navy would find of especial value. Life processes affect our effective naval action, whether through the influence on the mental and mechanical performance of men or the influence on equipment and materials. Knowledge of how they operate may provide us with the ability to control them, to imitate them or to avoid their consequences if need be.

We hope that the readers of this volume, who--for the most part are also those who have written it--will give some thought to their own areas of competence, asking what there may be in those areas that the Navy is particularly likely to need.

The program in calendar year 1968 covered studies in Biochemistry, Immunochemistry, and Pharmacology (with some rather special definitions of the content of these disciplines for the purpose of limiting the area of cognizance to the ONR mission). A short essay is appended to each group of reports, describing the program. Have we overemphasized anything? Have we missed anything?

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BIOCHEMISTRY STUDIES

The process of transforming the Biochemistry project from a program largely oriented toward the development of biochemical methods and the organic chemistry of compounds of potential biological and medical interest to one concerned with the understanding, control and possible application of selected life processes is nearly complete. The administrative view that the project is divisible into two parts; Marine Biochemistry and Metabolic Biochemistry, as it was presented in past years, is no longer meaningful. If subdivision is desirable, it might be more properly based on studies of biological functions on the one hand and biological products on the other, so far as the work reported in this year's abstracts is concerned.

Only three contracts dealing with enzyme structure-function relationships remained in the portfolio at the beginning of this fiscal year, and two of these were terminated so early that it was not appropriate to include reports. The "Biological Substances" which remain of concern are such items as Leucogenenol, fungistatic plant products and barnacle cement. "Biological Functions" range from bioluminescence through metabolic aspects of wound healing to the biochemical effects of environmental factors. Purposely, there is considerable overlap between the objectives of this part of the program and those described as Immunochemistry, the Biochemical Mechanisms of Toxic Environments, and "Pharmacology". If more than one interest is served, the probability of useful return is all the more certain.

To the program as outlined here has been added a new concern with the biochemical aspects of stress, involving the establishment of several contracts in the period which will be reported in next year's issue. This effort involves close interaction among the investigators in the several institutions and also with research in progress in naval in-house laboratories. If this kind of program planning proves to be as successful as early indications suggest, other central themes and team efforts may be the keynote in the future.

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PROPERTIES OF HYDROGENASE

David Rittenberg
Columbia University
New York, New York

ASSISTED BY

WORK UNIT NO. NR 108-047 **CONTRACT** Nonr 4259(02)

OBJECTIVES

(a) To investigate effect of wavelength on photochemical activation of CO inhibited hydrogenase, (b) to investigate the structure of the activated enzyme, (c) to investigate the relationships between structure and function in the deoxygenated enzyme inactivated by CO and not susceptible to subsequent photoactivation, and (d) to investigate the fractionation of deuterium in metabolic processes.

ABSTRACT

Contract terminated. No report requested.

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ENZYMATIC STUDIES

Hans Neurath
University of Washington
Seattle, Washington 98105

ASSISTED BY K. A. Walsh, R. A. Bradshaw, R. L. Stevens, W. P. Winter, A. Aboderin, R. W. Tye, and G. R. Reeck

WORK UNIT NO. NR 108-152 **CONTRACT** Nonr-477(35)

OBJECTIVES

To determine the relation between chemical structure, enzymatic function and phylogeny of proteolytic enzymes of various species, with particular emphasis on those of marine origin.

ABSTRACT

This program deals with comparison of the well-known proteolytic enzymes of higher animals with those of analogous enzymes obtained from marine organisms. The purpose of these investigations is to establish the relationship of the structure and function of enzymes, and the environment in which they act. The following projects have been carried out to this end during the period covered by this report:

1. Chemical structure of bovine pancreatic carboxypeptidase A. Chromatographic systems have been developed to separate and identify six different proteins corresponding in structure and function to carboxypeptidase A. Three of these differ from each other in the N-terminal region, representing different products of activation. Each of these three forms exists in two allotypic forms differing in two amino acid replacements in the molecule. All of these components have similar enzymatic properties. The work on amino acid sequence of carboxypeptidase A is nearing completion, including the 200 amino acid residue fragment which previously had resisted quantitative separation and identification of the various enzymatic fragments.

2. Proteolytic enzymes of the Pacific Spiny Dogfish. With the aid of local fishermen, a large supply of dogfish pancreas glands (14,000) has been obtained and subjected to large-scale separation procedures. It is anticipated to derive from these goodly amounts of chymotrypsinogen A, procarboxypeptidase A and B, as well as trypsinogen. The procarboxypeptidases and their corresponding enzymes will be subjected to detailed chemical and menzymatic characterization. Chymotrypsinogen and trypsinogen are currently under investigation.

The protein fractions of the pancreas of the African lungfish _procopterus_ have been separated chromatographically and many of them identified enzymatically. Continuing work will be directed toward the characterization of some of these enzymes.

Two trypsin-like enzymes have been isolated from the starfish _avasterias trochelii_.

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PLANS FOR FUTURE

Future work will be directed toward a more detailed characterization of the proteolytic enzymes of marine organisms, particularly those of the lungfish, starfish, and dogfish in comparison to those of mammalian pancreas.

CURRENT REPORTS AND PUBLICATIONS

(a) R. A. Kenner, K. A. Walsh, and Hans Neurath, "The reaction of tyrosyl residues of bovine trypsin and trypsinogen with tetranitromethane." Bioch. Biophys. Res. Commun., in press.

(b) H. Neurath, R. A. Bradshaw, L. H. Ericsson, D. R. Babin, P. H. Petra, and K. A. Walsh, "Current status of the chemical structure of bovine pancreatic carboxypeptidase A." Brookhaven Symposia, in press.

(c) P. H. Petra and H. Neurath (1968), "The heterogeneity of bovine carboxypeptidase A_{γ}." Fed. Proc. 27, No. 2.

(d) R. W. Tye, J. R. Uren, A. A. Aboderin, R. L. Stevens, R. Haynes, "Large scale preparation of pancreatic proteolytic enzymes and their zymogens from the spiny pacific dogfish." Pac. Slope Biochem. Conf.

(e) W. P. Winter and H. Neurath (1968), "Digestive proteases of the starfish." Fed. Proc. 27, No. 2.

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NUCLEIC ACIDS IN REGENERATING WOUND TISSUE

M. B. Williamson
Loyola University
Hines, Illinois

ASSISTED BY L. G. Wong

WORK UNIT NO. NR 108-315 **CONTRACT** Nonr 3502 (02)

OBJECTIVES

(a) To investigate the factors affecting the formation of wound tissue; (b) to study the formation of RNA in the cells of wound tissue, particularly in the nuclei of this tissue, with special reference to the synthesis of collagen; (c) to find the mechanism which utilizes the extensive amounts of cystine required for the formation of wound tissue.

ABSTRACT

Because the half-life of m-RNA in the cytoplasm is relatively short, the distribution of RNA in the nucleus has been studied. We have isolated fractions of nuclear RNA from regenerating wound tissue which have a relatively high G+C / A+U ratio. On the basis of the amino acid composition, the base code of the m-RNA required for the synthesis of both collagen chains might be expected to have a G+C / A+U ratio between 1.5 and 7.8, as outer limits.

Most of the nuclear RNA fractions isolated from wound tissue have a base ratio of less than one. However, the most abundant RNA fraction obtained has a ratio of about 1.5; other fractions with ratios up to 6.85 have been found. The relative abundance of these two fractions is 6:1. We postulate that the abundant nuclear RNA fraction is contaminated with RNA moieties from other fractions having a high G+C / A+U ratio. These relatively crude extracts presently are being fractionated further on Ecteola and sephadex columns. To obtain a clue as to their possible function, the rate of formation of these nuclear RNA sub-fractions will be studied at different stages of wound tissue development. The rate of ^{3}H-uridine incorporation will be the criterion to measure the rate of formation.

We have shown that half-cystine residues may be associated with proteins by means of both peptide and disulfide bonds or exclusively by a disulfide linkage. These two differently bound types of half-cystine residues can be distinguished by dialysis against Na_2CO_3 and Na_2SO_3. The turnover of the disulfide-bound half-cystine may occur spontaneously by means of an exchange reaction, but *in vivo*, appears to be controlled enzymatically. The

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rate of incorporation and turnover of both types of ^{35}S-cystine has been determined in the sub-cellular fractions of regenerating wound tissue. The greatest activity of disulfide-bound labeled cystine appears in the microsomal fraction, especially when collagen synthesis is at its greatest. The disulfide-exchange enzyme has been found to be most prevalent in the microsomes. We postulate a relationship between the disulfide turnover and protein synthesis. Experiments to test this postulation are presently in progress.

CURRENT REPORTS AND PUBLICATIONS

Utilization of cystine by wound tissue proteins. Fed. Proc. 26, 824 (1967).

Synthesis of nuclear RNA in regenerating wound tissue. Quart. J. Surg. Sci., 3, 105 (1967) (with T. Thachet).

Hétérogénéite de l'acide ribonucléique des noyaux céllulaires au cours de la cicatrisation des plaies. Arch. de Biochem. Cosm., 10, No. 98, 10 (1967) (with T. Thachet).

Inhibition of interaction of cystine with proteins by glutamic acid. Fed. Proc., 27, 392 (1968).

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EXPERIMENTAL STUDIES ON THE BIOCHEMISTRY AND BIOPHYSICS OF ENZYME MOLECULES

Thomas P. Singer
University of California
San Francisco, California

ASSISTED BY

WORK UNIT NO. NR 108-337 **CONTRACT** 5024(00)

OBJECTIVES

(a) The mechanism of the DPNH-Coenzyme Q reaction in mitochondria and in soluble enzymes, (b) to establish the relation of DPNH-ubiquinone reductase to cytochrome reductase, and (c) to isolate, characterize and determine the mechanism of cytoplasmic fumarate reductase.

ABSTRACT

Contract terminated. No report requested.

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FREE AMINO ACIDS AND RELATED SUBSTANCES IN MARINE ORGANISMS

Eugene Roberts and James S. Kittredge
City of Hope Medical Center
Duarte, California

ASSISTED BY Frank Sarinana

WORK UNIT NO. NR 108-458 **CONTRACT** Nonr-3001(00)

OBJECTIVES

(a) An investigation of the amino acids and related substances in marine organisms; (b) The detection, isolation and characterization of phosphonic acids; (c) The investigation of the mechanism of biosynthesis and metabolism of compounds containing covalent C-P bonds.

ABSTRACT

(a) We have isolated aminoalkylphosphonic acids from a planktonic amphipod, Amonyx nugax, and from a mixed net sample of phytoplankton and microzooplankton. The 2-aminoethylphosphonic acid phosphorus comprised 2.9 and 3.2%, respectively, of their total phosphorus. We then examined the possibility that crustacea may synthesize these compounds. The shore crab, Pachygraspus crassipes, did not incorporate $^{32}PO_4^{3-}$ into C-P compounds. The alternative explanation involved the synthesis of these compounds by the phytoplankton and assimilation by crustacea into their tissue lipids. We have, therefore, cultured various species of marine phytoplankton with $^{32}PO_4^{3-}$ and fractionated the phosphorus compounds that were stable to hydrolysis. We detected the synthesis of four aminophosphonic acids in each of four species of dinoflagellates and in two species of coccolithophorids. This work was repeated with the same positive results, but in each instance the total amount of phosphonic acids synthesized was low. We were unable to detect the synthesis of C-P compounds by a marine diatome, yeast nor bacteria. In the manuscript describing these observations, we point out that since the productivity of the oceans is a function of the rate of return of nitrate and phosphate to the euphotic zone, we must now re-evaluate the phosphorus cycle in the sea. The rather large amounts of aminophosphonic acids observed in several phyla, and the new finding that they are produced at the base of the food chain imply that this fraction of the total phosphorus may be removed from the cycle for a longer interim than ester phosphates.

(b) The resolution of the problems involved in the degradation of labeled 2-aminoethylphosphonic acid (2-AEP) allowed us to localize the ^{14}C label incorporated into 2-AEP from a number of ^{14}C labeled glycolytic intermediates which were likely precursors. The analysis of the data indicate that either phosphoenolpyruvate (PEP) or oxaloacetate (OAA) (which compounds are intraconvertable by phosphoenolpyruvate carboxykinase), are the likely carbon precursors in the biosynthesis of 2-AEP and that the 1-carbon of 2-AEP is derived from the 3-carbon of PEP or OAA and the 2-carbon is

derived from the 2-carbon of these compounds.

(c) During the course of the fractionation of labeled lipid extracts of the sea anemone, Anthopleura xanthogrammica, in a search for lipids containing the recently isolated N-methyl derivatives of 2-AEP, we observed the release during hydrolysis of one lipid fraction of a strongly anionic phosphonic acid. This fragment has now been tentatively identified as phosphonoacetic acid by its behavior during electrophoresis and GLC. A further isolate has been methylated and sent out for mass spectral identification. Since this observation fits remarkably well with the proposed route of biosynthesis of 2-AEP, i.e., phosphonoacetic acid would be the expected hydrolytic product, by decarboxylation, of a lipid containing phosphonopyruvate, we are now attempting to determine the relative rates of labeling of lipid-phosphonopyruvate and lipid-2-AEP in A. xanthogrammica exposed to $^{32}PO_4{}^{3-}$ for varying periods of time. The removal of water soluble contaminants from the lipid extract on Sephadex columns and fractionation of the lipid on DEAE columns followed by hydrolysis and fractionation of the products by paper electrophoresis has resulted in the desired fractions, but still containing traces of orthophosphate in the phosphonoacetic acid.

PLANS FOR FUTURE

(a) Examination of other species in an effort to obtain a cell-free system which will synthesize C-P compounds (e.g., preliminary examination of the sea hare, Aplisa californica, indicates a rapid synthesis of C-P compounds), (b) Isolation and characterization of the lipid yielding acetophosphonic acid on hydrolysis.

CURRENT PUBLICATIONS

(a) Roberts, E., Simonsen, D. G., Horiguchi, M., and Kittredge, J. S. Transamination of Aminoalkylphosphonic Acids with Alpha Ketoglutarate. Science 159, 886-888 (1968).

(b) Horiguchi, M., Kittredge, J. S., and Roberts, E. Biosynthesis of 2-Aminoethylphosphonic Acid in Tetrahymena. Biochim. Biophys. Acta 165, 164-166 (1968).

(c) Holden, J. T., van Balgooy, J. N. A., and Kittredge, J. S. Transport of Aminoalkylphosphonic Acids in Lactobacillus plantarum and Streptococcus faecalis. J. Bacteriol. 96, 950-957 (1968).

(d) Kittredge, J. S., Horiguchi, M., and Williams, P. M. Aminophosphonic Acids: Biosynthesis by Phytoplankton. Comp. Biochem. Physiol. in press.

(e) Kittredge, J. S., and Roberts, E. The Carbon-Phosphorus Bond in Nature. An invited review for Science, manuscript revised.

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BIOCHEMISTRY OF THE LUMINESCENCE OF MARINE ORGANISMS

J. Woodland Hastings
Harvard University
Cambridge, Massachusetts

ASSISTED BY George Mitchell, Joan Friedland, Anne Gunsalus, Margaret Fogel, Warren Duane, James Morin, Ruth Schmitter

WORK UNIT NO. NR 108-495 **CONTRACT** N00014-67-A-0298-0001

OBJECTIVES

(a) To elucidate the molecular mechanisms involved in bioluminescence of marine organisms where chemical energy is converted to light in enzymatic reactions, (b) to gain insight into the role and control of bioluminescence, and its special prevalence in marine forms.

ABSTRACT

Over the past years my laboratory has concentrated especially on two groups of marine organisms, the bacteria and the dinoflagellates. This year we successfully initiated work on Ctenophores and Coelenterates.

The biochemical systems responsible for bioluminescent flashing in _Mnemiopsis leidyi_ and three species of _Obelia_ (_bicuspidata_, _geniculata_ and _longissima_) were isolated and partially characterized. In each case the system involves a calcium activated protein having a M.W. of about 18,000. The active proteins were isolated by extraction in 0.04 M EDTA in 0.2 M Tris at pH 10, and after dialysis are stable for at least several weeks. Bioluminescence can be evoked simply by the addition of excess calcium. The light emission occurs as a flash having a duration of about one second. The identity of the energy-yielding chemical step is obscure. The reaction does not utilize or depend upon the presence of molecular oxygen. Although the protein isolated from _Mnemiopsis_ is similar in its response to calcium and in its M.W., it has the unusual property of being rapidly inactivated by exposure to light. Such is not the case in _Obelia_ even though light may inhibit the _in vivo_ bioluminescent flashing.

A study of the localization of light emission within the _Obelia geniculata_ and _O. longissima_ hydroid stage was achieved by photographs taken through an image intensifier. Luminescence was initiated by stimulating across the colony perisarc with fine silver-silver chloride electrodes. These studies show that the stolons, stems and pedicels are luminescent, while the hydranths and gonangia are not. The light emanates from discrete, irregularly spaced spots. Successive pictures show that the spots are in fixed positions along the coenosarc.

In studies with the bacterial system we have found that the color of the light-emission in the _in vitro_ reaction can be greatly altered using different flavins. The presumed natural substrate is reduced FMN, which results in an _in vitro_ emission peaking at about 490 nm, considerably shifted from the fluorescent emission at 530 nm. Bioluminescence stimulated with reduced iso-FMN has its peak emission at 470 nm, but this cannot be attri-

buted to the excited singlet as such, since fluorescence of iso-FMN peaks at about 550 nm. Another flavin of interest is the 2-thio analog, which though non-fluorescent, gives good bioluminescence activity, but with emission at 530 nm.

Bioluminescence can be initiated by flash excitation of luciferase without the addition of flavin. This has now been shown to be due to a modified luciferase with an altered subunit and a non-covalently bound small molecule; the latter can be reversibly removed and its identity is presently being investigated. These results were reported at the International Photobiology Congress in August.

A study has been undertaken of mutants having lesions in the luminescent system. In conjunction with this the in vivo control of the synthesis of luciferase is being studied, from which we have concluded that luciferase is an inducible enzyme. These results were reported at the 1968 American Society for Microbiology Meetings in Detroit.

Studies on the soluble and particulate bioluminescent systems of Gonyaulax continue to be fruitful. The subunit structure of the soluble luciferase was reported, showing that both the enzyme and its subunit are active. When the pH is lowered, the particles (termed scintillons) emit a bright short flash of light which closely resembles the flash of the living organism. We have now been able to obtain repeated flashes from the scintillons by "recharging" them with fresh substrate (luciferin). Flashing is thus envisioned to involve the utilization of luciferin in the scintillon followed by the exchange of the product for fresh substrate.

Ultrastructural (electron microscope) studies of scintillons are concurrently being undertaken, and a more complete description of this unusual bioluminescent system can be expected to be available in the near future.

PLANS FOR FUTURE

(a) To determine the molecular species involved in the emission of the Ctenophores and the way in which light inactivation of the protein occurs (b) To similarly determine the excited species in bacterial luminescence and the mechanism of the reaction. The further study of the light induced bioluminescence should be of special value in this regard. (c) To fully characterize the Gonyaulax luminescent system and the structure and role of the scintillons.

CURRENT REPORTS AND PUBLICATIONS

(a) R. DeSa and J. W. Hastings (1968), "The characterization of scintiloons: bioluminescent particles from the marine dinoflagellate, Gonyaulax polyedra." J. Gen. Physiol., 51, 105-122

(b) J. W. Hastings (1968), "Bioluminescence." An. Rev. Biochem. 37, 597-630

(c) K. Nealson, T. Platt and J.W. Hastings (1968), "Bacterial luciferase as an inducible enzyme." Bacteriol. Proc. 46

(d) N. Krieger and J.W. Hastings (1968), "Bioluminescence: pH activity profiles of related luciferase fractions." Science 161, 586-589

(e) J.W. Hastings and J.G. Morin (1968),"Calcium activated bioluminescent proteins from Ctenophores (Mnemiopsis) and colonial hydroids (Obelia)" Biol. Bull. 135, 422

(f) J.G. Morin, G.T. Reynolds and J.W. Hastings (1968), "Excitatory physiology and localization of bioluminescence in Obelia." Biol. Bull. 135, 429-430

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THE DEVELOPMENT OF FUNCTIONAL CELL STRAINS

Samuel Graff
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New York, New York

ASSISTED BY

WORK UNIT NO. NR 108-517 **CONTRACT** 266(76)

OBJECTIVES

(a) To study animal cell replication and intermediary metabolism, and (b) to utilize animal cell culture systems for the production of masses of epithelial cells for prospective post-burn therapy grafts, for the production of bone marrow cells for prospective post-irradiation replacement therapy and for the production of interferon.

ABSTRACT

Contract terminated. No report submitted.

BR 83002 DI.DP. IR.07.04 P101

CARBOHYDRATE BIOCHEMISTRY OF ALGAE

B. J. D. Meeuse
University of Washington
Seattle, Washington

ASSISTED BY

WORK UNIT NO. NR 108-535 **CONTRACT** Nonr 477(42)

OBJECTIVES

(a) To study the chemical nature of the reserve carbohydrates of the various groups of algae and members of the phytoplankton, (b) to investigate the biosynthesis and enzymatic breakdown of these compounds, (c) to establish the spectrum of carbohydrate-splitting enzymes present in various invertebrates feeding on algae, and (d) to study the nature and mode of action of some of these enzymes.

ABSTRACT

Permission to omit this year's abstract granted.

BR ... DP ... 07.01 F11

LYSOSOMES AND INTRACELLULAR DIGESTION IN SEA STARS

George S. Araki
San Francisco State College
San Francisco, California

ASSISTED BY

WORK UNIT NO. NR 108-688 **CONTRACT** N00014-66-C-0317

OBJECTIVES

To investigate intracellular digestion in the marine invertebrate Patiria miniata and to ascertain the involvement of lysosomes.

ABSTRACT

None received.

BR R3COC DL DP DR C3 C1 F11V

STUDIES ON PHOTOSYNTHESIS AND PHOTOTROPISM

H. Gaffron
Florida State University

ASSISTED BY G. H. Schmid, E. Kessler and G. Harnischfeger

WORK UNIT NO. NR 108-668 **CONTRACT** NONR 4641 (00)

OBJECTIVES

Use of instruments acquired under the now expired contract NONR 988 (10) for the measurement of photochemical responses in pigment-sensitized artificial and natural systems.

ABSTRACT

With the instruments acquired under contract NONR 988 (10) and enumerated in previous reports, we have followed the changes of absorption spectra and of photosynthetic capacities in leaves and algae with different chlorophyll contents. The newest results in the series of papers published by Schmid and Gaffron concern the nature of the so-called photosynthetic unit. The unit is defined as the inversion of the efficiency of carbon dioxide fixation or oxygen evolution per chlorophyll molecule by light saturating flashes of short duration. In the present experiments, the flashes were no longer than 40 μsec, and their intensity surpassed considerably those used years ago in other experiments of this kind. Measurements with algae, and particularly with healthy young leaves of various species of tobacco, revealed that there is no constant chlorophyll unit which is characteristic for the photosynthetic apparatus of the kind discussed since 1932 in the literature. Instead, experiments performed with saturating flashes superimposed on a continuous low background illumination revealed the existence of at least five sizes of units, ranging from 300 to 5000 chlorophyll molecules per CO_2 reduced. These units appeared to be well defined structural entities, which increased by doubling roughly from 300 to 600, 1200, 2400 and 5000 chlorophyll molecules. The old concept of the unit had better be abandoned and replaced by a term for flash efficiency. The smallest unit of the old definition simply means the highest efficiency per flash ever found in these experiments.

CURRENT REPORTS AND PUBLICATIONS

(a) G. H. Schmid (1967), "The Influence of Different Light Intensities on the Growth of the Tobacco Aurea Mutant Su/su." Planta 77, 77-94.

(b) P. H. Homann and G. H. Schmid (1967), "Photosynthetic Reactions of Chloroplasts with Unusual Structures." Plant Physiol. 42, 1619-32.

BR RILOC DL DP DR 07.09

(c) G. H. Schmid and H. Gaffron (1968), "Photosynthetic Units." J. Gen. Physiol. 52, 212-239.

(d) T. S. Stuart (1968), "Revival of Respiration and Photosynthesis in Dried Leaves of Polypodium polypodioides", Planta, 83, 185-206.

BR R3 ... DL DP. DR 01.04. F ...

APPLICATION OF MICROWAVE ABSORPTION AT 3000 MC FOR THE DETERMINATION OF ELECTROLYTE CONTENT OF SOLUTIONS

A. A. Albanese
The Winifred Masterson Burke Relief Foundation
White Plains, New York

ASSISTED BY L. A. Orto, E. H. Wein, and B. J. Hunter

WORK UNIT NO. NR 108-703 **CONTRACT** Nonr 5036 (00) M 1

OBJECTIVES

Development of microwave instrumentation for the quantitative determination of total electrolytes of body fluids in vitro and in situ and application of the procedures to problems of water balance in man and studies in marine biology and biochemistry.

ABSTRACT

During the past year further improvements were made in the microwave electrolyte analyzer. Sensitivity has been increased threefold. Size has been decreased progressively from 14 cu. ft. to 1 cu. ft. to 0.25 cu. ft. Portability has been achieved by use of a self-contained 24 V rechargable battery pack and reduction of total weight to approximately 6 lbs.

It should also be noted that the microwave absorption principle of electrolyte analyses is completely non-destructive and the instrument permits continuous analyses of flowing fluids. Further, this procedure avoids thermal and electrolytic dissociation of the sample and polarization of electrodes which prevail in conductivity analyses as well as sample degeneration encountered in cryoscopic analyses. This characteristic of microwave analyses affords unique advantages in the several biological applications now under consideration or study.

Specifically, replacement of the conductivity cell now employed in an available single patient dialysate system by the microwave analyzer would obviate problems of electrolytic alterations of circulating body fluids. This dialysate unit is employed in the clinical management of patients suffering from acute or chronic renal failure or shock of severe burns or other trauma.

Relevant applications such as the monitoring of ionic changes in stored whole blood and osmotic fragility of human erythrocytes are now underway. Microwave measurements on whole blood stored at 32°C have shown that incipient hemolysis of the sample results in an increased coefficient of total electrolyte content, apparently due to a liberation bound potassium from the lysis of red blood cells. Determination of human erythrocyte fragility by the present method has proved far simpler and more accurate than assays by conventional techniques. Studies on the effect of

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age, disease, and medications (anticoagulants) on erythrocyte fragility are in various stages of progress.

Since total electrolyte content of urine (Jacobsen, et al., Arch. Int. Med. 110: 83, 1962) has been found to have greater clinical significance, as a criterion of kidney function, than specific gravity measurement the microwave procedure is being employed routinely for urinalysis in our laboratory.

PLANS FOR FUTURE

(a) Achievement in size reduction of the apparatus from 14 cu. ft. to 0.25 cu. ft. with increased portability, sensitivity, and stability will now permit us to return to the original goal of this project, namely determination in situ of body water and electrolytes of test subjects. Development of this technique would be particularly useful in making such determinations which are required at frequent intervals in patients debilitated by secondary malnutrition, trauma, or chronic diseases. (b) Lastly, explorations are in progress to ascertain the feasibility of employing microwaves of 10,000 MC for electrolyte analyses. Operation at this frequency (x-band) affords the advantage of further miniaturization of instrumentation sample size or area.

CURRENT REPORTS AND PUBLICATIONS

(a) A. A. Albanese and L. A. Orto (1968), "The Proteins and Amino Acids." in "Modern Nutrition in Health and Disease." 4th Edition, (Wohl and Goodhart, editors), Lea and Febiger, Philadelphia.

(b) A. A. Albanese, E. J. Lorenze and L. A. Orto (1968), "Effect of Strokes on Carbohydrate Tolerance." Geriatrics 23:142-150, March.

(c) A. A. Albanese, A. H. Edelson, E. J. Lorenze, and E. H. Wein (1968), "Quantitative Radiographic Survey Technique for Detection of Bone Loss." Presentation at 25th Ann. Meet. of Am. Geriatric Soc., New Orleans April 25-28 ALSO: Geriatric Times 2:10, Oct. ALSO: J. of the Am. Geriatric Society, In Press.

(d) A. A. Albanese, E. J. Lorenze, L. A. Orto and E. H. Wein (1968), "The Effect of Anabolic Steroids on Hypercalciuria of Corticosteroid Therapy" Presentation at 5th Ann. Meet. of the Am. College of Clin. Pharmacology and Chemotherapy, Atlantic City, May 2-4.

(e) A. A. Albanese, E. J. Lorenze, L. A. Orto and E. H. Wein, (1968), "Nutritional and Metabolic Effects of Some Newer Steroids V Norbolethone." N. Y. State J. of Med. 68:2392-2406, Sept. 15.

(f) A. A. Albanese (1968), "Anticatabolic Applications of Newer Anabolic Steroids." Med. Times 96:871-881, Sept.

(g) A. A. Albanese, A. H. Edelson and E. J. Lorenze (1969), "Quantitative Radiographic Technique for the Detection of Bone Loss." Exhibit at Med. Soc. of State of N. Y., New York City, Feb. 9-13.

(h) A. A. Albanese, E. J. Lorenze, M. L. Woodhull, E. H. Wein, and L. A. Orto (1969), "A Preliminary Objective Evaluation of Therapeutic Agents for Management of Bone Loss." 6th Ann. Meet. of the Am. Soc. of Clin. Pharm. and Chem., Atlantic City, May 1-3.

BR F... ... F ... C2 ... F43V

HYDROSTATIC PRESSURE EFFECTS ON PROTEINS

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ASSISTED BY

WORK UNIT NO. NR 108-708 **CONTRACT** Nonr N00014-66-C0253A02

OBJECTIVES

(a) Ultimately to understand the marine biosphere in molecular terms, (b) To use macromolecular models to study the effect of temperature and hydrostatic pressure on subcellular components of living systems in the marine biosphere.

ABSTRACT

A system for studying hydrophobic interactions in proteins, the thermally induced aggregation of polyvalyl RNase, has been further studied under conditions of slower reaction. The conditions of 0.01 M phosphate buffer at pH 6.4 and temperatures of 20 to 30°C have yielded reaction rates slow enough for study in a high pressure optical cell. Under these conditions we have also been able to make estimates of the Arrhenius activation energy, E_a, for the aggregation reaction. The E_a appears to have a complex relationship to the ionic strength of the medium, such that a clear interpretation is not yet obtained. The E_a ranges from 50 kcal/mole PVRNase at 0.01 M salt to 128 kcal at 0.1 M salt. But the value drops to 88 kcal at 0.2 M salt and rises again to 120 kcal at 0.6 M salt.

Using the logarithmic relationship between reaction rate and pressure (Trans. Faraday Soc. 31, 875, 1935), we have calculated the activation volume, ΔV^*, for PVRNase aggregation under various conditions. Pressures up to 300 atm were used in these measurements. In agreement with earlier observations (Biochem. Biophys. Res. Comm. 22, 262, 1966), the ΔV^* decreased with increasing salt concentration (200 ml/mole at 0.15 M salt to 55 ml/mole at 0.4 M salt).

This might be explained by a change in the activity of water brought about by the added salt. The promotion of aggregation reaction by added salt is expected for hydrophobic interactions and may be contrasted to the effects of salts on the polymerization of myosin which has been attributed mainly to ionic interactions (R. Josephs and W. F. Harrington, Biochemistry 7, 2834, 1968). The change in activation volume for the PVRNase aggregation as a function of temperature was more dramatic. At pH 6.4 and 0.15 M NaCl the ΔV^* increased from 210 ml/mole at 30°C. More recently we have succeeded in obtaining difference spectra of RNase under high pressure. Native RNase appears to be stable to 900 bars, but partially unfolded RNase is further unfolded upon the

application of high pressures. However, this pressure enhancement of partially unfolded RNase seems to involve more than one spectrally observable change. There may be two reactions taking place under pressure, one reversible and the other irreversible.

PLANS FOR FUTURE

(a) Further characterize pressure-volume relationships in the transconformation reactions of biopolymers and in the association and dissociation reactions of biological macromolecule systems (b) extend studies to other biopolymer systems which will respond to high pressure.

CURRENT REPORTS AND PUBLICATIONS

(a) A. H. Nishikawa, R. Y. Morita, and R. R. Becker (1968), "Effect of the solvent medium on polyvalyl-ribonuclease aggregation." Biochemistry 7, 1506-1513.

(b) R. Y. Morita (1969), "Application of hydrostatic pressure to microbial cultures." In D. W. Ribbons and J. R. Norris (eds.), Methods in Microbiology, Academic Press (in press).

BR R7002 DC DP DR.07.04 F49V

STUDIES ON AMINE METABOLISM DURING ANAPHYLAXIS

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ASSISTED BY S. Nadackapadam and M. Grossman

WORK UNIT NO. NR 108-716 **CONTRACT** N00014-57-A-0397-0001

OBJECTIVES

(a) To isolate and purify the "slow reacting substance" (SRS) of Brocklehurst, (b) to determine which labelled metabolites are incorporated into SRS (when these metabolites are injected into immune guinea pigs) as a means of identifying the chemical nature of SRS.

ABSTRACT

Ovalbumin-sensitive guinea pigs were injected intraperitoneally with 200 μC/Kg body weight of N-acetyl-^{3}H-mannosamine and sacrificed one hour later. In a typical preparation, six animals were used and one lung from each animal was crushed between metal blocks chilled to the temperature of liquid nitrogen. The pool of frozen lungs served as a control to the other lungs which were sliced with a McIlwain tissue-chopper and incubated with ovalbumin (1 mg/ml) in Tyrode-bicarbonate solution for 10 minutes at 37°C with O_2/CO_2 (95/5).

The tissues were extracted by homogenization in Tyrode and deproteinized with zinc sulfate and sodium hydroxide. Extracts were concentrated and fractionated on one meter columns of Sephadex G-25 with distilled water.

Absorbance at 280 mμ of all fractions was measured as well as radioactivity, the latter by using Triton-X-100 in the scintillation counting fluid.

Out of a series of small peaks of optical density, only a narrow zone showed radioactivity. One of the radioactive peaks was found to be coincident with the only fraction giving a positive bioassay for slow-reacting substance (SRS), as measured by the contraction of guinea pig ileum.

The preparations of SRS described below were prepared by the method of Brocklehurst, that is, the lungs were perfused with warm Tyrode, and after challenge with antigen, were extracted by grinding with cold ethanol (3 ml/g) and sand. Each dried extract was dissolved in Tyrode and chromatographed on Sephadex G-25.

Injection of N-acetyl-^{3}H-mannosamine, as above, into the animals prior to sacrifice, similarly resulted in the appearance of radioactivity in the fraction giving bioassay.

Extracts from frozen non-challenged immune lung similarly showed radioactivity in the comparable peak material. These materials, however, gave little or no bioassay.

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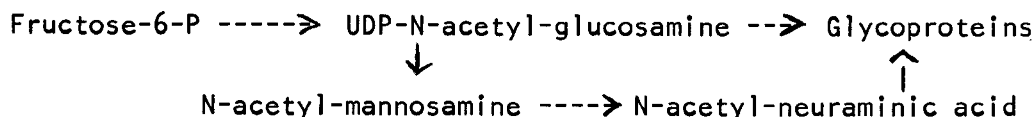
Injection of 40 μC/Kg body weight of Fructose-6-phosphate-^{14}C into batches of immune animals one hour prior to sacrifice, resulted in the appearance of radioactivity in a larger number of the fractions separated on columns of Sephadex G-25.

In these experiments the results indicated that a wide variety of carbohydrate-containing substances were labelled by Fructose-6-phosphate-^{14}C whereas the administration of N-acetyl-^{3}H-mannosamine led to a more exclusive labelling of the SRS. These data are consistent with the known metabolic pathways:



Hydrolysis of the SRS fraction and adjacent column fractions in 0.1N H_2SO_4 at 80°C for 2 hours led to the release of carbohydrates. Thin layer chromatography of these products on silica gel using propanol:H_2O (7:3) or propanol:ethyl acetate:H_2O:25% ammonia (60:10:30:10) followed by spraying with p-anisidine, acid resorcinol (Svennerholm, Biochim. Biophys. Acta <u>24</u>, 604, 1954) or the sequence of ninhydrin-Dragendorff reagent-molybdate indicated that glucosamine and galactosamine were present in fractions containing SRS.

Autoradiograms of the thin-layer chromatograms were obtained by exposing Kodak No-Screen film to the plates for 4-8 weeks prior to film development, and glucosamine and galactosamine moieties in SRS were shown to have become labelled as a result of administration of Fructose-6-phosphate-^{14}C to guinea pigs. The results suggest the presence of carbohydrate in SRS.

In similar experiments with immunized guinea pigs, intraperitoneal injection of 40 μC/Kg of Choline-(^{14}C-Methyl) one hour prior to sacrifice has shown that the SRS fraction (derived from challenged lungs) on Sephadex G-25 chromatography coincides with a well-defined peak of radioactivity.

Data from autoradiograms is not yet available.

PLANS FOR FUTURE

To study the metabolic fate of further radioactive compounds in guinea pig lung as a means of ascertaining the composition of SRS.

CURRENT REPORTS AND PUBLICATIONS

Manuscripts are in preparation.

BR R3C0C D. DP DR 07.01 545V

IMMUNOPHARMACOLOGICAL STUDY OF INFLAMMATION IN MICE

Edwin M. Uyeki
Department of Pharmacology, University of Kansas Medical Center
Kansas City, Kansas

ASSISTED BY D. E. Griswold

WORK UNIT NO. NR 108-745 **CONTRACT** N00014-67-C-0166

OBJECTIVES

To study the role of pharmacological mediators in exudative inflammation induced by prior immunizations (hypersensitivity) with different antigens.

ABSTRACT

I. General statement.

Using the paw edema assay in mice as an experimental model for exudative inflammation, we propose to study the role of pharmacological mediators in exudative inflammation induced by prior immunizations (hypersensitivity) with different antigens. Experimentally we recognize 4 types of paw edema swelling, classified on the basis of their temporal development and with the use of selective antigens. As a working hypothesis, we propose that paw edema which can be antigenically induced in mice is dependent on a proliferating pool of immunocompetent cells derived from precursor cells. These precursor cells respond to antigenic challenge by producing cell populations (either antibody-producing cells or cell-borne cells) which express their immunocompetence by the degree of paw swelling. Whether these arbitrarily classified types of paw edema swelling correspond to anaphylactic, immediate and delayed hypersensitivities will be one of the main objectives of the proposal.

II. Studies on pharmacological mediators on mouse and rat paw edema.

Our initial studies dealt with effects of various pharmacological mediators of inflammation in different strains of mice. The substances tested for reactivity in mouse paw swelling are (1) serotonin or 5-HT, (2) bradykinin or BK, (3) histamine and carrageenin. Following is a synopsis of our findings on paw edema in mice induced by subplantar footpad injections of 20 microliter quantities of the agents:

a. Ha/ICR strain of mice differs significantly in its response to 5-HT as compared to LAF_1 and A/He mice.

b. LAF_1 and CF_1 are significantly different from A/He in their response to dextran administration.

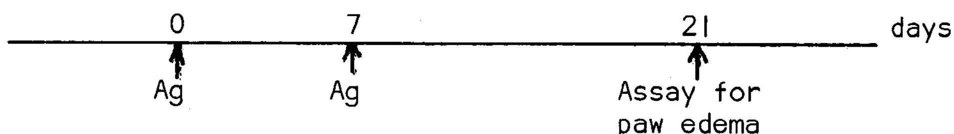
c. In response to subplantar injections of high doses (20 micrograms) of bradykinin, all of the mice strains respond well, as indicated by the 3-4 mm of pen deflection (one mm of pen deflection is equivalent to a volume of 25.5 microliters of paw swelling in our plethsmographic measurements). However, at lower concentrations of BK (viz., 2 micrograms) A/He is significantly higher than all other strains.

d. Although differences are observed with subplantar injections of histamine, all strains of mice respond poorly to histamine injections. The range of values shown on the chart indicates 0.6-1.5 mm of pen deflections. Moreover, the 500 microgram quantity of histamine represents an enormous dose.

e. Subplantar injections of various mediators into rat paws indicated that rats are generally much more responsive to mediators than mice. This investigation provides an important baseline for studies involving antigenically-stimulated edema formation and the possible relationship of pharmacological mediators to edema formation.

III. Studies on immediate response to subplantar injections of bovine serum albumin (BSA) in HA/ICR mice.

The protocol for the induction period is the following:



Antigen: a) One mg of bovine serum albumin (BSA) injected subscapularly two times. b) One mg of bovine serum albumin plus Complete Freunds Adjuvant (CFA) subscapularly.

Agent to elicit paw edema response: 200 µg of BSA in 20 µliter amounts injected into footpad.

In animals given BSA with CFA, approximately 50% succumb from anaphylactic shock, usually within one hour. Instead of 200 µgm, if 20 µgm are injected in footpads, there is immediate swelling. These animals show few symptoms of the shock-like state without accompanying mortalities. At first glance, it would appear that edema resulting from the lower dose (20 µg) "mirrors" the primed animal's propensity to go into the shocked state at higher doses. Mr. Griswold is currently devising experimental procedures to consider this viewpoint. Experiments dealing with BSA injections without CFA indicated that the resulting edema is susceptible to cyclophosphamide, prednisone and, to a lesser extent, amethopterin. These studies were interpreted as indicating that cellular proliferation is a prerequisite for eliciting the paw edema response. The action of Cinanserin, a potent inhibitor of serotonin and histamine, to partially inhibit paw swelling in this system is not understood. More experiments are needed to confirm this observation.

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BIOCHEMISTRY AND PHARMACOLOGICAL PROPERTIES OF LEUCOGENENOL

F. A. H. Rice
The American University
Washington, D. C.

ASSISTED BY

WORK UNIT NO. NR 108 772 **CONTRACT** N00014-67-C-0275

OBJECTIVES

To study the biochemical and pharmacological properties of leucogenenol, a metabolic product isolated from cultures of Penicillium gilmanii, that induces a leucocytosis when injected into animals.

ABSTRACT

Injection of leucogenenol intravenously into mice caused an increase in the number of myeloblasts in the bone marrow. There was a lesser increase in the intermediate forms leading to and including the mature neutrophil. Basophils, megakaryocytes and cells of the eosinophil series were not affected. There was also an increased number of cells in mitosis.

The myeloid and lymphoid tissues of irradiated mice injected with leucogenenol recovered more rapidly than uninjected controls.

Injection of leucogenenol decreased the latent period for hemolysin formation in irradiated mice.

Leucogenenol affected the respiration of lymphoblasts in tissue culture at a level of 10 to 100 molecules per cell.

Leucogenenol has been found to be a normal constituent of beef and human liver.

PLANS FOR FUTURE

Continue present studies.

CURRENT REPORTS AND PUBLICATIONS

(a) F. A. H. Rice (1966), "Isolation from Penicillium gilmanii of a substance that causes leucocytosis in rabbits." Proc. Soc. Exptl. Biol. & Med., 123, 189-192.

(b) F. A. H. Rice and Meredith Barrow (1967), "Chemical determination of leucogenenol and its production by Penicillium gilmanii." J. Applied Microbiology, 15, 790-793.

(c) F. A. H. Rice (1968), "Leucocyte response to the

BK RJGOC DL DP DK 07 01 F17

injection of leucogenenol in rabbits and mice." J. Infect. Dis., 118, 76-84.

(d) F. A. H. Rice and J. H. Darden (1968), "Effect of intravenous injection of leucogenenol on the blood cells of the bone marrow." J. Infect. Dis., 118, 289-292.

(e) F. A. H. Rice, Joan Lepick and J. H. Darden (1968), "Studies of the action of leucogenenol on the myeloid and lymphoid tissues of the sublethally irradiated mouse." Radiation Research, 36, 144-157.

(f) F. A. H. Rice (1969), "Effect of saponin on the average size and distribution of leukocytes in blood from normal and irradiated mice." Radiation Research, subm.

(g) F. A. H. Rice, Joan Lepick and Patricia Patterson (1969), "Effect of leucogenenol on hemolysin formation in the irradiated mouse." Radiation Research, subm.

(h) F. A. H. Rice and A. Rene (1969), "Action of leucogenenol on the respiration of lymphoblasts in tissue culture." Proc. Soc. Exptl. Biol. & Med., subm.

BR R:02 DL DP DR 07.01 F1+V

PRODUCTION OF PHYTOALEXINS BY THE ORCHIDACEAE

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Irvine, California 92664

ASSISTED BY Brigitta Flick

WORK UNIT NO. NR 108-796 **CONTRACT** ONR 00014-67-0323-0002

OBJECTIVES

(a) To investigate the reaction of certain angiosperms (members of the Orchidaceae) following fungal attacks on infection. (b) Elucidate the nature of the fungus-produced, phytoalexin-inducing compound(s). (c) Isolate and identify phytoalexin(s) produced by orchids. (d) Assess the value of orchid phytoalexins in the treatment of fungal infections and protection of material.

ABSTRACT

Orchids are universally associated with mycorrhizal fungi. To establish successful symbiotic relationships the fungus must be present, but its growth controlled to the extent of not allowing it to spread beyond a defined region of plant cells. Such control depends on the ability of the plant to produce fungistatic compounds, i. e., phytoalexins. This in turn hinges on production by the fungus of phytoalexin inducers. Some fungi apparently cannot induce phytoalexins, and, if pathogenic, completely parasitise and eventually kill their hosts, whereas others simply exist side by side with the orchid neither attacking nor being inhibited. The first step in our program was therefore to cross test several fungi and orchids for phytoalexin induction.

A total of 20 different fungal species from various sources were employed, including some used in previous investigations by other workers, and several isolated from plants used in our experiments. Pseudobulb tissues of *Cymbidium* and *Cattleya* orchids were employed as potential sources of phytoalexins. These tissues generally contain deep seated bacteria and require double decontamination procedures. Pseudobulbs were peeled and exposed for one hour to gaseous propylene oxide followed by immersion in saturated calcium hypochlorite for 30 min. Tissue cylinders and slices were exposed to fungi in petri dishes on potato dextrose agar. All of the fungi tested are extremely slow growing and each test or assay requires about a four week period. Of over forty orchid/fungus combinations tested to date, clear and distinct halos were observed only in two instances (Fig. 1A, 1B) both involving *Cymbidium* tissues, but two separate strains of *Rhizoctonia repens*, both isolated from orchid roots. *Cattleya* tissues did not, as a rule, respond clearly to most fungi although in at least one instance fungal inhibition may have been due to tissues and their extracts.

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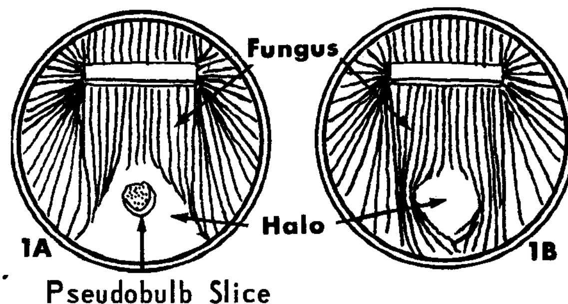
Following formation of halos (Fig. 1A) the tissues were removed and extracted. Even 10 days after removal of the tissues no encroachments of the fungus into clear areas could be noted (Fig. 1B). We interpret this to mean that phytoalexin(s) release by the tissues did occur and that the compound(s) is (are) relatively stable. These assumptions are now being tested utilizing extracts of the tissues which produced the halos. Tissues which have not been exposed to fungi are used as controls.



In parallel with phytoalexin induction assays we have also invested time in developing methods for the culture of orchid tissues. The purpose of this is to enable us to isolate and maintain tissue clones which are especially suitable for studies, or as sources of phytoalexins. To date we have tested approximately 75 culture medium-tissue combinations and have established pilot cultures of root tips which seem to grow well, albeit slowly (in the manner of all orchids); maintained root sections green for long periods of time and are on the verge of producing protocorms and what appear to be normal seedlings from leaf tips.

PLANS FOR FUTURE

As a first order of business we intend to repeat our successful phytoalexin induction tests. This will be followed by isolation and purification of phytoalexin(s) and their inducer(s). After that our aim is to investigate: (a) the use of orchid phytoalexins in treatment and/or prevention of fungal infections and preservations of materials, (b) the mode of action of orchid phytoalexin(s) and (c) the process involved in their production. As our tissue culture methods become established we plan to start utilizing defined tissue clones in future work.

CURRENT REPORTS AND PUBLICATIONS

(a) J. Arditti (1966), "The production of fungal growth regulating compounds by orchids." Orch. Dig. Sci. Suppl., 30 (3): 88-90.

(b) J. Arditti (1966), "Orchids." Scientific American, 214 (1): 70-78.

(c) J. Arditti. "Phytoalexins." [In process of discussions and negotiations with Scientific American.]

BR R:COC DL DP LR. 7 01. F4

LOCALIZATION OF A METABOLIC BLOCK IN THE GRANULATION TISSUE AT PROGRESSIVE STAGES OF WOUND HEALING

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University of Texas Medical Branch
Galveston, Texas 77550

ASSISTED BY Dr. Shizue Maekawa

WORK UNIT NO. NR 108 810 **CONTRACT** N00014-68-A-0105-0001

OBJECTIVES

A block in the Embden-Meyerhof pathway at the height of phospho-fructokinase was found earlier and only little pyruvate is formed. However, a complete Kreb's cycle was demonstrated, our objectives therefore are: (a) In which of the three components of granulation tissue (aseptic inflammation, fibroblasts, connective tissue) the block occurs? (b) What are the sources of substrates for the Kreb's cycle, if so little pyruvate is formed from glucose?

ABSTRACT

We devised a special technique, based on the Lowry methods, which enabled us to dissect lyophylized 20μ sections into four portions (inflammatory part, the fibroblast, the connective tissue and the underlying normal muscle.) Each portion was incubated anaerobically in a specially constructed ultramicro vessel with glucose as substrate. The lactate formed was estimated enzymatically with ultramicro techniques that enable us to estimate 0.45 μmoles or less. The presence of the block was detected in the fibroblasts and in the connective tissue, but not in the normal muscle which produced lactate at a very high rate.

The activity of phosphofructokinase was found to be considerably higher in muscle than in the granulation tissue. We also showed that glucosamine has an inhibitory effect on phosphofructokinase.

The nature and biological significance of the block has not yet been elucidated but we suspect that it may be connected with the synthesis of mucopolysaccharides.

PLANS FOR FUTURE

We are at present studying the steady state of substrates of the Embden-Meyerhof pathway in order to have a better insight into their synthesis and the role of alternate pathways. We also intend to find out the source of the substrates for the Krebs cycle, i.e., whether glucogenic amino acids or fatty acids are involved and how the production of ATP in granulation tissue compares with that of the liver.

BR ... DL DP DK 07 01.F19

LUMINESCENCE OF BIOPOLYMERS AND MICROORGANISMS

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Bethesda, Maryland

ASSISTED BY R. Kolinski

WORK UNIT NO. NR 108-815 **CONTRACT**

OBJECTIVES

(a) To examine the luminescence of proteins and model compounds in order to correlate the luminescence properties of proteins with the microenvironments of the emitting groups, thereby permitting structural conclusions to be drawn from the luminescence pattern, (b) to study the luminescence properties of viruses in order to identify the chief emitting centers and the role of virus structure in modifying their fluorescence and phosphorescence

ABSTRACT

It has been demonstrated that transfer of excitation energy occurs with high efficiency from un-ionized to ionized tyrosine in oligomers of tyrosine. The ionization of a single tyrosine in hexatyrosine results in the appearance of the fluorescence and phosphorescence characteristic of ionized tyrosine, while the contribution of neutral tyrosine is suppressed. The transfer appears to occur at the singlet level by a Forster-type mechanism.

The effects of chemical modification upon the fluorescence and phosphorescence properties of a series of tryptophan derivatives have been examined. The quantum yield and excited lifetime of fluorescence at 25°C are very sensitive to any modification of the α-carboxyl or α-amino groups. Acetylation, esterification, and peptide bond formation reduce both yield and lifetime. The ratio of lifetime to yield is constant, indicating that all quenching processes are first order with respect to the excited state. In contrast, at 77°K in a rigid medium, the differences in yield and lifetime disappear, indicating that the non-radiative processes competitive with emission by the excited state are temperature- and viscosity-controlled.

The non-radiative processes of interest include proton quenching, electron transfer from the excited state, and "tunnelling" from the excited to the ground state. The second of these, of which the first may be a special case, has been studied in deal. A series of chemically diverse compounds which share the property of being efficient electron scavengers have been found to act as effective quenchers of the fluorescence of tryptophan and other indole derivatives. It is believed that the quenching mechanism involves electron transfer from the excited indole to quencher.

A computer program has been developed for analyzing complex phosphorescence decay curves, greatly enhancing the potentialities of phosphorescence for analyzing mixtures. At present, work is centered about

defining the resolving power and limitations of the method, which will be applied to proteins and viruses.

PLANS FOR FUTURE

(a) To examine the importance of the non-radiative processes described above in determining the luminescence properties of proteins and to apply computer analysis to the complex phosphorescence decay curves of proteins.

CURRENT REPORTS AND PUBLICATIONS

(a) R. F. Steiner (1968), "Luminescence of tyrosine oligopeptides." Biochem. Biophys. Res. Commun., 30, 502.

(b) R. F. Steiner and R. Kolinski (1968), "Luminescence of oligopeptides containing tryptophan and tyrosine." Biochemistry, 7, 1014.

(c) I. Weinryb and R. F. Steiner (1968), "Luminescence of tryptophan and tyrosine derivatives." Biochemistry, 17, 2488.

(d) R. F. Steiner and E. Kirby, "Quenching of the fluorescence of indole derivatives by electron scavengers." In preparation.

BR R000 ... DR ... F20

EFFECT OF LIGHT STIMULATION ON THE RNA OF RETINAL GANGLION CELLS

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ASSISTED BY Sandra Fonville

WORK UNIT NO. NR 108-833 **CONTRACT** N00014-68-0391

OBJECTIVES

To investigate quantitative and qualitative changes in the macromolecular constituents of the nervous system, mainly of RNA, taking place as a result of light stimulation with light of different wavelength and intensity.

ABSTRACT

This work is just beginning. The initial period has been devoted to setting up the techniques and equipment necessary for this investigation: (a) design, construction and testing of light proof boxes adequate for rearing guinea pigs in total light deprivation for several months from birth; (b) determination of dietary and environmental requirements of guinea pigs kept in light deprivation over extended periods of time; (c) setting up the optic system to provide stimulation with monochromatic light to the experimental animals; (d) daily two hour long stimulation with monochromatic light to the guinea pigs; (e) histological preparation and examination of the retina of control (reared in total light deprivation) and experimental animals (light stimulated); (f) dissection of retinal ganglion cells, from fixed tissue, in the oil chamber under a standard Zeiss microscope with phase-contrast with the aid of the deFonbrune micromanipulator; (g) preliminary determination of the total RNA content and its base ratios of isolated retinal ganglion cells by the ultraviolet microspectrophotometric techniques of Edstrom (Meth. Cell Phys. 1: 417, 1964); (h) dissection of the brain of *Limulus polyphemus*; (i) histological and electrophysiological identification of the central optic areas corresponding to the lateral eye of the *Limulus polyphemus*; (j) preliminary determinations of total RNA content in the lateral eye nerve and isolated neurons of the central optic areas of the *Limulus*.

BR RJ[illegible] DL DP. DR. [illegible] 01 F[illegible]

PLANS FOR FUTURE

(a) To determine the total RNA content, and its base ratios, in isolated retinal ganglion cells of control and experimental guinea pigs and pigeons reared from birth in total light deprivation and stimulated daily with light of different wavelength and intensity; (b) to determine the total RNA content, and its base ratios, in isolated neurons of the central optic areas and lateral eye nerve and axons in control and light-stimulated *Limulus polyphemus*.

CURRENT REPORTS AND PUBLICATIONS

(a) I.W.M. Barbato (September 1968, Portland, Ore.), "RNA Content in the Lateral Eye Nerve and in Isolated Single Neurons of the Brain Optic Lobe of the *Limulus polyphemus*." West.Sect.Meeting of Assn. Res. Ophth.

(b) I.W.M. Barbato (1968), "RNA Content in the Lateral Eye Nerve and in Isolated Single Neurons of the Brain Optic Lobe of the *Limulus polyphemus*." Invest. Ophth. (in press).

BR R... DL.DP.DR 07.07 F21

STUDIES ON THE BIOLOGY OF THE BARNACLES: A HISTOLOGICAL COMPARISON OF THE CEMENT APPARATUS OF THE CIRRIPEDIA

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ASSISTED BY

WORK UNIT NO. NR 108 834 **CONTRACT** N00014-68-C-0334

OBJECTIVES

(a) To clearly describe the cement apparatus of the Cirripedia (b) and to compare the histological characteristics of the tissues responsible for the elaboration of the cement among various species of barnacles. (c) To determine the biochemical pathway of cement synthesis and the mechanism of adhesion. (d) To isolate, purify and identify the chemical nature of the cement.

ABSTRACT

The cement apparatus of the Cirripedia consists of unicellular glandular structures which communicate with a series of tubular canals. Cement used in the process of attachment, is elaborated in the glands and transported to the points of attachment through the canals. Certain histological differences in the cement glands of the Lepadidae and the Balanidae have been described in the present study and morphological differences in the canal system have been discussed. The mechanism for the origin of the cement and its conduction to the points of attachment has been determined in *Lepas anatifera* and *Balanus tintinnabulum*. In addition, the origin of the unicellular glands and the canals have been described for these species.

PLANS FOR FUTURE

Differences in the location of the glands and the architecture of the canal system appear to be species specific. In order to properly describe the mechanism involved in fouling caused by these organisms the cement apparatus of various species of barnacles must be studied histologically, microchemically and biochemically. If the biosynthetic pathway in the production of cement can be shown to be similar in all forms, measures can be studied to eliminate fouling by these organisms using antimetabolic procedures.

BR R... D. DP DR ... F AV

CURRENT REPORTS AND PUBLICATIONS

(a) Dyrce Lacombe (1968), "Histologia, histoquimica e ultra-estrutura das glandulas de cimento e seus canais em *B. tintinnabulum*." Publicacao No. 017 of the Instituto de Pesquisas da Marinha, Rio de Janeiro, Brazil

(b) Dyrce Lacombe and V. R. Liguori (1968), "A histological comparison of the cement apparatus of the Cirripedia." In press

BR R100C 01 DP LR 09 09 F20

BIOCHEMICAL MECHANISMS IN TOXIC ENVIRONMENTS

Excellent in-house facilities exist in the Navy for the handling of the toxicological problems which arise in the course of on-going operations and, to a considerable extent, those which are foreseeable in the light of planned technology. The Office of Naval Research is delighted to supply basic research efforts clearly identifiable with known naval needs, but its program planning should be designed to explore new fields, to anticipate trouble so that it can be avoided, and to provide rational grounds for planning and design.

These considerations amount to specifications for a type of toxicology which is not a matter of running LD_{50} determinations on a specified list of compounds of "Naval Interest". To be basic research in toxicology, investigation must be closely akin to pharmacology. To be naval, it must deal with the special conditions likely to be common in the naval duty stations of the future; with hyperbaric conditions, exotic atmospheres, self-contained sealed environments which may be "conditioned" to perfection, but within which low levels of unwanted compounds on their way to the disposal system must be inhaled constantly over periods of weeks or months. To be maximally useful, it must be possible to rate hazards and deal with them, not in terms of mortalities or overt illness, but in terms of reduction of effective performance and the long-term consequences of chronic exposure.

The re-naming of the former "Toxicology" program, and the inclusion of such research as the intermediary metabolism of oxygen, are efforts on our part to illustrate our desire for more sound proposals designed to serve the needs outlined above.

BK R... L..LP DR 0?.?? F...

ADVISORY CENTER ON TOXICOLOGY

National Academy of Sciences
National Research Council
Washington, D.C.

ASSISTED BY Committee on Toxicology

WORK UNIT NO. NR 303 366 **CONTRACT** Nonr 2300(29),NR303-366

OBJECTIVES

(a) to act as s source of information and advice on toxicology problems submitted by the sponsors: Army, Navy, Air Force, Atomic Energy Commission, National Aeronautics and Space Administration, Coast Guard, Public Health Service, Federal Aviation Administration; (b) to act as a medium of communication between the sponsors and the NRC Committee on Toxicology and to provide technical support to the Committee; (c) to operate a system for storage and retrieval of toxicological information from published and unpublished sources for use in the foregoing.

ABSTRACT

(a) A record total of eight-four requests for information and advice requiring significant effort have been received so far this calendar year from the various sponsoring agencies. In addition, there have been 154 minor inquiries. The subject matter of these requests for assistance are more broad than in the past due to the addition of two new sponsoring agencies. The Committee on Toxicology has continued to respond to those problems involving policy matters as well as those requiring criteria or standards for human exposures to potentially harmful materials. (b) The Center continues to receive excellent cooperation from industrial, academic, and governmental organizations and frequently has performed a useful role in coordinating efforts in the field of toxicology. (c) The Director has accepted several invitations to participate in symposia and other scientific meetings of governmental agencies and professional societies. This has provided a mechanism for further dissemination of information generated by the Center and the Committee.

PLANS FOR FUTURE

The Advisory Center on Toxicology plans (a) to continue to provide assistance on toxicological matters to its sponsoring agencies,(b) to expand its coverage of domestic and foreign literature,(c) to serve as a focal point for coordination of toxicological research.

BR R... DR 07.01 F23

ADVISORY CENTER ON TOXICOLOGY

National Academy of Sciences
National Research Council
Washington, D.C.

ASSISTED BY Director and Staff

WORK UNIT NO. NR 303 450 **CONTRACT** Nonr-2300(15),NR303-450

OBJECTIVES

To provide information and advice on the toxicity of materials of concern to the Naval Ship Engineering Center.

ABSTRACT

Information and comment has been provided by the Center and the Committee on Toxicology concerning the toxicological aspects of materials for use in aircraft, ships, and submarines.

PLANS FOR FUTURE

To continue to aid the Naval Ship Engineering Center by reviewing the toxicological aspects of proposed and accepted materials of construction and by providing liaison to the Committee on Toxicology for problems involving human exposure.

BR R. .OC L. . DP. PF 03 04. F25 /

CELLULAR ACTION OF PHARMACODYNAMIC AGENTS UNDER HYPERBARIC CONDITIONS

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ASSISTED BY George K. Hanasono

WORK UNIT NO. NR 303-774 **CONTRACT** PO 7-0036

OBJECTIVES

(a) To define the actions of drugs on cellular and subcellular systems under the hyperbaric conditions of the Navy's underwater operations, (b) to study the effects of carrier gases (e.g., helium) in cellular and subcellular membranes, (c) to determine effects of the hyperbaric atmosphere on drug absorption, distribution, metabolism and excretion.

ABSTRACT

The effects of the helium/oxygen atmosphere at various pressures has been studied on the ability of isolated microsomal enzyme systems to degrade hexobarbital. This liver enzyme system is responsible for the biotransformation of a large percentage of the drugs used clinically. A 9000 x g x 20 minutes supernatant from rat liver homogenate was utilized. Tests were made in pressure vessels developed in this laboratory and which allow for the addition of reagents while the system is pressurized. All studies were made at 37° and a PO_2 of 0.3 ATA balanced to the appropriate pressure with helium. Each pressure study was paired with a corresponding control run at 1.0 ATA in 70%/30% helium/oxygen. Control and pressure determinations were made with enzyme systems prepared from the same liver homogenate. The initial concentration of hexobarbital was 0.6 mM. All samples were preincubated for 2 minutes before the drug was added to allow for saturation by the atmosphere. Comparisons were made by the paired $\underline{t}$-test. The following table summarizes the data obtained in these studies.

Pressure	n	Incubation Time	ΔMetabolism* (mμmoles/mg protein) ± SEM
5 ATA	8	5	1.7 ± 0.9
	7	10	0.3 ± 0.5
	8	15	0.8 ± 1.3
	8	20	0.6 ± 1.1
	8	25	-1.1 ± 1.2
10 ATA	8	5	2.0 ± 0.6**
	8	10	1.5 ± 0.8
	8	15	0.6 ± 1.0
	8	20	-0.2 ± 1.0
	8	25	1.0 ± 0.5

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20 ATA	8	5	1.7 ± 0.7 **
	7	10	1.1 ± 0.3 **
	8	15	0.2 ± 1.3
	8	20	0.4 ± 1.0
	8	25	0.3 ± 0.7

* Δ Metabolism = (metabolism at pressure - metabolism at 1 ATA).
**Significant difference at a level of P=0.05 or less between metabolism at pressure and metabolism at 1 ATA.

An effect of pressure was noted in the early phases of biotransformation at 10 and 20 ATA. This effect was manifested as an increase in the metabolism of hexobarbital under the hyperbaric conditions. The mechanism of this effect is unknown but may be related to a greater degree of enzyme saturation during the early periods of high substrate concentration. Such an influence of pressure, if confirmed by further studies, has possible significance to the use of drugs during saturation during operations, since the pressures studied are within the range of those used during such operations.

PLANS FOR FUTURE

(a) These studies will be expanded to include variation in initial substrate concentrations and the investigation of possible changes in Michaelis-Menten kinetics. (b) The in vivo effects of hyperbaric conditions on microsomal enzyme systems will be studied by exposing rats to pressures encountered in saturation during conditions and examining the activity of the liver fractions with respect to the biotransfromation of hexobarbital.

BR K3OC L DP DR 04.01 F 241

Oxygen Metabolism: Metabolic Implications in Hyperbaric Systems

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ASSISTED BY A. Widman; A. Iannone, N. Spinelli

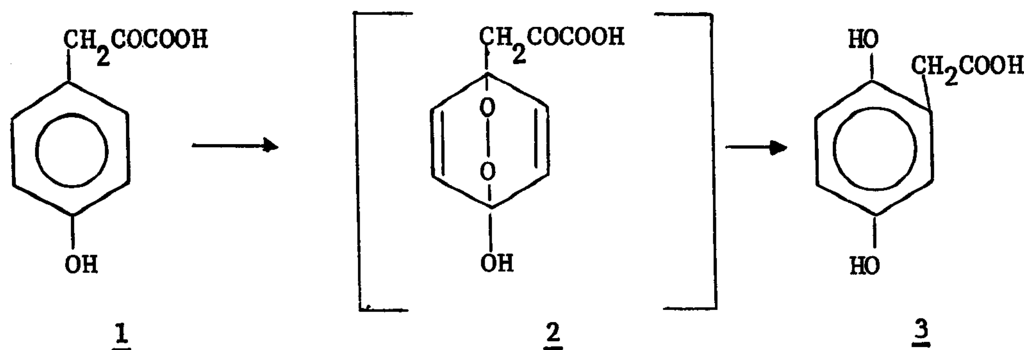
WORK UNIT NO. NR 303-811 **CONTRACT** 8171

OBJECTIVES

The basic objective of this research is a determination of the mechanism by which oxygen becomes incorporated into biochemical substrates and more especially aromatic systems. As an initial approach we have undertaken to determine whether endo-peroxides are transient intermediates in aromatic hydroxylation reactions, and if so, the implications such biologically-active components have for hyperbaric conditions.

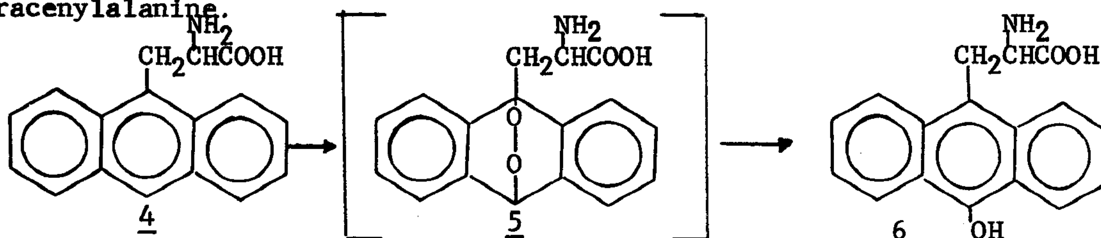
ABSTRACT

The synthetic scheme for the preparation of p-hydroxy (^{18}O) phenylpyruvic acid has been perfected as well as the method for purifying this substrate. Thin-layer chromatographic (t.l.c.) techniques have been developed for the separation of the expected components following enzymatic conversion of the substrate to homogentisate. Preparative procedures are underway to permit the isolation of sufficient product for mass spectrographic analysis. Conditions have been established for obtaining suitable spectra with one-mg samples. The fragmentation pattern of homogentisate has been carried out (M.M. Bursey, University of North Carolina) and with this background a determination of the position of the ^{18}O-label in homogentisate appears to be entirely feasible. The objective of this work will be to determine the lability of the hydroxyl function in the conversion of p-hydroxyphenylpyruvate to homogentisate. This will be done by comparing the ^{18}O: ^{16}O ratio of this hydroxyl group with the 5 hydroxyl group in the product.



Enzymatic studies are underway to work out the conditions which will be applied to the ^{18}O-labeled substrate.

Concomitantly we have undertaken to study as a model system the possible conversion of 9-anthracenylalanine to 10-hydroxy-9-anthracenylalanine.



The possibility that such a transformation occurs, similar to the conversion of phenylalanine to tyrosine, may permit the isolation of an intermediate 9, 10 peroxido-9-anthracenylalanine in this biological hydroxylation. 9-Anthracenylalanine has been synthesized and the preparation of the two other components in this system is planned. The rationale for the use of the anthracene analogue is based on the probability that such a transannular peroxide would show greater stability than would be the case for the benzene analogue.

At a recent meeting of the Stress Study Group in Johnsville, results from Dr. Houlihan's laboratory were presented, pointing to the strong possibility that oxygen toxicity is associated with the metabolism of the catecholamine to the melanins. The following mechanisms are possible pathways by which both epinephrine and 3,4-dihydroxyphenylalamine (DOPA) can be transformed into the melanins.

(Insert schemes)
(Schemes will be sent upon request)

Peroxides, such as structures 7 and 8 are presented as initial intermediates in this reaction sequence and it is conceivable that under hyperbaric conditions such transformations become preferred pathways. The described studies which may give definitive evidence for the formation of peroxides in aromatic systems would be supportive of this hypothesis.

BR R3C0C DL DP DR 07.01 F25V

EARLY DETECTION OF TOXIC EFFECTS OF ORGANIC CHEMICALS

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ASSISTED BY H. Shah, S. Puri, A. Olshan, J. Zabik, M. Roffman

WORK UNIT NO. NR 303-812 **CONTRACT** N00014-68-A-0215

OBJECTIVES

To study the biochemical and pharmacological alterations resulting, prior to the appearance of gross pathological lesions, from chronic inhalation of organic chemicals. Methylchloroform was selected for present investigation because of its wide-spread use in naval installations and industry.

ABSTRACT

Our previous studies with male mice demonstrated that prolonged inhalation of methylchloroform induced hepatic microsomal enzymes which are responsible for the side-chain oxidation of hexobarbital and demethylation of aminopyrine. The biochemical changes were accompanied by the significant reduction in the duration of hypnosis induced by short-acting barbiturates. During the period of this report these studies were extended to young male rats. Twenty-four hour (time dependent effect) inhalation of methylchloroform (approximately 3000 ppm; concentration dependent effect) significantly reduced the sleeping time of hexobarbital and enhanced in vitro metabolism of hexobarbital as well as demethylation of aminopyrine by hepatic microsomal enzymes. All effects were measured 24 hours after the termination of methylchloroform inhalation. Demethylation of p-chloro-N-methylaniline, a new substrate discovered in our laboratory as a tool for measuring induction of hepatic demethylase activity, was not altered by methylchloroform inhalation. Methylchloroform inhalation also shortened the duration of loss of righting reflex induced by meprobamate or zoxazolamine, and these effects were not altered by pretreatment with morphine (20 mg/kg, i.p., daily, for 3 days prior to methylchloroform inhalation or by adrenalectomy prior to methylchloroform exposure, indicating no role of stress in producing these effects. Methylchloroform inhalation did not alter total body weight but significantly increased liver weight of the exposed animals. The effects of methylchloroform inhalation on hepatic drug metabolism were reversible and did not interfere with further development of hepatic microsomal enzymes.

Further investigations on methylchloroform inhalation in com-

bination with other toxic agents were initiated. It was found that pretreatment with high doses of ethionine prolonged markedly the hexobarbital sleeping time and reduced the rate of hepatic metabolism of hexobarbital after methylchloroform exposure. In addition, methylchloroform inhalation sensitized the rats to the toxic effects of carbon tetrachloride inhalation.

Measurement of behavioral effects of methylchloroform inhalation was initiated. Those concentrations of methylchloroform which produced significant alteration of hepatic microsomal enzymes did not affect the learning ability or the performance of learned discriminative tasks in the operant conditioning experiments. Higher concentrations of methylchloroform depressed the operant behavior markedly.

PLANS FOR FUTURE

The future biochemical studies will attempt to localize the site of methylchloroform action by investigating the changes in components of microsomal electron transport system in the exposed animals. They will include NADH-Cytochrome C Reductase and P-450. Other studies would determine the biological effects due to the interaction of methylchloroform inhalation with the inhalation of other chlorinated compounds such as carbon tetrachloride or chloroform. In addition, the effects of methylchloroform inhalation on hepatic metabolism of drugs will be studied in hyperbaric environments and under physical or psychological stresses.

The tests which can be used clinically to detect the prepathological effects of methylchloroform inhalation will be explored.

CURRENT REPORTS AND PUBLICATIONS

(a) H. Lal and H. Shah (1967), "Effects of prolonged methylchloroform inhalation on drug induced CNS activity." Toxicol. Appl. Pharmacol., 10, 389.

(b) H. Lal and H. Shah (1968), "Reduced sleeping time and increased microsomal oxidation of hexobarbital after methylchloroform inhalation in mice." Pharmacologist, 10, 153.

(c) H. Lal and H. Shah (in press), " Effects of ethionine, dimethylsulfoxide and route of administration on methylchloroform-induced alteration of barbiturate-action." Toxicol. Appl. Pharmacol.

(d) H. Lal, A. Olshan, H. Shah, S. Puri, and G. Fuller (in press), "Enhancement of hepatic drug-metabolism in rats after methylchloroform inhalation." Toxicol. Appl. Pharmacol.

BR RJCOC DL DP.DR 01.01.F

AEROSOL BEHAVIOR IN HIGH PRESSURE ENVIRONMENTS

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Bolt Beranek and Newman Inc.
Cambridge, Massachusetts

ASSISTED BY E. Ahern and R. C. Wanta

WORK UNIT NO. NR 303-829 **CONTRACT** N00014-68-C-0271

OBJECTIVES

(a) To determine how the behavior of fine particles suspended in the atmosphere of a deep submergence vessel are affected by both the composition and pressures involved, (b) to consider how aerosol removal devices are affected by the atmosphere, and (c) to consider how the effects of aerosols upon man might be altered by the composition of the atmosphere.

ABSTRACT

The literature has been searched for information on aerosol behavior under high pressures. Virtually nothing has been found which is directly applicable to this project. However, a great deal of material on nuclear submarines has been read and formed into an annotated bibliography (to be presented as part of the final report). Calculations have been made detailing the viscosity, density, and molecular mean free path length of the atmosphere with increasing pressure. Similarly, aerosol calculations have been made considering the rates of diffusion, sedimentation and agglomeration as well as the slip correction factor. Consideration has been given to the problems of electrostatic precipitation, filtration, droplet growth and evaporation. Initial findings are that electrostatic precipitation is difficult if not impossible at the higher pressures, filtration is only slightly affected, the formation of liquid aerosols from the bulk material is inhibited but particles once formed have significantly increased lifetimes.

PLANS FOR FUTURE

(a) To consider how pulmonary deposition is affected by the pressure and composition of the atmosphere, (b) to perform an experiment verifying the calculations on electrostatic precipitation, and (c) to detail a series of necessary experiments for future studies.

CURRENT REPORTS AND PUBLICATIONS

(a) R. A. Gussman (1968), "Aerosol behavior in high pressure environments." First Quart. Prog. Rpt., Bolt Beranek and Newman Inc., Cambridge, Mass., 1 Mar.-30 Apr.

BR RJCOC DL DP DR 07 01 F24

(b) R. A. Gussman (1968), "Aerosol behavior in high pressure environments." Second Quart. Prog. Rpt., Bolt Beranek and Newman Inc., Cambridge, Mass., 1 May-31 Jul.

(c) R. A. Gussman (1968), "Aerosol behavior in high pressure environments." Third Quart. Prog. Rpt., Bolt Beranek and Newman Inc., Cambridge, Mass., 1 Aug-31 Oct.

BR RIOOC DL.DP.DR 01.01.F24V

IMMUNOCHEMISTRY

In FY 1968, a major part of the Immunochemistry program remained a matter of investigating the underlying molecular structure, function and biosynthesis of the reagents of immunity. Some effort was given to biochemical accounts of the effects of immune reactions in particular clinical situations, and to logical methods of therapy. In view of the large investment by other agencies and the importance of the subject to general medicine rather than a specific naval need, we have resisted passage on the bandwagons of protein synthesis and immunosuppression to a considerable degree.

The specifically naval needs in immunochemistry, as we see it, stem from the need for multiple immunizations for the protection of globally dispersed personnel, the special naval capabilities for the storage of preserved cells and tissues and their application in replacement therapy, and the visionary hope that the principle of serological specificity may find application in such diverse fields as the control of fouling and marine pests as well as medicine if an antibody-like capability can be built into biologically active materials.

The existence of the naval skin bank capability and frozen blood banks do lend pertinence to studies of immunosuppression. We have begun to support some work in this area in FY 69, and will be phasing out some of the very excellent but public health oriented work with which this program has been associated for the past decade.

BR RJCOC D. LP DR 07 01 F28

MECHANISM OF ANTIGEN-ANTIBODY REACTIONS AND SIMILAR NONSPECIFIC REACTIONS BETWEEN PROTEINS AND OTHER MACROMOLECULES

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ASSISTED BY G. Kamat, Judy Ramaley, Isis Nawar, Eudora Tharp, D. Johnson, and M. Dunlap

WORK UNIT NO. NR 106-035 **CONTRACT** N00014-67-A-0289-0001

OBJECTIVES

(a) To obtain more information concerning the structure of antibodies, and particularly that of their combining sites; (b) to compare the structure and other properties of rabbit antibodies with that of chickens; (c) to gain more insight into the mechanism of antibody biosynthesis.

ABSTRACT

Previous experiments had shown that chicken antibodies against an acidic antigenic determinant, As(=p-azophenylarsonate), and those formed in the same animal against a basic determinant R_4N(=p-azophenyl-N-trimethylammonium), after digestion by trypsin, yielded the same peptide maps, and very similar starch gel electropherograms. However, amino acid analyses revealed important differences between the two antibodies. The chicken anti-As antibodies contained more serine and glutamic acid and less phenylalanine than the anti-R_4N antibodies. The amino acid composition of both types of chicken antibodies differed significantly from those of the analogous rabbit antibodies directed against the same antigenic determinants. This difference in the amino acid compositions shows that large parts of the antibody molecule are not involved in the specific combination with the antigenic determinant. This is in agreement with our earlier conclusion that the specific combining sites of the antibody molecules are small and combine with only a small determinant patch on the surface of the antigen molecule.

Injection of tritiated dinitrophenyl-bovine serum albumin into mice revealed that the isotopically labelled material was concentrated chiefly in the mitochondrial fraction of the spleen and liver. Only a very small amount of protein-bound tritium was found in the ribosomal fraction and the endoplasmic reticulum. The highest specific activity was found shortly after injection in the endoplasmic reticulum of the spleen, later in the mitochondrial fraction. Since we know from earlier histological experiments that the bulk of the injected antigen is trapped in macrophages, it seems now that the mitochondria of these serve as deposits for the antigen or its determinant fragments.

BR RJCOC DI DP. DR 07.01 F28V

PLANS FOR FUTURE

We intend to extend our investigation of whole antibody molecules to that of their parts which contain the specific combining sites, particularly their Fab fragments and the C-1 peptide obtained by the action of cyanogen bromide.

CURRENT REPORTS AND PUBLICATIONS

1. F. Haurowitz: Struttura e Formazione degli Anticorpi (Structure and Formation of Antibodies), La Ricerca in Clinica e Laboratorio, no.4. 61-82, 1967 (received 1968).

2. F. Haurowitz: The Evolution of Selective and Instructive Theories of Antibody Formation, Cold Spring Harbor Symposia on Quantitative Biology, 32, 559-567, 1967 (published 1968).

3. F. Haurowitz, Immunochemistry and the Biosynthesis of Antibodies, Interscience Division of John Wiley and Sons, New York, 1968. XVII and 301 pages.

BR R3OC DI DP. DR 07 01 F29

IMMUNOCHEMICAL CRITERIA OF PURITY OF PROTEINS AND POLYSACCHARIDES

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New York, New York

ASSISTED BY F. G. Gruezo, E. Licerio, K. O. Lloyd

WORK UNIT NO. NR 106-100 **CONTRACT** Nonr 266 (13)

OBJECTIVES

(a) To evaluate existing methods and to develop new methods for establishing purity of proteins and polysaccharides, (b) to study fundamental mechanisms of antigen-antibody combination and (c) to correlate structure of polysaccharides with immunochemical specificity.

ABSTRACT

Alkaline degradation with $NaOD-NaBD_4$ of Le^a ovarian cyst fluid obtained from Nigeria yielded a series of oligosaccharides which made it possible to propose for the first time an overall structure for the carbohydrate moiety of the human blood group A, B, H, Le^a and Le^b substances (1). A most important feature of this structure is that the two types of oligosaccharide determinants recognized in the blood group substances are linked to a single galactose to form a branched structure. This finding raises the possibility of cooperative effects in the interaction of these two determinants with antibody. The ORD and CD spectra of these oligosaccharides have been published (2).

From a consideration of amino acid analyses of purified human antibodies to polysaccharides which represent selected populations of γG immunoglobulin molecules and of sequence data on Bence Jones proteins, it could be shown that most of the variation in amino acid composition of the antibodies originated from the variable region of the molecules (3). Further examination of the invariant residues of human K, L and mouse K Bence Jones proteins revealed a substantial number of invariant and almost invariant glycines in the variable region while the constant region contained no invariant glycines but did contain invariant hydrophobic leucines, valines, alanines and histidines. The glycines were thought to provide the flexibility needed in the variable region to permit the substitutions which occur at the variable positions while the hydrophobic invariant residues in the constant region could be involved in interaction of the heavy and light chains (4).

BR R3002 DL DP DR 07 09 F29V

PLANS FOR FUTURE

Work is continuing on the isolation and elucidation of the structures of other oligosaccharides from blood group substances. An ovarian cyst from a Nigerian who is a non-secretor of A, B, H, Le^a and Le^b substances has been obtained and will be studied.

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BR RJC0C DL. DP. DR. F30

NATURE OF CYTOTOXIC REACTIONS MEDIATED BY ANTIBODY AND COMPLEMENT, AND RELATED PHENOMENA

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WORK UNIT NO. NR 106-104 **CONTRACT** Nonr 248(60)

OBJECTIVES

Investigation of the cytolytic and inflammatory activities of the complement system.

ABSTRACT

Specific Purification of C'2: We have developed a method for isolation of pure C'2 by adsorption on EAC'4 followed by elution. The final product yields a single band on disc electrophoresis as judged by staining and assay for C'2 activity. A single arc is obtained on immunoelectrophoresis with an antiserum against whole guinea pig serum. Tests for functional purity indicate that the material contains less than three units of any of the other eight complement components in 100,000 units of C'2. The stability at 37°C over a period of three hours is excellent and even after 20 hours only slight loss has been observed. The final yield is about 10%. The absolute efficiency of specifically purified C'2 is about 20%, i.e., approximately 5 molecules of C'2 are needed to produce one hemolytically active SAC'4,2a site. Presumably, the other 4 molecules are wasted in side reactions.

Biologically Active Fragments of C'3 and C'5: C'3 is cleaved by EAC'4,2a. One of the products is a small fragment, C'3a, molecular weight about 10,000, which is spasmogenic for guinea pig ileum. The C'5 molecule is cleaved by EAC'4,2a,3. One of the fragments, C'5a, molecular weight about 15,000 is spasmogenic for guinea pig ileum and chemotactic for rabbit polymorphonuclear leukocytes.

C'6: Guinea pig serum C'6 has been obtained in a functionally purified state by column chromatography and disc electrophoresis on polyacrylamide gel. Titration data, as well as kinetic analyses of C'6 fixation, are compatible with the assumption of a one-step reaction mechanism. Consumption of C'6 and formation of C'6 sites on the cellular precursor intermediate are favored by low ionic strength and elevated temperature.

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C'9: Partially purified preparations of C'9 from guinea pig serum have been obtained by chromatography on DEAE cellulose, hydroxyapatite and Sephadex G-200. Relative to serum, the product has been purified 1100-fold with a yield of about 23%.

Endotoxin: Endotoxic lipopolysaccharide reacts with whole guinea pig serum causing destruction or consumption of the six terminal complement components, C'3 to C'9. There is no detectable loss of C'1, C'4 and C'2. During this process, spasmogenic and chemotactic activities are generated. The physical properties of these activities suggest that they may be due to the C'3a and C'5a fragments.

Cobra Venom: A purified factor isolated from cobra venom reacts with guinea pig serum in a fashion similar to that of endotoxic lipopolysaccharide. Complement components C'3 to C'9 are consumed without detectable loss of C'1, C'4 and C'2. Spasmogenic and chemotactic activities are generated, possibly due to the formation of the C'3a and C'5a fragments.

PLANS FOR FUTURE

(a) Further studies on the cleavage of C'2 by C'1a (b) further work on the cleavage of C'3 by C'4, 2a (c) further work on the cleavage of C'5 by C'4, 2a, 3 (d) further studies on the mechanism of reaction of C'6 with the C'4, 2a, 3, 5 complex (e) studies of the mechanism of action of C'9 (f) further work on purification of C'4, C'6, C'7, C'8 and C'9.

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(b) Shin, H. S. and Mayer, M. M. The Third Component of the Guinea Pig Complement System. II. Kinetic Study of the Reaction of EAC'4, 2a with Guinea Pig C'3. Enzymatic Nature of C'3 Consumption, Multiphasic Character of Fixation, and Hemolytic Titration of C'3. Biochemistry, 7:2997, 1968.

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BR R... DL DP. DR. 07 01 F31

THE MECHANISM OF ANTIBODY FORMATION

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WORK UNIT NO. NR 106-509 **CONTRACT** Nonr-1834(37)

OBJECTIVES

(a) To follow the development of natural antibodies in mice as a function of age, (b) to compare the antigenic structure of rabbit IgG and IgM bearing the same heavy chain allotype, (c) to study the antigenic relationship among avian lysozymes.

ABSTRACT

(a) The finding from this laboratory (Science, 155:1514, 1967) that newborn offspring of immunologically paralyzed mice are more susceptible to the induction of both immunologic paralysis and immunity to pneumococcal polysaccharide type III than newborn normal mice, has been extended to mice of various ages. The quantity of polysaccharide required to induce immunity or paralysis in offspring of normal mice decreased from birth to two weeks of age. This increase in susceptibility to immunity and paralysis induction was attributed to the elimination of maternal natural antibodies specific for the polysaccharide. By 3 weeks of age, the offspring of normal mice were paralyzed or immunized with the same doses of polysaccharide required for adult mice. The doses of polysaccharide necessary to paralyze or immunize offspring of paralyzed mice remained constant through 2 weeks of age, but increased during the following 2 weeks and reached adult levels by the 4th week of age. Administration of one milliliter of normal mouse serum to offspring of paralyzed mice brought their susceptibility to immunity and paralysis to the level of that of offspring of normal mice.

(b) Sheep RBC sensitized with a sub-hemolytic dose of anti-sheep RBC IgG of Aa1Ab4 allotypic specificity did not hemolyze upon addition of complement, but did hemolyze upon successive treatment with anti-Aa1 and complement. When RBC so sensitized were incubated in agar together with spleen cells from an Aa3Ab4 rabbit which had been immunized with Aa1Ab4 IgG, hemolytic, complement-dependent plaques appeared around cells secreting anti-Aa1 antibodies. Plaques were specifically inhibited by Ig bearing Aa1 allotypic specificity. Thus, it was possible to compare Aa1 IgG with Aa1 IgM for efficiency of plaque inhibition. The molar concentration of IgG required to inhibit 50% of the plaques was about 30-fold smaller than that of intact IgM (calculated on the basis of the mol. wt. of the IgM subunit), and 100-fold smaller than that of reduced and alkylated IgM. These results indicated that antibody produced by single cells had greater average affinity for the Aa1 allotype of IgG,

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which was the antigen used for immunization, than for the cross-reacting allotype of IgM. The polymeric nature of IgM did not seem to be responsible for the difference in efficiency of plaque inhibition, since reduced and alkylated IgM did not inhibit any better than intact IgM. As IgM, used in sufficient concentration, caused 100% plaque inhibition, there was no evidence of cells producing antibody specific for a determinant present only on IgG. All experiments were done with a single batch of immune spleen cells, which were kept frozen in liquid nitrogen.

(c) Hemolytic plaques have been obtained by plating in agar spleen cells from mice immunized against chicken lysozyme together with sheep RBC sensitized with the IgG fraction of commercial hemolysin covalently linked to chicken lysozyme. Such plaques were specifically inhibited by soluble chicken lysozyme incorporated into the agar. Nanogram amounts of chicken lysozyme inhibited plaques obtained during the secondary response to lysozyme, while greater amounts were required to inhibit plaques obtained during the primary response. This probably reflected the greater antigen-binding ability of secondary response antibody. Lysozymes from ring-necked pheasant and bobwhite quail have been obtained, and will be compared with chicken lysozyme with respect to efficiency of inhibition of anti-chicken lysozyme plaques.

PLANS FOR FUTURE

(a) The effect of administration of measured amounts of purified anti-pneumococcal polysaccharide type III on the susceptibility of offspring of immunologically paralyzed mice to the induction of paralysis and immunity will be studied. The quantity of antibody required to restore the level of susceptibility of offspring of paralyzed mice to that of normal mice should provide a measure of the natural antibody specific for the polysaccharide transferred from mother to offspring.
(b) The technique of plaque formation by cells secreting antibodies against protein antigens will be extended to antibodies against haptens. The technique will be used to explore several immunologic phenomena, including the possible existence of single cells producing antibodies of different specificity, the phenomenon of allotype suppression, and the change in binding affinity of antibodies produced at different times during immunization.

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(e) D. Segre and M. Segre (1968), "Hemolytic Plaque Formation by Mouse Spleen Cells Producing Antibodies to Ovalbumin." Immunochemistry, 5, 206-212.

BR R3000 DL DR DR 07 01 F3

IMMUNITY UNDER STRESS

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ASSISTED BY

WORK UNIT NO. NR 106-599 **CONTRACT** Nonr 3545(00)

OBJECTIVES

(a) To investigate biochemical and physiological factors involved in antibody synthesis and decay, and (b) to investigate further molecular changes which may occur in antibodies produced by animals subjected to stress.

ABSTRACT

Contract terminated. No report requested.

BR RSCOC DL DP. DR 03 04 F32V

STRUCTURAL AND IMMUNOCHEMICAL STUDIES ON MYOGLOBINS AND HEMOGLOBINS

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ASSISTED BY

WORK UNIT NO. NR 106-620 **CONTRACT** Nonr-4564(00)

OBJECTIVES

(a) To develop new chemical procedures for specific modification and cleavage of proteins, (b) to carry out studies on the chemistry of myoglobins and hemoglobins, and (c) to study the correlation of structure of these proteins with their antigenic properties.

ABSTRACT

Work previously reported has now been published (1-4). Apomyoglobin has been nitrated completely and specifically at the three tyrosine residues. Nitromyoglobin prepared from nitrated apomyoglobin and unmodified ferriheme exhibited electrophoretic and spectral differences but had conformational parameters identical with those of metmyoglobin X. Nitrated apomyoglobin and nitrated metmyoglobin possessed lower antigenic reactivities relative to apomyoglobin and to metmyoglobin X. Fragments 56-131 and 132-153, obtained by cleavage of apomyoglobin at methionines 56 and 131, were also nitrated. Peptide 56-131 and its derivative nitrated at tyrosine 103 showed identical reactivities with antisera to metmyoglobin X. It was therefore concluded that tyrosine 103 is not located in an antigenic reactive region in metmyoglobin. On nitration of tyrosines 146 and 151 the inhibitory activity of peptide 132-153 was completely abolished. The inhibitory activity lost compared well with the decrease in antigenic reactivity observed with nitrated apomyoglobin and nitrated metmyoglobin. Also, nitrated metmyoglobin did not react with antisera to fragment 132-153 (2).

These findings suggest that one or both of tyrosines 146 and 151 are present in a reactive region in metmyoglobin (5).

The preparation of globin complexes with zinc metalloporphyrin, protoporphyrin IX and ferriheme dipyrid-4-ylpropyl ester is described and their physicochemical properties investigated. Reconstitution of hemoglobin from globin and resynthesized heme (i.e. from ferric iron and protoporphyrin IX) gave a preparation having conformational parameters and immunochemical behavior identical with those of the native protein. Hemoglobin derivative prepared with protoporphyrin IX showed a great increase in the Stokes radius which was accompanied by an enhancement of the antigenic reactivity of the derivative (relative to native hemoglobin).

Similar increase in antigenic reactivity was obtained with hemoglobin containing heme dipyrid-4-ylpropyl ester, although only relatively

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slight changes in conformational parameters were observed. The Stokes radius of the hemoglobin derivative prepared with zinc metalloporphyrin was greatly increased and reacted poorly with antisera to hemoglobin (6).

PLANS FOR FUTURE

(a) To continue our work on the development of new chemical procedures for specific modification and cleavage of proteins, and (b) to continue our investigations on the correlation of structures of hemoglobin and myoglobin with their antigenic properties.

CURRENT REPORTS AND PUBLICATIONS

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(2) Atassi, M. Z. and Saplin, B. J. "Immunochemistry of Sperm Whale Myoglobin. I. The Specific Interaction of Some Tryptic Peptides and of Peptides Containing all the Reactive Regions of the Antigen." Biochemistry 7, (1968) 688.

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BR R3COC DL DR DR.03.04.1 ...

BIOCHEMICAL PROPERTIES OF ANTIBODIES TO PURINE AND PYRIMIDINE RIBONUCLEOTIDES

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ASSISTED BY

WORK UNIT NO. NR 108-646 **CONTRACT** Nonr 4259(11)

OBJECTIVES

To study the biochemical activities of antibodies specific for ribonucleotides and ribonucleosides.

ABSTRACT

Studies are continuing on the biological and biochemical properties of antibodies to ribonucleosides and ribonucleotides.

Antibodies specific for thymine and guanine nucleosides reacted (immunochemically) with denatured but not with native DNA. These antibodies inhibited the ability of calf thymus DNA to serve as a primer for DNA polymerase extracted from chick embryos. No inhibition of DNA synthesis was observed with anti-bovine serum albumin or normal sheep globulin. Anti-T was more inhibitory than anti-G whether _Bacillus cereus_, _Micrococcus lysodeikticus_ or calf thymus DNA preparations were used as primers. Purified anti-T inhibited the priming of DNA to the same extent, relative to antibody content as the globulin fraction anti-T. The data were consistent with the hypothesis that binding of the anti-nucleoside antibodies to the exposed bases in denatured DNA prevented these masked regions from acting as templates for the DNA polymerase.

The reaction of anti-cytidine and anti-thymidine antibodies with denatured DNA preparations varying in G-C content was studied. If the DNA preparations were denatured with formaldehyde, the order of reactivity with anti-T antibodies coincided with DNA base composition, i.e. clostridum $>$ calf thymus $>$ micrococcus. Anti-C produced the expected reaction pattern with the bacterial DNA's (micrococcus $>$ clostridium); however, calf thymus DNA did not react as expected. The deviant pattern observed with anti-C may be an effect of the formaldehyde denaturation since formaldehyde reacts with the 6-amino group of cytosine, possibly altering its antigenic identity. Anti-C and anti-T antibody were also tested as probes for the detection of non base-paired regions within native DNA. Such regions might occur

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during DNA transcription. As a model system non base-paired lesions were introduced into native DNA molecules by methylene blue photo-oxidation. This procedure exposed cytosine residues which reacted with anti-C antibodies. Anti-T antibodies failed to react with photo-oxidized DNA until approximately 25% of the guanines had been photo-oxidized or the DNA had been subjected to further denaturing conditions. The reaction of the antisera indicated that the initial photooxidative lesions produced only limited DNA strand separation.

Studies are now being initiated to determine the effect of purified antibodies on a DNA dependent RNA polymerase system.

PLANS FOR FUTURE

Future plans include the preparation of conjugates of proteins with the following nucleosides: 5-Iodouridine, 5-bromouridine, 6-azouridine, and 6-methyluridine. These conjugates will be used to stimulate specific antibody and their specificities will be examined. Our plans also include the examination of their activities in various biochemical systems dependent on DNA or RNA. Purification of the various anti-nucleoside antibodies will be continued.

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BR RICOC DL DP DR. 07 01 F34V

IMMUNOLOGICAL FUNCTION IN SEVERE THERMAL INJURY

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WORK UNIT NO. NR 106-733 **CONTRACT** Nonr-4503 (00)

OBJECTIVES

To evaluate specific changes in the immunological status of experimental animals following severe thermal injury, with particular reference to the ability of the burned host to develop and exhibit delayed hypersensitivity and/or autoimmune-like responses.

ABSTRACT

It has been suggested that thermal injury may induce the formation of toxins, and that the release of such materials into the host may stimulate antibody responses. A number of investigators have, however, failed to demonstrate the presence of either new antigens or antibodies characteristic of severe thermal damage. In the course of this study, rabbits were immunized with extracts of burned guinea pig skin excised from the donors at various time intervals after injury, and the antigenic properties of the specimens were examined by analysis of their reactions with the resulting rabbit antisera.

Male Hartley guinea pigs were subjected to full-thickness skin burns by contact with a thermal-regulated metal surface maintained at 250°C; the animals were sacrificed at 2 hours, 1 day, 2 days, and 6 days after injury, and the burned skin was excised. Control skin segments obtained from normal animals were subjected to similar thermal injury in vitro; an additional set of normal unburned skin specimens was also studied. The antigenic preparations were washed in cold saline solution, and forced through an X-press by the technique of Edebo. The resulting suspension was centrifuged at 57,348 G and the supernatant extract was decanted and collected at 4°C. This extract was mixed with equal volumes of complete Freund's Adjuvant, and injected intradermally into Albino rabbits. Immunological tests performed included double diffusion gel precipitation, immunoelectrophoresis, absorption of sera with burned or normal skin specimens, and with pooled serum and plasma from normal guinea pigs.

Double diffusion gel precipitation tests using rabbit antiserum against burned guinea pig skin excised six days after injury yielded a line of precipitation against extracts of burned skin, but failed to react with normal skin extract. Similar precipitation lines were observed when antisera were obtained by injection of rabbits with extracts of post-op day 2 and post-op day 1 burned skin specimens. This "burn" antigen was absent, or present only in trace amounts in skin specimens removed with-

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in two hours after thermal injury.

Absorption experiments with selected antisera indicated that normal skin extract could not absorb out the reactive principle, but normal guinea pig plasma completely eliminated the activity of such antisera. The "specific burn antigen" isolated in burned skin was absent, however, in normal guinea pig serum.

These results indicate that, under the experimental conditions described, burned skin contains an antigenic component which is absent from normal skin. Detection of this antigen is dependent upon the continued contact of the injured tissues with the living host. Normal guinea pig plasma contains an antigen similar or identical to this antigen, but normal guinea pig serum does not contain this antigen. This observation strongly suggests a relationship between the detected "burn antigen" and fibrinogen.

The mechanisms whereby in vivo thermal injury causes an unusual concentration of fibrinogen or fibrinogen-like materials at the burn site are not clear at present. It is possible, however, that this might be a reflection of the increase in capillary permeability which has been reported as a sequela of thermal injury. The possible relevance of this observation to the course and development of other immune manifestations of thermal injury is currently under study.

PLANS FOR FUTURE

(a) To identify further the fibrinogen-like material which appears at burned skin sites (b) to analyze the possible relevance of this observation upon reports of "toxic" effects of burned tissues upon cells in tissue culture systems (c) to utilize this model for further studies of the course and development of autoimmune manifestations of thermal injury.

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BR R3C0C DL DP DR 07.01 F35V

STUDIES ON THE HEREDITARY HUMAN GAMMA GLOBULIN (Gm) GROUPS

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ASSISTED BY

WORK UNIT NO. NR 106-741 **CONTRACT** Nonr 3656 (12)

OBJECTIVES

(a) Continuing investigation of all facets of γ-globulin, including chemical structure, genetically determined antigenic properties, regulation of quantitative aspects of its synthesis and qualitative aberrations in various disease states associated with too little or too much immune globulin, (b) broad studies of the biochemical basis of genetic difference in γ-globulins and antibodies from one individual to another, (c) studies of the biological consequences of such genetic diversity, and (d) continuing investigations of the interaction of γ-globulin with complement.

ABSTRACT

A new passive hemagglutination technique employing isolated γ-globulins and myeloma proteins of known genetic types coated onto human group O cells by the $CrCl_3$ method has been developed. Cells thus coated have been successfully used for Gm and Inv typing of human sera, eliminating the need for rare anti-Rh coats of a given type for Gm typing (a,b,c).

Human antibodies to IgA globulin have been demonstrated in human sera by the passive hemagglutination method described above using as antigens IgA myeloma proteins coupled to neat indicator red cells. The vast majority of sera containing such agglutinators occur in patients with little or no serum IgA and normal levels of IgG and/or IgM. Specificity of the antibodies was shown by inhibition of agglutination. The antibodies of different sera appear directed toward different antigenic sites in the alpha chains. Such sera may prove useful in demonstrating allotypic genetically determined antigens in IgA molecules analogous to the Gm factors of IgG molecules (d,e,f,g,h,i).

Eighteen different primate species have been typed for Gm factors present at the human γ_{2b} and γ_{2c} loci. Gm factors are absent in lower primates. The Gm factors associated with γ_{2c} molecules, namely, Gm(s), (c^5), (b^0) evolve first in the Cercopithecoidea species. Gm(z) occurs together with Gm(a) on human γ_{2b} molecules, but in baboon sera Gm(z) occurs in the absence of Gm(a). The simultaneous occurrence of Gm(a) and (z) in the same sera is established in Hominoidea. Gm(b^3) is present only in gorillas, orangutans, and chimpanzees; while Gm(b^4) and (c^3) were detected in chimpanzees. Polymorphism for the factors studied exists only in orangutans and chimpanzees. The data show a close correlation between taxonomic classification and the evolution of Gm factors. Tryptic peptide maps demonstrated the presence of the "a" peptide in Gm(a+) non-human primate IgG (j,k,l).

We have also undertaken a phylogenetic study of (1) the heavy chain antigens which distinguish the μ, γ, and α chains; (2) the kappa and lambda subclass antigens of all of the light polypeptide chains and the

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$\gamma 2_a$ (γG_2), $\gamma 2_b$ (γG_1), $\gamma 2_c$ (γG_3), and $\gamma 2_d$ (γG_4) subgroup antigens of the heavy chains of γG immunoglobulins; and (3) the hereditary antigens, the various Gm and Inv factors which are associated with the heavy and light chains respectively. The cross-reactivity of γ, α and μ heavy chains and the kappa and lambda light chain antigens of humans and various non-human primates, has been related to evolutionary status of the various species (m,n).

Leukocytes from 9 patients with "acquired" agammaglobulinemia were studied _in vitro_ in a new synchronized cell culture system. Synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) induced by phytohemagglutinin was measured by determination of the degree of incorporation of labelled precursor. Synthesis of both DNA and RNA was decreased in the agammaglobulinemic cells. The presence of an inhibitor in the patients' sera could not be demonstrated. These results suggest that the basic defect in agammaglobulinemia is cellular rather than humoral. Further, study of lymphocytes of asymptomatic parents of patients with acquired agammaglobulinemia demonstrated a similar defect in DNA and RNA synthesis; thus, showing that "acquired" agammaglobulinemia is genetically determined. In the same system, addition of minute doses of hydrocortisone equivalent to blood levels present _in vivo_ in patients in therapy, resulted in a decrease in DNA synthesis. Hence, the effect of such immunosuppressive agents appears to be due to lymphocyte replication rather than to lymphocytolysis. Studies of lymphocytes from patients with various autoimmune diseases have also been initiated. Other work on autoimmune disease and especially the genetic predisposition thereto is summarized in two invited review chapters (o,p).

PLANS FOR FUTURE

(a) To continue investigation of "autoantibodies" and their significance in human disease (b) to find genetic markers in human IgA and IgM globulins analogous to the Gm factors in IgG (c) to establish biochemical bases, by amino and sequence analysis of appropriate peptides, of the genetic differences between gamma globulins of differing genetic types.

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BR R?COC DL DP DR 01 01 F37

SWITCHING MECHANISMS FOR TYROSINE METABOLISM IN DEVELOPING CELL SYSTEMS. THE EFFECT OF POLYSOMAL PROTEIN ON IMMUNOGLOBULIN SYNTHESIS

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ASSISTED BY D. Santomtonio, D. Coates

WORK UNIT NO. NR 106 758 **CONTRACT** N00014-67-A-0120-0003

OBJECTIVES

(a) To study the mechanisms that determine the pattern of metabolism of tyrosine in developing cell systems. (b) to test the possibility that immunoglobulin production may be controlled, in part, at the translational level and that polysomal proteins are affectors.

ABSTRACT

During cellular differentiation tyrosine utilization becomes variable as a function of cell type. Thus the end-product of this pathway may be melanin, nonadrenaline, adrenaline or acetoacetate and fumarate in cells of different developmental fates. Switching in this pathway could be accomplished alone or in concert at any of several positions. One possibility would be the selective modulation of enzyme activity at branch points in the pathway. Another would be selective production of enzymes at the branch points. This latter alternate could be accomplished at either the transcriptional or translational levels.

The first possibility has been tested by isolating and characterizing enzymes at branch points in the pathway with a view toward learning whether such agencies as metabolite modulation (for example end-product inhibition) could operate as switching devices. Earlier work was concentrated on the oxidases for DOPA, DOPAMINE and Tyrosine. These are a single family of enzymes each of which may oxidize these three substrates. More recently the decarboxylase for DOPA and Tryptophan (again a single family of molecules with multiple substrate specificy) was studied. All available metabolites in the pathway have been tested for their possible roles as modulators of enzyme activity, and both inhibitors and accelerators have been found. These data were used to produce a computor model. The computor predicted that switching could <u>not</u> be accomplished through metabolite modulation and that switching could be achieved <u>only</u> by two agencies acting together-(1) selective variations in the <u>total</u> amount of <u>active</u> Tyrosine-Dopa-Dopamine oxidase (TDD oxidase) complex as a function of cell type, and (2) selective alterations in the substrate specificity of this enzyme as a function of cell type.

We have begun to study the possibility that TDD oxidase may be controlled, in part, at the translational level by isolating and characterizing the polysomes that participate in the synthesis of this

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enzyme. Polysomes from skin were isolated by centrifugation and treated with an antibody preparation specific for TDD oxidase. A precipitate is formed which retains the ability to synthesize protein. We postulated that the precipitate was an anti- TDD oxidase- TDD oxidase polysome complex, and our principal efforts in recent months have been to establish this as fact. These experiments support our hypothesis.

First, unfractionated polysomes have TDD oxidase activity after treatment with trypsin. When whole polysomes are resolved by centrifugation in a sucrose gradient the enzyme activity is found in two positions, at the top of the gradient and at a position corresponding to about 250 S. This would correspond to a polysome carrying 7-9 ribosomes, and would code for a peptide of about 30,000 M.W. TDD oxidase is a tetramer of about 120,000 M.W. so the polysome is of an appropriate size for the enzyme. Furthermore if TDD oxidase antiserum is used as a reagent to detect TDD oxidase antigens in the sucrose gradient eluant, a peak of antigenicity is found in the 250S region and at the top of the gradient.

The most convincing experiment was to isolate polysomes with TDD-oxidase antiserum and use these as a primer for a cell protein synthesizing system using C^{14}-amino acids as substrate. After incubation this system (along with carrier TDD-oxidase) was used as antigen for gel diffusion (ochterlony plate) studies. Precipitin arcs formed, and after removing soluble protein, the dried plate was autoradiographed. The precipitin arcs were radioactive indicating that the polysomes were priming for TDD oxidase synthesis. We may now use this technique to make a quantitative estimate of TDD oxidase m-RNA in various cell types. If there is a lack of correlation between rate of TDD oxidase production and amount of polysomes capable of priming that synthesis, then the possibility may be entertained that switching occurs at the translational level. Similar experiments on cells undergoing cellular differentiation will hopefully give a correlation between the time of appearance of the message and the protein that it codes.

Our experiments on the substrate specificty of tyrosine have led to the observation that inactive TDD oxidase may be activated for all three substrates by gentle trypsinization and activated for Dopa alone by irradiation with U.V. light. Titration with mercurials has the same relative degree of inhibition on the three substrate specificities. One observation leads us to believe that the membrane bound form of the enzyme has a substrate specificity different from that of the soluble form. This possibility is being investigated further.

Earlier experiments in this laboratory had established that basic proteins extracted from polysomes have an inhibitory effect on the rate of protein synthesis in cell free systems containing liver polysomes. The inhibition was shown to be operative both to polysome and S-RNA functions. Investigations are now being conducted to determine whether or not polysomes derived from antibody producing tissues are affected similarly.

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PLANS FOR FUTURE

(a) To make quantitative estimates of TDD oxidase m-RNA in various types and in cells undergoing cellular differentiation. (b) To produce computor models which test the possibility that switching in the tyrosine pathway during development may occur at the translational level. (c) To test further the idea that the substrate specificity of TDD oxidase may be altered by association with different prosthetic groups - e.g. lipids and other membrane components.

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P. Reid and E. Triplett (1968) Observations on the immune systems of Micropterus Salmoides. Transplantation 6: 338.

BR R3COC DL.DP.DR.07.07. F33V

THE ROLE OF THE THYMUS IN THE IMMUNE RESPONSE

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ASSISTED BY

WORK UNIT NO. NR 106-765 CONTRACT N00014-67-A-0128-0001

OBJECTIVES

(a) To study the role of the thymus in determining the genetic constitution of antibody, (b) to study the role of chronic thymectomy in allograft rejection.

ABSTRACT

In a previous abstract (1968) we outlined our reasons for suspecting that the thymus might play a regulatory role in determining the genetic constitution of antibody. An experiment to test this hypothesis was also outlined. Intrauterine rabbits, in the 23rd day of gestation are thymectomized and implanted with an allotypically different thymus, or implanted with an allotypically different thymus without being thymectomized. The implanted embryos are then returned to the uterus of the mother to continue intrauterine life until day 30-31 when they are delivered by Caeserian section.

In this abstract, no intrauterine thymectomized animals have been studied. Non thymectomized implanted animals, studied 8-10 weeks after birth, however, do not show antibody of donor allotype, do not have donor cells present (determined by karyotyping male-female partners) and are not stimulated into division by antiallotype sera (performed in vitro). The donor thymus, however, was not recovered at post mortem. The necessity of recovering the donor thymus in order to make any conclusion is apparent. Further experiments of this nature will be attempted in intrauterine thymectomized rabbits. A second approach to this question is underway. C3H and C57 black mice have different gamma globulin types easily identified by gel diffusion techniques. A small percentage of neonatally thymectomized C3H animals do survive although completely thymectomized and immunologically incompetent into adulthood. Their immune capabilities can be restored by pregnancy. Neonatally thymectomized C3H animals

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at 28 days of life are mated with C57 Black animals. After delivery, the C3H animal is immunized and the gamma globulin type is determined. Small percentages of differing gamma globulin types and the host animals are easily detectable in control studies. To date only one animal which did not show the donor gamma globulin type has been studied. More animals are now being prepared.

Our second objective is to study the role of chronic thymectomy in allograft rejection. It has been noted that chronically thymectomized dogs show a decreased ability to respond to an antigen when the response is measured in mixed leukocyte culture. Allograft survival rates are being studied and compared with non thymectomized dogs showing similar antigen response in mixed leukocyte culture.

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BR R..OC LL EP. DR. 07.04 F39V

ANTIGENICITY OF CONJUGATED PEPTIDES AND RELATED TOXIC MOLECULES

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WORK UNIT NO. NR NR 106-782 **CONTRACT** N00014-67-C-0513

OBJECTIVES

(1) To develop methods for increasing antigenicity of otherwise non-antigenic toxins of animal origin. (2) To produce antibodies with strong toxin neutralizing properties.

ABSTRACT

Many of the naturally occurring toxins are not highly antigenic and hence elicit production of antisera having very low neutralizing titer, if indeed, there are any antibodies produced at all. The molecular size of these toxin molecules, while not the only reason for low antigenicity, certainly contributes to the production of low titer antisera. A second and very important reason for poor antigenicity of these poisons is that they usually are so highly toxic it is impossible to administer doses large enough to elicit good production of antibodies. Therefore, development of methods for increasing molecular size and decreasing toxicity of these poisons would be a valuable contribution to prevention of intoxications by these substances.

Two types of compounds have been selected for this study: (1) paralytic shellfish poison (saxitoxin), and (2) hypotensive peptides, bradykinin, eledoisin, and a hexapeptide closely related in physiological properties to eledoisin. Bradykinin was used as a type compound because other investigators have produced antibodies to this peptide. Because of its ready availability, the hexapeptide has been most widely used in the studies to date.

We have followed two approaches for increasing the antigenicity of our test compounds. The first approach was to use finely divided polymethylmethacrylate particles as adsorbents for the antigens. Berglund used these particles as effective antigen carriers in eliciting antibody production to a fibrino-peptide containing 14 amino acids. We injected rabbits subcutaneously with antigen-carrier mixture in Freund's adjuvant. Following a prolonged course of subcutaneous administration of the antigen, the animals were finally injected intravenously with antigen-carrier only. We were unable to demonstrate any increase in antibody titer as

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measured by the precipitin reaction and by neutralization. Therefore, we have concluded that the polymethylmethacrylate particles frequently used in the agglutination test for rheumatoid-arthritis serum factor and employed by Berglund to enhance antigenicity of a fibrino-peptide are not effective as antigen carrier for the hypotensive peptides. Experiments we have carried out using the hexapeptide and the plastic particles show that the peptide is not strongly adsorbed by the particles. Thus, we confirmed observations we have made with antisera.

The second approach to increasing antigenicity is chemical coupling to a relatively non-antigenic protein molecule or polyamino acid. Because there is some evidence in the literature that polylysine is relatively non-antigenic, we used this polyamino acid as carrier. The studies were carried out initially using the hexapeptide coupled to polylysine (MW 150,000) by the carbodiimide reaction. The resulting preparation was used as antigen after dialysis to remove the unreacted hexapeptide and the reagent. Antibodies to polylysine were produced by rabbits immunized with the conjugate but we have been unable to show the presence of antibodies to the hexapeptide itself.

One primary purpose for carrying out the present investigation was to produce effective neutralizing antibodies against paralytic shellfish poison. Until recently, the part of the investigation dealing with saxitoxin was delayed because of availability of the poison. Recently, however, the principal investigator went to Alaska and collected sufficient Alaska butter clams (Saxidomus giganteus) to yield 100 lb. of clam siphons which assay approximately 7,000 mouse units per 100 g of meat. We are currently isolating saxitoxin from these siphons. The isolated material will then be used in coupling to a large protein molecule probably homologous serum albumen (rabbit serum albumen). The coupled compound will then be used as an antigen.

A second approach to producing antibodies to saxitoxin is based upon evidence from observations by others that the poison is bound to the tissue of the clam siphons. Therefore, we are using these tissues and crude extracts of the siphons as antigens. We are currently still in the process of immunizing the rabbits and consequently there is, as yet, no information available about the level of antibody production.

PLANS FOR FUTURE

In continuing the studies, the investigation is to be directed completely toward increasing antigenicity of paralytic shellfish poison by the procedures referred to in the abstract. These procedures consist of the following: (1) chemically coupling the poison to a protein molecule as carrier and using this conjugate as antigen; (2) utilizing the naturally bound toxin present in the clam meats as antigen; (3) because of the basicity of the toxin, as acidic adsorbent (i.e., ion exchange resin) of small particle size, 0.5 micron or less, may be tried as a possible antigen carrier.

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THE ACTION AND INTERACTION OF PURIFIED LYMPHOID CELLS IN THE REJECTION OF SKIN GRAFTS

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WORK UNIT NO. NR 106-813 CONTRACT N00014-68-C-0287

OBJECTIVES

(a) To delineate the cells active in the production of humoral and cellular immunity, (b) to purify and identify various morphologic and/or physiologic cell populations involved in the production of humoral antibodies and graft rejection, (c) to study the interaction among the different populations of purified cells in these processes.

ABSTRACT

Skin grafting is an area in which both humoral and cellular antibodies have been implicated. The specific roles of the various cell types in rejecting such grafts is not defined.

Initial efforts have been directed toward fractionating cells involved in the production of circulating antibodies. For this purpose the double aldehyde procedure of stabilizing erythrocytes for passive hemagglutination (Hirata J. Immun. 100:641, 1968) has been adapted to our system. We have prepared large batches of cells which have been sensitized to hemocyanin or BSA, as well as the sulfanilate hapten conjugated to the protein and coupled to the erythrocytes as sulfanilate-azo-protein of either BSA or hemocyanin. These sesitized cells are being stored in liquid nitrogen and in this state are stable for prolonged periods of time.

Since we are interested in determining the antibody-producing functions of different populations of cells maintained in the same tissue culture medium, it was necessary to develop the hemolytic plaque technique. Although the aldehyde treatment of erythrocytes is satisfactory for passive hemagglutination, it is not applicable to this extremely sensitive technique. Consequently, the Carbodiimide sensitization procedure for coupling proteins to erythrocytes has been adapted for this purpose. It was found that although sheep erythrocytes are not as satisfactory as rabbit erythrocytes for sensitization with BSA, comparable results were obtained when sensitizing cells from either species with sodium sulfanilate. This hapten has been coupled directly to erythrocytes without need of

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a protein carrier. In order to obtain greater uniformity and shorten the time necessary for preparation of sensitized cells, a procedure was developed for freezing these cells and maintaining them in liquid nitrogen until needed.

The tissue culture technique is being utilized in an effort to discern the effect of different physiologic environments on the antibody producing capacity of dispersed spleen cells. The primary response of rabbits to sheep erythrocytes is being studied with this system. A perfusion chamber designed and developed by one of the investigators (HA) is being employed in these studies. Aliquots of media as well as cells have been removed at periodic intervals after explanting and tested for soluble antibodies and the plaque forming population of cells. A method is being developed whereby the aliquots of cells can be removed without disturbing the general milieu of the remaining population.

PLANS FOR FUTURE

(a) To maintain cells from immunized animals under different culture conditions and observe the effect on survival, and the ability to produce antibodies, (b) to delineate the populations of cells maintained under the different conditions, (c) to study the interaction of these cells in the production of circulating antibodies, (d) to relate these conditions and cells to the rejection process.

CURRENT REPORTS AND PUBLICATIONS

H. Ainis and N. Biskup, Double Aldehyde Stabilization of Erythrocytes in Passive Hemagglutination of Hemocyanin and Azo-proteins. Federation Proceedings: 27; 259, 1968

BR R... DR.07.01 F41V

PHARMACOLOGY

Research in basic biology associated with naval operations inevitably brings contact with some relatively unexplored areas whose significance to naval needs must be problematic because they are unexplored. Many biological products of considerable physiological potency exist, some of which may present hazards we should anticipate in our planning and some of which may have great value to the Navy in a number of ways. Besides the obvious concern with venomous pests and flesh unfit for consumption, it is probable that many compounds elaborated by organisms to their own advantage in the competition for food and space could be useful as pest controls, or could suggest synthetic materials employing similar principals. Obviously, a number of medically useful compounds ranging from Peruvian Bark to antibiotics have been the gift of recognition of the metabolic performance of a wide range of organisms. In addition, study of the metabolic synthesis and fate of pharmacologically potent materials may be expected to provide new insight into the nature of some disabilities and into logical methods of dealing with them.

We began this program in the name of Toxinology, but find the field wanders a bit too far from the central theme of Biochemistry. The revision of the name to Pharmacology is intended to provide a statement of interest in the same general class of research effort as was represented by the Toxinology studies in the past, but with the relegation of a part of the interest to the cognizance of the oceanic biologists in another division of ONR.

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A STUDY OF IMMEDIATE SENSITIZATION OF TISSUES BY ANTIGENS AND ANTIBODIES, *IN VITRO*

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WORK UNIT NO. NR 305-720 **CONTRACT** NONR 225(46)

OBJECTIVES

(a) to find improved fractionation procedures for the production of the non-spasmogenic principle in tetanus toxin, (b) to continue studies of the properties and mode of action of other releasers: antigen-antibody reactions, bacterial toxins, and animal venoms, (c) to continue studies on the fractionation and proteolytic behavior of sea urchin toxin.

ABSTRACT

A. Toxins

1. Tetanus. A search for improved methods of fractionating the MEPP-rich starting material to isolate the non-spasmogenic factor in active form resulted in the finding that this condition could be fulfilled by using hydroxylapatite as the chromatographic adsorbent. This material proved to have much greater sample capacity and a higher degree of resolution than Sephadex. Six clearly defined fractions were obtained. The physical and biological properties are presently under study.

2. Streptolysin. The present work has dealt with the double insult problem, *i.e.* with the analysis of the combined toxic and anaphylactic reaction of the sensitized heart challenged with "inactive" (oxidized) and "active" (reduced) streptolysin O. Guinea pigs were actively or passively sensitized in the first instance by subcutaneous inoculation of 1 mg of oxidized SLO in complete Freund's adjuvant, and in the latter instance by the *in vivo* or *in vitro* exposure to rabbit antibodies produced by active immunization with the same antigen. Challenge of the actively sensitized whole heart with 0.1 mg/ml of inactive toxin produced only the typical anaphylactic response: increased rate and amplitude of contraction, AV block, and a concomitant release of histamine. The responses of actively or passively sensitized atria to oxidized toxin were also of the usual kind we have described for atrial anaphylaxis (1). The response consisted of an increased rate and force of beat accompanying the copious release of histamine by the preparation. The character of the response changes radically when sensitized tissues are challenged with *active* toxin. In that case, the initial event is a reduction in the force of atrial contraction which, subsequently, is followed by the typical anaphylactic increase in rate and force. The presence of atropine selectively blocks the early depression in the sensitized atrium challenged with active toxin, but has no effect on the later inotropic and chronotropic reactions to the anaphylactic component. Pyribenzamine has no influence on the early phase, but blocks the later anaphylactic

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response. When both blocking agents are present together during the challenge maneuver only a slight transient depression is observed in the early reaction, but there is no secondary anaphylactic reaction. Thus, oxidized streptolysin O elicits the formation of homo- and heterocytotropic antibodies; cardiac tissues of guinea pigs sensitized with these antibodies are not protected against either the toxic or anaphylactic actions of the active toxin. The aggravation of the toxic action by tissue-fixed antibodies makes this a useful model for studies of the functional cardiac changes in rheumatic heart disease.

3. Sea Urchin Toxin. Several large batches of kinin were prepared by treating heated, acidified, human plasma with SUT or trypsin. The peptides produced by this digestion were recovered by precipitating the reaction mixture with ethanol, extracting the dried ethanolic residue with acidified *n*-butanol and precipitating the butanolic extract with ether. Selected samples were further purified by gel filtration on Sephadex G-25 and then chromatographed on paper along with synthetic bradykinin. Paper chromatography of the HTP-SUT digest gave five distinct spots, 3 of them having biological (kinin) activity; one of these corresponded in mobility to authentic bradykinin. Pharmacologically, the active product formed by trypsin seemed to be almost entirely bradykinin-like, but in the case of SUT an additional active material was present.

A survey of the esteratic activities of the 3 Sephadex fractions and 6 hydroxylapatite preparations was made by determining whether they could break down the synthetic substrates TAME and ATEE. None of the preparations attacked TAME and only three-Sephadex I and hydroxylapatite I & II- attacked ATEE. These experiments exhibited classical Michaelis kinetics with respect to enzyme and substrate concentration. The K_m values were Seph. I, 2×10^{-3} M; HOA I 3.04×10^{-3} M; and HOA II 1.33×10^{-2} M.

B. *In Vitro* Anaphylaxis

Reverse passive sensitization of guinea pig hearts with penicilloyl conjugated rabbit γ-globulin gives a typical anaphylactic response including a decrease in coronary perfusion rate and the liberation of histamine. Direct passive sensitization with penicilloyl-conjugated rabbit antiovalbumin does not result in histamine liberation when the preparation is challenged with the Pen group; instead of decreasing the flow rate increases, although challenge with ovalbumin produces the usual anaphylactic reaction complex. An experimental analysis of this problem showed that the anti Pen-RγG incites the formation of γ_1 antibodies in the rabbit. A direct test was made in which rabbit antiovalbumin was fractionated into γ_1 and γ_2 species. On challenge, those hearts sensitized with γ_2 antibodies released histamine and decreased the coronary flow, whereas similar tests on γ_1-sensitized hearts produced no histamine and increased the flow, presumably because of the liberation of a completely different mediator.

PLANS FOR FUTURE

(a) to continue studies on the isolation, purification, and characterization of components from tetanus and sea urchin toxins, (b) to study their biochemical and immunological behavior with particular reference to anaphylaxis, (c) to continue studies on the anaphylactic selectivity of electrophoretically distinct immunoglobulins.

BR R3COC DL DP DR. 07.01. F43

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BR RJCOC DL.DP.DR 07.01 F43V

Biophysical and Biochemical Studies of Marine Toxins

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ASSISTED BY Z. Paster, A. Dutta

WORK UNIT NO. NR 305-743 **CONTRACT** NONR 3985(02)

OBJECTIVES

(a) To study naturally occurring toxins, (b) to investigate the biophysics of excitable membranes and the effects of toxins on the tissues.

ABSTRACT

1) Toxic Algae. Drs. Dutta and Paster studied the photo inactivation of toxin from *Prymnesium parvum* at a series of temperatures, in oxygen and nitrogen, in light and dark. Photooxidation by pigment impurities was demonstrated.

2) Nemertive worms are carnivorous with a prominent proboscis used to capture and poison prey. A paralytic toxin Amphiporine was described by Z. M. Bacz in 1936. Under this contract Mr. Kem has identified this toxin as a graduate student under my supervision and in collaboration with Dr. Coates at the University of Illinois. Isolation, purification, identification and synthesis is reported. a) 10,000 Paranemertes worms were collected and extracted in alcohol. b) Purification: Biological activity could be localized as a zone on a thin layer chromatograph. Ehrlich's reagent (p-dimethyl amino benzaldehyde) visualized the zone. Purification was achieved by sequential chromatography with a series of increasingly polar solvent Benzene-ether, Ether, Ether-Chloroform, Chloroform and Chloroform-ethanol. Most of the toxic principle washed out in Ether and was collected. Crystals of the pricrate salt were prepared. c) Biological Assays were made using Crayfish. Animals were placed on their backs. That fraction not righting themselves within 2 minutes was plotted against log dose and median paralytic dose gave a measure for biological activity. d) Identification followed studies on UV, IR, NMR and Mass Spectra. Elemental analysis showed a composition of 42.66% C, 2.98%H, 18.41%N. The other analyses indicated the compound to be 2-(3-pyridyl)-3,4,5,6 tetrahydropyridine. This is the first time this compound has been observed as a natural compound. The nearest relative in nature is a reduction product, 2-(3-pyridyle)-p-peridine, called anabasine, a tobacco alkaloid.

Synthesis. The compound was synthesized and proved to be identical with the purified Paranamertes toxin.

This work is incorporated into the thesis presented by Mr. Kem for a Ph.D. at the University of Illinois 1969 and is being published.

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3) Biophysical studies of excitable membranes. The olfactory nerve of the garfish has been successfully used for heat measurements during a single action potential at 0°C which lasts for more than ½ second. Heat is given out during the depolarization, absorbed during the overshoot and reabsorbed also during the final repolarization. This is important in relation to the membrane capacity and activation.

PLANS FOR FUTURE

1) Axenic cultures of the toxic alga Gymnodinium breve, have been obtained with the antibiotic Kanamycin. Growth rates will be studied with a variety of nutrients, especially amino acids. This will enable us to proceed with the accumulation of enough toxin to undertake purification. 2) Studies will be continued on the mode of operation of toxic substances on excitable membranes. The research will be undertaken in the Allan Hancock Foundation, University of Southern California.

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BIOCHEMISTRY OF PHYSIOLOGICALLY ACTIVE PRINCIPLES IN ECHINODERMS

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ASSISTED BY

WORK UNIT NO. NR 305-757 **CONTRACT** 3128(00)

OBJECTIVES

(a) To investigate the relationships of the chemical structure of steroid glycosides from echinoderms and their biological activity, and (b) to elucidate the structure of holothurin, a physiologically active glycoside from the sea-cucumber Actinopyga agassizi.

ABSTRACT

None received.

BR RJCOC DL DP. DR. 04.01. P49

STUDIES ON THE COAGULANT ENZYMES OF FRESH SNAKE VENOMS

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ASSISTED BY J. Bonner, P. Ohlmeyer and M.T. Schwartz

WORK UNIT NO. NR 305 776 **CONTRACT** NOOO14-67-A-0449-0001

OBJECTIVES

This investigation will study venoms from various land and marine snakes for enzymes acting directly on fibrinogen. A major study is being made on the coagulant enzyme of Bothrops atrox and Bothrops jararaca. A method of isolating the Bothrops atrox Coagulant Enzyme from fresh whole venom frozen in liquid nitrogen, is being developed. The isolated enzyme will be characterized chemically and some of its physiological activities will be studied. A survey study of various marine and terrestrial snake venoms for enzymes acting directly on fibrinogen is planned to begin early in 1969.

ABSTRACT

The focus of this research to date has been on the venom of Bothrops atrox. The Bothrops atrox Coagulant Enzyme (BaCE) which acts directly on fibrinogen by hydrolyzing a single arginyl-glycyl bond, releasing fibrinopeptide A, is the special object of interest. The venom from 15 to 20 single extractions of Bothrops atrox is pooled and shot-frozen within 30 minutes of extraction in liquid nitrogen (lN_2). These and other venoms are kept in vapor-phase storage over lN_2 until used. The whole venom has been characterized by wet chemical, spectrophotometric, immunochemical, electrophoretic and microbiological methods. The whole venom has been subjected to chromatographic separation on various ion exchange materials, various permeation gels and hydroxylapatite. A third stage of fractionation is currently under investigation. However, the enzyme activity is not yet resolved into a single component.

We have confirmed that the BaCE, like the Bothrops jararaca coagulant enzyme, hydrolyses principally the fibrinopeptide A bond of fibrinogen. The resultant fibrin monomer will form complexes with fibrinogen.

Frequent sequelae of Bothropic envenomation are thrombotic manifestations, such as massive thrombi in the large blood vessels, dry gangrene or focal necrosis. Another manifestation of envenomation is circulatory shock. A major by-product of our research on the BaCE has been its development as a definable enzymatic model for the study of the hemorheological changes characteristic of circulatory shock. The slow intravenous injection of small quantities of a BaCE preparation in dogs resulted in hemodynamic changes characteristic of circulatory shock. The quantity of enzyme injected was sufficient to cause fibrinogen-fibrin complexes (FFC) to form but would not clot or defibrinate the blood. The shear stress-shear rate measurements strongly suggested that a

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marked yield value occurs after the injection due to FFC formation. In ex vivo blood systems similar results were obtained.

While the BaCE in low concentration will form FFC, leading to an increase in blood viscosity and a resistance to blood flow, at higher concentrations a coagulum will form. Thus, the mode of the venom's toxicity may be related to both the quantity and rate of the venom's entry into the circulation. These studies may provide a new rationale for the development of new ways of treatment of snake bite from Bothrops atrox and jararaca, or possibly, by extension, from species of Crotalus, Agkistrodon or other genera, where envenomation induces profound circulatory disturbances leading, for instance, as mentioned above, to shock, thrombosis, necrosis or gangrene.

PLANS FOR FUTURE

(a) Continuation of fractionation studies to obtain the isolated Bothrops atrox Coagulant Enzyme (BaCE). (b) Physicochemical characterization of the isolated BaCE. (c) Kinetic and specificity studies on the isolated BaCE in comparison to thrombin. (d) Investigation of the mechanism of action of BaCE on fibrinogen. (e) Initiation of a survey on coagulant enzymes of snake venoms, other than Bothrops atrox, which act directly on fibrinogen.

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TOXIC PRINCIPLES OF SEA SNAKE VENOMS FROM SOUTHEAST ASIA AND THE FAR EAST

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WORK UNIT NO. NR 305-780 **CONTRACT** N00014-67-A-0299-0005

OBJECTIVES

(a) To isolate the toxic principles of sea snake venoms, (b) to determine chemical properties of toxic principles, (c) to determine the role of enzymes in venom toxicity.

ABSTRACT

Enhydrinia schistosa (common sea snake) venom from the Strait of Malacca was resolved into 7 fractions by isoelectric focusing. Only one toxic fraction was isolated. The isoelectric distribution of sea snake venom was markedly different from that of the land snake. When *Agkistrodon rhodostoma* (Malayan pit viper) venom was fractionated by the same technique, 15 fractions were obtained.

Toxicity of *Laticauda semifasciata* venom from the Philippines was stable over the pH range of 2 to 11. The venom toxicity was not affected by iodoacetate, however, the toxicity disappeared when the venom was treated with o-methylisourea and 1,2-cyclohexanedione. This may suggest that the histidine residue is not essential for toxicity while lysine and tryptophane are important. Therefore, the venom was fractionated and the toxic fraction was isolated. Similar experiments using o-methylisourea and 1,2-cyclohexanedione will be carried out on the isolated toxin.

Phospholipase A was isolated from *Laticauda semifasciata* venom using carboxymethyl cellulose column chromatography and separated from the toxic fraction. Chemical properties of this enzyme will be investigated.

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PLANS FOR FUTURE

(a) Snake venoms will be fractionated and the toxic principles will be isolated for additional study. (b) Chemical modification of the toxin will be made in order to determine which amino acid residues are essential for venom toxicity.

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BR K3COC DI DP DR. 07.01. F47

THE CHARACTERIZATION OF CHIRONEX FLECKERI NEMATOCYST TOXIN

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WORK UNIT NO. NR 305-785/12-29-66 **CONTRACT** N00014-67-C-0408

OBJECTIVES

(a) To obtain pure suspensions of undischarged nematocysts of Chironex fleckeri, (b) to discharge nematocysts thereby obtaining a solution of pure toxin, (c) to define a satisfactory assay procedure for testing toxin strength,(d) to establish chemical identity of toxin and number of components contained in it, (e) to establish the primary structure of the chief toxin component.

ABSTRACT

Solutions of Chironex toxin electrically milked from living Chironex tentacles (obtained from Dr J.H. Barnes, Cairns, Queensland) have been used to establish a satisfactory bioassay procedure and to study the stability of the toxin under different conditions.

Of a number of bioassay procedures tested, the rat blood pressure preparation was found to be the most satisfactory method. In rats anaesthetized with intraperitoneal injections of urethane, sublethal doses of Chironex toxin injected into the tail vein produce a rapid rise in blood pressure followed by a less rapid return to normal. Such sublethal doses can be given repetitively at 10 minute intervals, and the response, as measured by the increase in blood pressure, can be used in a two by two assay of standard and test solutions at two dose levels. Lethal doses of Chironex toxin (about twice the highest sublethal dose used in the assay) produce somewhat larger increases in blood pressure followed by a very rapid fall to near zero pressure. Direct examination of the heart, and ECG's recorded after injection of lethal doses, failed to show any specific effects, although AV block, with auricular fibrillation and slow ventricular beats, was the most general finding. ECG's taken during injections of sublethal doses showed only slight rate changes and no changes in pattern.

The action of the toxin on the rat appears to be central since pithed/anaesthetized rats respond to normal blood pressure-elevating doses with negligible rises in blood pressure. Rats given light ether anaesthesia, and then pithed, show an intermediate response to such a dose. The lethal dose of toxin for unanaesthetized, urethane-anaesthetized, pithed, and urethane-anaesthetized pithed rats have been determined and appear to be significantly different from one another.

LD_{50}'s for numerous different toxin preparations have been deter-

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mined by injecting the toxin intravenously into mature male mice. The method of Litchfield and Wilcoxon (1949) was used to evaluate these experiments. LD_{50}'s have been determined on three different inbred strains of white mice.

Aqueous toxin solutions stored at 4°C gradually lose their activity over a period of three or four weeks. Partial stabilization of the toxin was obtained by dialysing it against 0.9% sodium chloride and almost complete stabilization by adding 1% bovine serum albumin after dialysis. Addition of calcium (.11M) to dialysed toxin rapidly destroyed its activity.

It has been shown that different dilutions of toxin have different activities. The potency ratio between 1/200 and 1/40 toxin, injected immediately after dilution, was 2. 1/200 dilutions were shown to lose activity on dilution, the potency ratio between a solution injected immediately upon dilution, and one injected after 90 minutes dilution was 1.5. A 1/40 dilution loses activity less rapidly.

A series of dose-death-time titration curves have been established by plotting the reciprocal mean death times (in seconds) vs. the corresponding mouse units (where 1 mouse unit is the calculated LD_{50} dilution dose). It is hoped that the mean death time for a given dose, determined on a relatively small number of mice, can be used to assay toxin strength.

PLANS FOR FUTURE

(a) to study further the problems of stabilizing the toxin in dilute solutions so that conventional techniques of protein purification can be used to separate the various toxin factors from one another, (b) to purify the lethal factor(s), and (c) to determine the primary structure of the lethal factor.

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SOME CHEMICAL AND ZOOTOXICOLOGICAL PROPERTIES OF STINGRAY VENOM

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WORK UNIT NO. NR 305-786 **CONTRACT** N00014-67-C-0391

OBJECTIVES

To isolate and characterize the venom from the stingray.

ABSTRACT

Previous studies have shown that the venom (extracts of spine sheaths) from the stingray, Urobatis halleri, contains many components, both micro- and macromolecules, some of which are biologically active. Emphasis in the present study has been placed on the nature of the toxin, the conditions for its stability, and the role of the macromolecules in the production of the deleterious effects of the whole venom.

By means of gel filtration and ion-exchange chromatography it has been possible to separate active protein fractions from inactive ones, and to achieve a ten-fold increase in the order of lethality. The active fractions obtained to date possess several protein components, one or more of which are responsible for the lethality in mice, and which also produce pain and cardiovascular changes in other mammals. While lethality in mice to various extracts is the usual criterion used for the assessment of activity, it has been shown that the fraction which is lethal to mice has no effect on either the mammalian or the crustacean nerve-muscle preparation. The active fraction which is responsible for the hypotensive crisis is currently under investigation. Refinements in chromatographic methodology are also underway in an attempt to separate the various proteins which comprise the active fraction.

Because the active protein fraction is unstable and because the usual methods for preparation or storage, such as lyophilization, ammonium sulfate precipitation, etc., are not applicable, a great deal of effort has been expended in an attempt to find conditions most favorable for its stability. Solutions of the venom are unstable. The proteins are thermolabile and are totally inactivated in a matter of hours at room temperature and in a few days at refrigeration

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temperatures. Freezing, at least at -80°C, decreases the rate of activity loss. It was also found that the rate of decay was dependent on the age of the spines from which the extracts were made so that extracts made from spine sheaths of freshly collected stingrays retained their lethality much longer than extracts made from material that had been stored frozen (-60°C) for extended periods of time. Although the rate of decay seems to be directly proportional to the length of storage, the initial magnitude of toxicity does not seem to be so related. Neither the adjustment of the pH and/or the salt concentration of the extracts, nor the addition of substances such as EDTA, DMSO, cysteine and glutathione to the extracts stabilized the venom. However, the addition of a small quantity of toluene did in several cases appreciably slow the rate of lethality decay. This decrease in decay rate cannot be accounted for entirely on the basis of the bacteriostatic properties of toluene *per se*, but is probably related to the interaction of the toluene with some enzymic system in the extract which is responsible for the degradation of the active components of the venom.

PLANS FOR FUTURE

(a) To continue the study of the factors which contribute to the stability of the venom. (b) To isolate and characterize the various fractions of the venom having deleterious effects.

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STUDIES ON THE SCULPIN *SCORPAENA GUTTATA*

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WORK UNIT NO. NR 305-793 **CONTRACT** N00014-67-C-0390

OBJECTIVES

(a) To study certain biological activities of the sculpin and its predator-prey relationships, (b) to isolate and characterize its venom, and (c) to develop suitable countermeasures against envenomation.

ABSTRACT

(a) Studies on the behavior of the sculpin have shown that they do not use their venomous dorsal, ventral and anal fin spines in the capture of prey, but do use them as defensive weapons against large-mouth predators. The octopus, Octopus bimaculatus, is one of several animals which sometimes prey on the sculpin. The sculpin's venomous spines are relatively ineffectual against octopuses because of the latter's pliability. The sculpin has developed an evasive behavior to evade octopuses under most circumstances. This behavior is in contrast to the characteristic "stand fast" behavior displayed by the sculpin to many other predators, including certain large fishes, seals and SCUBA divers. The sculpin discriminates predatory behavior from other types of behavior in octopuses, which makes it seem likely that the ability is learned rather than innate. It appears that the number of sculpins in a habitat is related to the available space and the presence and number of octopuses. In relation to the sculpin problem around an artificial reef or a submerged object, the introduction of octopuses into the new habitat might reduce the number of sculpins but is not likely to eliminate them. The ability of the sculpin to distinguish between kinds of behavior in octopuses and the habituate to movements rules out the use of mock octopuses or other mechanical devices employing movement or form to repel these fish.

(b) The venom (extracts of dorsal, ventral or anal fin spines) contains many components, both micro- and macromolecules, some of which are biologically active. The deleterious manifestations of the venom (acute hypotension, respiratory distress, pain and death) appear to be due to the activities of certain of the macromolecules. The whole venom, as well as fractions which have been obtained by purification procedures, are relatively unstable at both room and refrigerator temperatures, and to some extent at -18°C. Activity

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can be retained for longer periods of time at -80°C. These preparations can be more effectively preserved by lyophilization, provided that the process is begun immediately after the extracts have been **shell frozen.** Marked losses in lethality occur on fractionation of the whole venom by gel filtration on either modified dextrans or agarose, and while loss also occurs when the proteins are precipitated out of solution with ammonium sulfate, this latter method can be used for concentrating crude extracts.

PLANS FOR FUTURE

(a) To study certain movements of individual and "schooled" sculpins to and from artificial reefs. (b) To continue the chemical studies on the isolation and purification of the toxin and on its physiopharmacological properties.

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THE CHEMISTRY OF COELENTERATE TOXINS AND THEIR MODE OF ACTION

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ASSISTED BY

WORK UNIT NO. NR **CONTRACT** N00014-67A-0298-0014

OBJECTIVES

a) Determine site of mode of action of coelenterate toxins, b) purify the toxins, c) compare action of toxins with other molecules having similar effects.

ABSTRACT

The action of anemone toxin has been investigated by application to voltage-clamped crayfish axons. The toxin irreversibly and selectively increases the time constant of sodium inactivation without any effect on resting sodium or potassium currents, peak sodium or potassium currents, or the kinetics of sodium or potassium activation. The exclusive action of toxin on sodium inactivation makes this toxin unique. The two agents most similar in effect to it, scorpion toxin and DDT, in addition reduce and delay potassium activation.

Anemone toxin is unlike DDT in that it has no effect on calcium-spiking mechanisms, whereas DDT appears to be a competitive blocker of calcium spikes.

Several anemone toxins have similar actions, but the action of Scyphozoan toxins appears to be somewhat different.

Purification of anemone toxin can be improved, particularly for batchwise processing, by ammonium sulfate precipitation. This is then followed by the gel filtration and ion exchange chromatography previously described by the author.

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