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PROGRESS REPORT ABSTRACTS

BIOCHEMISTRY BRANCH

Office of Naval Research

JANUARY 1967

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FOREWORD

Investigators supported by the Biochemistry Branch of the Office of Naval Research are both the authors and the readers of this annual volume of Abstracts. The assembled abstracts, reproduced without editing, serve as a thumbnail sketch of the overall Branch Program, and it is to be hoped that year-to-year comparison may permit some concept of the way in which the program changes in response to changing Naval needs.

The Abstracts do not constitute publication in the usual sense, and should be treated as PRIVILEGED PERSONAL COMMUNICATIONS. Reference to material in this volume should not be made without the express permission of the author.

The investigators and their associates have been most helpful in preparing these reports and in giving consideration to the means for making the Biochemistry Branch program what it should be. We are most grateful for this assistance, and for the excellence of the research reported in these abstracts.

Robert K. Jennings

ROBERT K. JENNINGS
Head, Biochemistry Branch
Biological Sciences Division

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I N T R O D U C T I O N

For the first time in some years, it is possible to present this volume as a fair approximation of the actual on-going program. There has been a period of transition during which the inclusion of many reports on contracts already terminated and the omission of reports of work just started made the whole less than representative for the year.

Due to changing needs for basic research in the foreseeable shape of naval operations and to changing aspects of government support for basic research, it is appropriate for ONR to contribute less to the development of the science of biochemistry and more to those aspects of biochemical investigation which promise to serve primarily the man aboard ship. We remain that Naval agency charged with the task of finding out things no one anticipated a need for on the basis of past experience. But what is needed is a knowledge of biological factors which might affect naval operations, the possible methods of controlling them, avoiding their limiting effects or taking advantage of their useful characteristics, and possibly of imitating nature's methods of dealing with the marine environment.

The emerging program is intended to foster the exploration of areas of ignorance which are of prime concern to the Navy. The biochemically related disciplines which may serve this purpose, at least for the moment, are considered to be five in number: marine biochemistry; metabolic biochemistry; immunochemistry; toxicology, and toxinology. The following abstracts have been grouped into these five categories. The key thought in reading them should be: "Do these subelements in the Branch program represent an optimal effort to explore the biochemical aspects of marine life and naval life?" To the reader who is himself a member of the team, the answer may be helpful in planning his future research, making it as productive as possible of useful information. We hope that it may also stimulate comments on the neglect of certain areas which may be ripe for exploration, or the existence of redundancies in the present effort, as judged by those most expert in the field.

The thinking which has gone into the selection of each of the five subdivisions of the FY 67 Branch program is described at the beginning of each of the five groups of abstracts. A few of the abstracts still deal with work which was completed during the year or will soon terminate, but we feel that the general trend of current ONR interest in Biochemistry is perceptible in the material here presented.

MARINE BIOCHEMISTRY

The sea is living tissue, having a physiological fluid matrix, living cells, a metabolism, and undoubtedly wastes and by-products produced by the living elements and having their influence on other cells. The Navy has operated first from a platform resting on this tissue, then as an encapsulated body within the tissue itself. With Sealab and Scuba diving, the duties of naval personnel make them similar to invaders--hopefully saprophytic--of a blood stream. We need to know how the normal elements in this environment operate and how they may affect invaders. Since we are concerned with basic research, we need to learn all we can about this environment and its biochemical effects if only to say with assurance, "that fact is negligible," rather than "we suppose that fact is negligible!"

The sea also offers a library of information to the biologist in the form of a great variety and a wide evolutionary range of successful organisms. It is a promising field for new exploration with the expectation of getting new insights into complex biological performances by man and other descendants of the marine life, as well as of new biological products useful to mankind in general and to the Navy in particular. There is certainly no reason to neglect this opportunity for making a valuable contribution to science and humanity as a by-product of navy-oriented basic research.

STUDIES OF PROTEOLYTIC ENZYMES

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ASSISTED BY K. A. Walsh, L. H. Ericsson, D. R. Babin, J. H. Freisheim, A. G. Lacko, W. P. Winter

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

OBJECTIVES

To determine the relation between the chemical structure and enzymatic functions of proteolytic enzymes and to elucidate their phylogenetic relationships.

ABSTRACT

In continuation of the work previously reported, the following investigations have been carried out in the past year:

1. Chemical structure of bovine pancreatic carboxypeptidase A - The determination of the complete amino acid sequence of this protein, containing 307 amino acid residues in a single chain, is the ultimate goal of this investigation, which is now in its third year. It is hoped that this goal will be reached sometime in 1967.

We have previously reported that an amino acid replacement occurs in the carboxyl terminal region of carboxypeptidase. Investigation of the enzymes obtained from 14 individual pancreas glands has provided evidence that these variations are due to allelomorphism, the distribution following the genetic laws. By means of a pancreatic fistula, pancreatic juice has been collected from individual animals, one of them containing the pure "leucine" enzyme and the other one the pure "valine" trait. These two enzymes are identical in enzymatic function, but differ considerably in their conformational stability.

2. The activation of procarboxypeptidase A - By means of succinylation with succinic anhydride, disaggregation of bovine pancreatic carboxypeptidase A has been achieved and the immediate precursor of carboxypeptidase, succinyl fraction I, has been isolated. The mechanism of the conversion of succinyl fraction I to succinyl carboxypeptidase has been investigated in detail. Chemical modification (with acetylimidazole) and metal replacements have provided evidence that certain aspects of the active site, presumably concerned with substrate binding, are preexistent in the precursor. These studies have provided for the first time a system for the investigation of the formation of bovine carboxypeptidase A from its immediate precursor.

3. Isolation and mechanism of activation of procarboxypeptidase A from dogfish pancreas - Procarboxypeptidase A occurs in this tissue in a monomolecular form rather than as a trimolecular aggregate as in the case of the bovine. The enzyme precursor has been isolated, purified and

characterized by amino acid analysis, end groups, and molecular weight, and the mechanism of activation has been investigated. Activation is accompanied by a decrease in molecular weight from 41,000 to 36,500. The active enzyme, dogfish carboxypeptidase A, is likewise being characterized by amino acid analysis, by enzymatic specificity. The effects of modification by chemical reagents (acetylimidazole) and by metal replacement have been found to be analogous to that of the bovine enzyme.

4. Proteolytic enzymes of the starfish (Evasterias Troschelii) - The search for a phylogenetic progenitor of trypsin and other serine proteases has been extended in a preliminary investigation to the peloric ceca of the starfish. It has been found that this tissue contains proteolytic digestive enzymes, active toward casein, hemoglobin and toward synthetic substrates for trypsin and chymotrypsin respectively. These investigations are now being extended to a more definitive characterization of the trypsin- and chymotrypsin-like enzymes of this tissue.

PLANS FOR FUTURE

In future work, the amino acid sequence of bovine pancreatic carboxypeptidase will be completed. Present investigations on the bovine and dogfish pancreatic enzymes will be continued, as will be the investigations on the proteolytic enzymes of the peloric ceca of the starfish.

CURRENT REPORTS AND PUBLICATIONS

a) James W. Prahl and Hans Neurath
Pancreatic Enzymes of the Spiny Pacific Dogfish I. Cationic Chymotrypsinogen and Chymotrypsin.
Biochemistry 5, 2131 (1966).

b) James W. Prahl and Hans Neurath
Pancreatic Enzymes of the Spiny Pacific Dogfish II. Procarboxypeptidase B and Carboxypeptidase B
Biochemistry 5 (1966), in press.

c) Kenneth A. Walsh, Lowell H. Ericsson and H. Neurath
Bovine Carboxypeptidase A Variants Resulting From Allelomorphism
Proc. Nat. Acad. Sci. 56, No. 4 (1966).

d) Hans Neurath and James H. Freisheim
Activation of Procarboxypeptidase A
Fed. Proc. 25, No. 2 (1966).

EXPERIMENTAL STUDIES ON THE BIOCHEMISTRY AND BIOPHYSICS OF ENZYME MOLECULES

Thomas P. Singer
University of California
San Francisco, California

ASSISTED BY J. Luthy, H. Mersmann, J. Salach, H. Tisdale, P. Tuzini

WORK UNIT NO. NR 108-337 **CONTRACT** Nonr-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, (c) to isolate, characterize and determine the mechanism of action of cytoplasmic fumarate reductases.

ABSTRACT

(a) By means of the specific inhibitors of DPNH oxidation by CoQ, such as rotenone, this reaction has been compared in mitochondria and intact particles with soluble preparations. Certain clear-cut differences have been found (both quantitative and qualitative) which suggests that the reaction as it occurs in soluble flavoprotein preparations is probably not at the same site as the physiological event in mitochondria. A specific inhibitor for the reaction has been isolated from mitochondria of heart muscle, which abolishes DPNH oxidation by CoQ_{10} at exceedingly low concentrations: it is of high molecular weight, protein in nature, although it appears to be heat stable, and lipid free. This inhibitor is effective only at much higher concentrations in abolishing the DPNH-CoQ reaction in phosphorylating particles. Further, the physiological event is strictly lipid dependent, whereas the reaction in soluble purified preparations is independent of lipids.

(b) The oxidation of long-chain CoQ homologues has been studied in heat-acid-ethanol extracts of ETP_H (DPNH-ubiquinone reductase) and of ETP (DPNH-cytochrome c reductase). No difference could be detected either in reaction rates or in inhibition by rotenone or amytal. These data and other systematic comparison previously published from this laboratory suggest that the two preparations do not differ in a discernible manner.

The highly purified, soluble DPNH dehydrogenase isolated in this laboratory has been quantitatively converted into a low-molecular weight flavoprotein, with the same high activity toward CoQ_{10} as shown by genuine DPNH-ubiquinone reductase. The parent enzyme has no such activity and is not inhibited by rotenone or amytal; the transformation product is inhibitor sensitive, however. This suggests that the DPNH-CoQ reaction observed in soluble preparations is the result of a conformation change incurred during the extraction procedure.

(c) Fumarate reductase from yeast cytoplasm has been obtained in highly purified form and shown to be a copper-FAD enzyme, the first genuine copper flavo-

protein hitherto described. It occurs in the form of several isoenzymes differing in charge and in some catalytic properties; the molecular weight is 62,000 to 64,000. There is also another species present with a molecular weight of one-half this value. All these forms appear to be under the same genetic control, but are independent in biosynthetic mechanism from mitochondrial succinate dehydrogenase in the same cell.

PLANS FOR FUTURE

(a) To isolate and characterize the new inhibitor of CoQ reductase from mitochondria, (b) to determine the site of rotenone binding in the respiratory chain, (c) to study the role of fumarate reductases in adaptation to low O_2 tension, and (d) to explore the possibility of using marine organisms in the study of succinate dehydrogenases and fumarate reductases as dominant factors in the adaptation of organisms to varying O_2 tension.

CURRENT REPORTS AND PUBLICATIONS

(a) Fumarate Reductase, Succinate and NADH Dehydrogenase of Yeast: Properties and Biosynthesis, T. P. Singer, Eugenio Rocca, E. B. Kearney in International Symposium on Flavins and Flavoproteins (Ed. by E. Slater), Elsevier Press, Amsterdam, BBA Library, Vol. 8, p. 391 (1966).

(b) On the Diversity of -SH Groups in DPNH Dehydrogenase and their Tentative Localization, Harry Mersmann, James Luthy, and Thomas P. Singer, Biochem. Biophys. Res. Communs., 25, 43 (1966).

(c) Transformation of DPNH Dehydrogenase into DPNH-CoQ Reductase, Thomas P. Singer, J. Salach and H. Tisdale, Abstracts, VIIth International Congress of Biochemistry, Tokyo, August, 1967, in press.

(d) Conversion of DPNH Dehydrogenase to DPNH-Ubiquinone Reductase, Thomas P. Singer, J. Salach, and P. Bader, Federation Proc., 26 (1967) in press.

(e) Fumarate Reductase - a Copper Flavoprotein, H. Tisdale, J. Hauber, and J. Prager, Federation Proc., 26 (1967) in press.

(f) Respiratory Chain-linked Dehydrogenases, Thomas P. Singer, in Biological Oxidations, (Thomas P. Singer, Ed.), J. Wiley and Sons, Inc., in press.

BIOCHEMISTRY OF MARINE ORGANISMS

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Corvallis, Oregon

ASSISTED BY

WORK UNIT NO. NR 108-402 **CONTRACT** Nonr-1286 (06)

OBJECTIVES

(a) To compare oxidative metabolism of marine organisms with that in well studied systems such as cardiac tissue, (b) to "cross reconstitute" the respiratory chain by components from marine organisms with those from mammalian heart, and (c) to search for new methodology for studying oxidative metabolism.

ABSTRACT

A new artificial electron acceptor has been found for the determination of flavin-linked dehydrogenases. This acceptor is Wurster's blue (WB), a semiquinone of tetramethyl-p-phenylenediamine (TMPD). To our knowledge, this is the first time of using a free radical as an acceptor for a practical purpose. WB is active for those enzymes with flavin as a prosthetic group, such as succinate dehydrogenase, DPNH dehydrogenases, glucose oxidase, lactate dehydrogenase (flavin-linked), D-amino acid oxidase, L-amino acid oxidase, lipoic dehydrogenase, TPNH cytochrome c reductase, aldehyde oxidase, xanthine oxidase (from several species), etc. It is inactive for lactate dehydrogenase (DPN-linked), alcohol dehydrogenase, galactose oxidase (Cu-linked), uricase (Cu-linked), glucose-6-phosphate dehydrogenase, etc.

Reconstitution of the succinate oxidase system by using the components obtained by sequential fragmentation of succinate oxidase of cardiac muscle has been successfully demonstrated. One of the reconstitution systems for succinate oxidase is made from soluble succinate dehydrogenase and a dehydrogenase-free particle. The latter particle contains all other respiratory components such as cytochromes and is prepared by alkali-treatment of the Keilin-Hartree heart muscle preparation. However, the succinate dehydrogenase prepared from bay mussel (*Mytilus edulis*) does not react with the dehydrogenase-free particle in contrast to the "interchangeability" of three mammalian species studied.

Recently the mechanism of the alkali (pH 9-10) action on cardiac succinate oxidase has been clarified. The succinate dehydrogenase protein is found to be dissociated from the particulate succinate oxidase (the heart muscle preparation) by alkali treatment. The dissociation occurs at the same rate as the inactivation of succinate oxidase. Data from numerous kinetic experiments (ref. j) indicate that the original site for binding of the dehydrogenase to the particle is available to link

active succinate dehydrogenase in the reconstitution of the succinate oxidase system derived from mammalian heart. The failure to use Mytilus succinate dehydrogenase for the reconstitution suggests that the structure or configuration of the invertebrate dehydrogenase may differ from that of the mammalian enzyme.

Others are detailed in publications.

CURRENT REPORTS AND PUBLICATIONS

(a) K. S. Nickel, D. F. Wilson, R. L. Howard and T. E. King (1964), "The effect of aging of the heart-muscle preparation on its riboflavin-5-phosphate content." Biochim. Biophys. Acta, 81, 597-599

(b) T. E. King, K. S. Nickel and D. R. Jensen (1964), "Iron, copper, cytochrome and lipid contents of heart muscle preparation and heart mitochondria." J. Biol. Chem., 239, 1989-1994

(c) D. F. Wilson and T. E. King (1964), "The determination of the acid-nonextractable flavin in mitochondrial preparations from heart muscle." J. Biol. Chem., 239, 2683-2690

(d) S. Takemori and T. E. King (1964), "Reconstitution of respiratory chain enzyme systems. XIII. Sequential fragmentation of succinate oxidase: Preparation and properties of succinate-cytochrome c reductase and the cytochrome b-c_1 particle." J. Biol. Chem., 239, 3546-3558

(e) T. E. King and S. Takemori (1964), "Reconstitution of respiratory chain enzyme systems. XIV. Reconstitution of succinate-cytochrome c reductase from soluble succinate dehydrogenase and the cytochrome b-c_1 particle." J. Biol. Chem., 239, 3559-3569

(f) D. F. Wilson and T. E. King (1964), "Reconstitution of respiratory chain enzyme systems. XV. Reconstitution of succinate oxidase using soluble succinate dehydrogenase and a 'cyanide particle'." Biochim. Biophys. Acta, 92, 173-175

(g) M. Kuboyama and T. E. King (1964), "Reconstitution of cytochrome oxidase from cytochrome a and hematin a." Biochim. Biophys. Acta, 92, 618-621

(h) S. Takemori and T. E. King (1965), "Effect of alkali and borohydride on cardiac cytochrome oxidase. Formation of Schiff base." J. Biol. Chem., 240, 504-513

(i) P. W. Camerino and T. E. King (1966), "Studies on cytochrome oxidase. II. A reaction of cyanide with cytochrome oxidase in soluble and particulate forms." J. Biol. Chem., 241, 970-979

(j) D. F. Wilson and T. E. King (1966), "Reconstitution of respiratory chain enzyme systems. XVI. The effect of alkali on the succinate oxidase system." Biochim. Biophys. Acta (in the press)

SULFUR IN ALGAE

Raymond F. Jones
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ASSISTED BY

WORK UNIT NO. NR 108-448 **CONTRACT** Nonr 4716(00)

OBJECTIVES

This is an investigation of the sulfur metabolism of red algae. Objectives this year included isotopic studies of the synthesis and metabolic significance of taurine in Porphyridium cruentum. Studies on the extra cellular polysaccharide and other cell wall materials continue with emphasis on the factors regulating synthesis and excretion of such compounds.

ABSTRACT

None received.

FREE AMINO ACIDS AND RELATED SUBSTANCES IN MARINE ORGANISMS

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ASSISTED BY Masaaki Horiguchi

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

During a survey of the "free" amino acids of marine invertebrates, the extracts of some 400 tissues of a taxonomically diverse collection of organisms were examined. Several unique compounds were detected. Two of these, from the sea anemone, Anthopleura elegantissima, were isolated and proved to be 2-aminoethylphosphonic acid (2-AEP) and its glycerol ester. Analogy suggested the possible occurrence of 2-amino-3-phosphonopropionic acid. This compound was detected in the zoanthid, Zoanthus sociatus, and its biosynthesis from orthophosphate was demonstrated in the ciliate, Tetrahymena pyriformis. A limited survey of twenty species of Coelenterates was conducted to determine the distribution of these two aminophosphonic acids. This survey demonstrated not only the wide distribution of these compounds but revealed the presence of five new aminophosphonic acids.

(a) Two of the new phosphonic acids which were detected have been isolated from six kilograms of the sea anemone, Anthopleura xanthogrammica. The structure of these compounds, N-methyl-2-aminoethylphosphonic acid and N,N,N-trimethyl-2-aminoethylphosphonic acid betaine (trimethyl 2-phosphonoethylammonium hydroxide inner salt), has been established by comparing the IR and NMR spectra with those of the synthetic compounds. A small amount of the N,N-dimethyl-2-aminoethylphosphonic acid was detected during the isolation. We have now established the biological occurrence of the phosphonic acid analogs of the three major nitrogenous constituents of phospholipids.

(b) A strongly basic phosphonic acid was detected during the above isolation. We have now isolated a few milligrams of this compound in crystalline form.

(c) We have isolated 2-AEP from a marine amphipod, Amonyx nugax, thus establishing the presence of this phosphonic acid in another phylum.

(d) A survey of marine micro-organisms and phytoplankton for the ability to synthesize 2-AEP demonstrated that three species of dinoflagellates and one coccolithophore can synthesize 2-AEP from ortho-

phosphate, while a marine diatome, two yeasts and a bacteria gave no evidence of producing these compounds. Since the dinoflagellates form a major part of the base of the food chain in the oceans, this observation indicates the large part that C-P compounds must play in the metabolism of marine animals.

(e) Utilizing a copper chelate resin, we were unable to detect 2-AEP in four 25-gallon samples of sea water.

(f) No 2-AEP was detected in two marine sediment samples.

(g) Since 2-AEP is the phosphonic analog of taurine, we investigated the possibility that it might have a similar role in the emulsifier in crab gastric juice. We could not detect 2-AEP in the isolate emulsifier nor could we detect synthesis of 2-AEP but we found five other phosphonic acids.

(h) We found that the pulmonate gastropod, Helix pomata, synthesizes 2-AEP and that 5.35% of the total phosphorus occurs in this compound.

(i) The unique synthesis of cysteic acid by chick embryos suggested a possible parallel route to phosphonoalanine. The injection of $^{32}PO_4$ yielded no C-P compounds.

(j) Incubation of Tetrahymena homogenates with ^{32}P-ATP did not result in any incorporation into 2-AEP. Examination of the extracts for reduced oxyphosphorus acids did not yield evidence for reduction.

(k) The possibility that the biosynthetic route to the C-P bond may involve reduced phosphorus and an α-β insertion into serine was investigated. Incubation of ^{14}C-serine in four buffers containing reduced phosphorus compounds yielded no incorporation into C-P compounds.

PLANS FOR FUTURE

(a) We are proceeding with the exploration of the mechanism of biosynthesis of the C-P bond along two routes, the search for the carbon precursor and the investigation of the reduction of orthophosphate.

(b) We are examining the transamination of aminophosphonic acids in a number of tissues.

(c) We have succeeded in converting 2-AEP to its trimethylsylil derivative and soon should have a rapid GLC assay method to accelerate our biosynthetic investigations.

(d) In order to investigate the physiological consequences of the incorporation of 2-AEP into mammalian membrane lipids, we have synthesized 130 g of 2-AEP.

CURRENT REPORTS AND PUBLICATIONS

J. S. Kittredge, A. F. Isbell and R. R. Hughes (1966), "Isolation and Characterization of the N-methyl Derivatives of 2-Aminoethylphosphonic Acid from the Sea Anemone, Anthopleura xanthogrammica." Biochemistry (in press).

BIOCHEMISTRY OF THE LUMINESCENCE OF MARINE ORGANISMS

J. Woodland Hastings
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ASSISTED BY Q.H.Gibson, H.Clemencon, J.Massa, M.Vergin & J.Friedland

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

OBJECTIVES

The study of basic biochemical mechanisms involved in the creation of excited states in bioluminescent reactions and its relationship to cellular function.

ABSTRACT

Two distinctly different systems from marine organisms are under study: (1) The bacterial reaction, in which reduced FMN is oxidized by a soluble system with the concomitant emission of blue-green (490 mμ) light; and (2) the marine dinoflagellate (Gonyaulax polyedra) in which emission occurs as a short flash from a sedimentable particle when the pH is rapidly lowered from pH 8 to pH 5.7. This particle has been termed the scintillon, and it is associated with a birhombohedral guanine crystal.

In the bacterial reaction we have undertaken a study of the enzyme, which has a molecular weight of about 60,000. It is composed of subunits, probably four, on the basis of amino acid composition. Subunits have not yet been isolated, but they may be detected by centrifugation. Their existence may also be inferred from the kinetics of reactivation of enzyme treated with 8M guanidine HCl, which is dependent on protein concentration. The enzyme contains per subunit, two free-SH groups, one tryptophane, but no disulfides. The loss of enzyme activity by reaction withPMB or other-SH reagents is reversible. All activity is lost in high concentrations of urea or guanidine HCl, and virtually 100% may be recovered. No information concerning the emitting molecule was obtained. ORD studies indicate a helix content of about 32%.

It has been shown that bacterial bioluminescence may actually be initiated by light and additional studies have been concerned with this, particularly with regard to the role of oxygen. Oxygen has been found to be essential, so

that discrete chemical steps involving the entry of oxygen must be involved in the light-induced pathway.

Studies with scintillons have been directed towards elucidating its structure and composition. Its association with a guanine crystal has been something of a stumbling block, since the scintillon is not a guanine crystal as such. The hypothesis that the scintillon is a guanine crystal with some additional material associated with it has been supported by measurements of its sedimentation constant (S_0 = 20,000) and by direct visualization of the flash in the image intensifier. In the latter a photon hield of about 500 per particle has been obtained, a value which is close to that obtained for the average yield in the basis of the number of guanine crystals. Work on the chemical nature of the added component is in progress.

PLANS FOR FUTURE

(a) To complete the study of the structure of bacterial luciferase and elucidate the enzyme intermediates involved in the reaction. (b) To determine the chemical composition of the components of the scintillon and define the nature of the light emitting system.

CURRENT REPORTS AND PUBLICATIONS

(a) Hastings, J. W., Gibson, Q. H., and Greenwood, C., Evidence for high energy storage intermediates in bioluminescence, Photochemistry and Photobiology, 4 1227-1241 (1965).

(b) Gibson, Q. H., Hastings, J. W., Weber, G., Duane, W., and Massa, J., The interaction of flavin mononucleotide with an enzyme system from Photobacterium fischeri, in "Flavins and flavoproteins" (E. C. Slater, Editor), Elsevier Publishing Co, Amsterdam (1966).

(c) Hastings, J. W., Vergin, M., and De Sa, R., Scintillons: The biochemistry of dinoflagellate bioluminescence, in "Bioluminescence in Progress" (F. H. Johnson and Y. Haneda, Editors), Princeton University Press (1966).

(d) Hastings, J. W., Gibson, Q. H., Friedland J., and Spudich, J., Molecular mechanisms in bacterial bioluminescence: On energy storage intermediates and the role of aldehyde in the reaction, in "Bioluminescence in Progress" (F. H. Johnson and Y. Haneda, Editors), Princeton University Press (1966).

(e) Gibson, Q. H., and Hastings, J. W., Light-initiated bioluminescence in Progress" (F. H. Johnson and Y. Haneda, Editors), Princeton University Press (1966).

(f) Hastings, J. W., and Reynolds, G. T., The preparation and standardization by different methods of liquid light sources, in "Bioluminescence in Progress" (F. H.

Johnson and Y. Haneda, Editors), Princeton University Press (1966).

(g) Hastings, J. W., The chemistry of bioluminescence, in "Recent Advances in Bioenergetics", Vol. I., (D. R. Sanadi, Editor), Academic Press, New York (1966).

(h) Reynolds, George T., Hastings, J. W., Sato, H. and Sweeney, A. R. The identity and photon yield of scintillons of Gonyaulax polyedra. Biol. Bull 131 403 (1966).

(i) Hastings, J. W. and Gibson, Q. H. The role of oxygen in light induced bioluminescence. J. Biol. Chem. (in press, Jan., 1967).

CARBOHYDRATE BIOCHEMISTRY OF ALGAE

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TASK 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, (d) to study the nature and mode of action of some of these enzymes.

ABSTRACT

A beginning has been made with the study of the reserve material of a marine Dinoflagellate, Thecadinium spec., an organism that could be grown in seawater fortified with Provasoli's enrichment medium. Granules of the reserve product, in its native state, could be isolated with a high degree of purity. Preliminary tests show that they are composed of a glucan which is predominantly α-1:4-linked, like starch; they also display a blue iodine-reaction. In addition, a number of bluegreen and red algae were screened for the presence of trehalose. This sugar, common in the animal kingdom (insect muscle!), shows a rather "spotty"distribution in plants; it is frequently found in yeasts, molds (Neurospora) and mushrooms, but to the best of our knowledge the only group of terrestrial green plants accumulating trehalose in appreciable quantities are the Selaginellas which include the famous resurrection plants of South American deserts. The sugar has been reported in freshwater red algae and in some bluegreens (Rivularia, Calothrix) and seems to be one of the main products of photosynthesis in the coralline red algae, as demonstrated by a group of workers in Halifax, Nova Scotia. The synthesis of trehalose has been elucidated only in yeast, where uridine diphosphate glucose (UDPG) is involved.

(b) A beginning has been made with the study of trehalose - and starch synthesis in red algae. In collaboration with Dr. T. Akazawa of Nagoya University, tests were made for the presence of ADPG - starch synthetase in (or on) starch granules isolated from Constantinea. In higher plants, it has been found that the enzyme is very tightly associated with the amylose-fraction of starch, hence it tends to remain with the starch-granules when methods of fractionnated centrifugation are applied. Plants with "glutinous" starch (which lacks amylose!) form an exception, in that the ADPG-starch synthetase here tends to remain in the soluble fraction. It may be significant that no activity was found in the Constantinea starch granules, which according to our earlier studies are composed only of amylopectin (like glutinous starch). However, more experiments are needed. In connection with this, methods for growing various red algae

on a fairly large scale in converted, illuminated refrigerators, have been developed, following the lead of Dr. Richard Norris and Dr. John West.
(c) Previous studies on the spectrum of carbohydrate-splitting enzymes present in the digestive juices of marine invertebrates were expanded, with special emphasis on a comparison of Cryptochiton stelleri with Katharina, a closely related form which has different feeding habits and may obtain most of its food (diatoms?) by scraping off rocks. So far, no striking differences have been found.
(d) The bulk of our efforts has been directed towards purification and characterization of the alpha-amylase in the sugar-gland of Cryptochiton stelleri. The specific activity of the purified amylase represented a 10-fold increase over the original juice, while no appreciable activity towards laminarin and maltose remained. Evidence was obtained for the presence and essentiality of Ca in the enzyme molecule. Sulfhydryl group inhibitors had no noticeable effect on its activity. The essentiality of zinc could not yet be established beyond doubt. Amylose was broken down to maltose and glucose, while in the early stages of breakdown of amylopectin maltotetraose seemed to predominate. Potato β-limit dextrin was hydrolized to an extent of 56%. In many of its features, Cryptochiton amylase thus resembles mammalian salivary amylase. Comparison of sugar gland juice and a phosphate buffer extract of Cryptochiton's hepatopancreas for activity towards amylopectin, laminarin and maltose (respectively) shows that the activity ratios are roughly the same for the two fluids. This provides some evidence for the opinion that the alpha-amylase, in this case, is produced by the animal itself and not by symbiotic microorganisms living in the sugar gland fluid.

PLANS FOR FUTURE

(a) To study the biosynthesis of floridean starch in selected members of the Rhodophyceae (red algae), more specifically to investigate the possible participation in this process of ADPG-starch synthetase, (b) to establish the chemical nature of the reserve product of certain Dinoflagellates such as Thecadinium and Amphidinium, (c) to investigate occurrence, role, and biosynthesis of the disaccharide trehalose in certain red and bluegreen algae, (d) to continue the investigation on the nature and the mode of action of the alpha-amylase of Cryptochiton stelleri (Middendorff).

CURRENT REPORTS AND PUBLICATIONS

M. Snel (1966), "A study of the amylase found in the sugar-gland of Cryptochiton stelleri (Middendorff) (Polyplacophora, Mollusca)." Master of Science Thesis, University of Washington, Seattle.

COMPARATIVE CARBOHYDRATE METABOLISM
OF MARINE MOLLUSCS.

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ASSISTED BY R. Bennett, Jr.

WORK UNIT NO. NR 108-642 CONTRACT Nonr 4222(08)

OBJECTIVES

(a) To examine the carbohydrate metabolism of several species of molluscs to determine the extent of common pathways such as glycolysis and the pentose phosphate pathway, (b) to learn whether or not unique metabolic sequences play an important role in the life of these animals.

ABSTRACT

Using differentially labeled sugars and comparing the quantity of $C^{14}O_2$ expired, we have tentatively determined that the molluscs, Mytilus californianus and Haliotus rufescens utilizes both the Embden-Meyerhof-Parnas Pathway and the Pentose phosphate pathway. The presence of these pathways have been confirmed in crude cell free preparations by demonstrating the presence of key enzymes of both pathways in both organisms. The pattern of enzyme activities indicate that the operation of glycolysis may differ considerably in molluscs as compared to higher animals. Enzymes of triose phosphate metabolism are limiting whereas α-glycerophosphate dehydrogenases were very high. Phosphofructokinase appears to be controlled allosterically by ATP and AMP and cyclic- 3;5'AMP.

Plans for the Future

(a) Determine the pathway for α-glycerophosphate metabolism in molluscs. (b) Determine whether the carbohydrate metabolic pattern found in the above two molluscs is a general property of all molluscs. (c) Examine the ways by which such organisms control their metabolic rate. (d) Assay for enzymes of galactose metabolism-especially those leading towards galactogen formation.

Current Reports and Publications

a) R. Bennett, Jr. and H. I. Nakada, "Comparative Carbohydrate metabolism of Molluscs". Pacific Slope Biochemical Conference 1966.

b) Two papers presently in preparation.

LYSOSOMES AND INTRACELLULAR DIGESTION IN SEA STARS

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ASSISTED BY J. Baker

WORK UNIT NO. NR 108-688 CONTRACT N00014-66-C0317

OBJECTIVES

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

ABSTRACT

Lysosomes are recently found intracellular constituents, bound by a membrane, and housing a variety of acid hydrolases, prominently, acid phosphatase. Heterophagic digestion as described by De Duve is based on fusion of primary lysosomes with phagosomes. Thus far, only a few mammalian cell types and protozoans bear out this hypothesis. The present investigation is directed at testing the sea star intestinal tract, which is characterized by intracellular digestion, for the heterophagic digestion hypothesis.

Initial results indicate relatively large vacuoles in epithelial cells of the intestinal and pyloric caeca of the sea star. These vacuoles are absent from the cardiac stomach. Positive acid phosphatase activity as determined histochemically by the azo-coupling technique is localized within the same vacuoles, thus suggesting the possibility that these are heterophagic vacuoles. It is not yet clear whether other enzymes are also localized within these vacuoles. Azoalbumin and other pigmented substances accumulate in the vacuoles. Primary lysosomes, however, have not been demonstrated yet.

PLANS FOR FUTURE

a) to continue characterization of the vacuoles histochemically, b) to isolate the vacuoles by differential centrifugation and characterize their enzymatic components biochemically, and c) to trace protein digestion within vacuoles.

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

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

OBJECTIVES

(a) To quantitate and correlate electrolyte and non-electrolyte content of biological fluids in man; (b) to evaluate applications of this analytical principle to problems of marine biology and biochemistry.

ABSTRACT

Determinations of total electrolyte content of various sea water samples by the microwave procedure were continued. In general, the observed values of open sea waters tested were in good agreement with the published data of Wolf (Wolf, A. V., "Aqueous Solutions and Body Fluids," Hoeber Medical Division, Harper & Row, New York, N.Y.,1966). Shore waters showed considerable variations from the norm. It has become apparent from these observations that a flow type cell and miniaturization of the microwave apparatus is needed in order to realize the full practical potential of this method. A practical cell for continuous measurements has been designed, and development of a compact microwave unit is in progress. It is expected that the new unit will permit a two- to three-times reduction in sample size.

The method has also been applied to studies in man on the effects of various hormones on total urinary electrolyte output and its relationship to total nitrogen and calcium excretion. In this manner, some heretofore unsuspected electrolyte imbalances induced by adrenal substances have been uncovered--namely, the fact that steroid induced calcium losses are compensated by the retention of sodium and potassium.

PLANS FOR FUTURE

(a) Complete development, standardization, and calibration of a compact and mobile microwave apparatus; (b) application of the new unit to problems of marine biology, e.g., correlation of total electrolyte content to density of micro-organisms; (c) extension of studies on the application of microwave absorption techniques to problems of human metabolism and electrolyte changes in blood preserved by freezing.

CURRENT REPORTS AND PUBLICATIONS

(a) A. A. Albanese (1966), "Clinical techniques for evaluating parenteral feeding." Presentation at a Postgraduate Course on Animal and Clinical Pharmacologic Techniques in Drug Evaluation. Hahnemann Medical College and Hospital, Philadelphia, Part I, January 24-28.

(b) A. A. Albanese (1966), "Clinical techniques for evaluating anabolic agents." Presentation at a Postgraduate Course on Animal and Clinical Pharmacologic Techniques in Drug Evaluation. Hahnemann Medical College and Hospital, Philadelphia, Part II, May 16-20.

(c) A. A. Albanese (1966), "Effect of exercise on nutritional requirements." Presentation at Gordon Research Food and Nutrition Conference, New London, New Hampshire, July 18-22.

(d) A. A. Albanese and L. A. Orto (In press), "Urinary excretion of amino acids," in "Newer Methods of Nutritional Biochemistry," Vol. 3 (A. A. Albanese, editor), Academic Press, New York.

(e) A. A. Albanese, E. J. Lorenze, and L. A. Orto (1966), "Carbohydrate tolerance in mature adults." Presentation at the 19th Annual Meeting of the Gerontological Society, New York, N. Y., Nov. 3-6.

(f) A. A. Albanese and L. A. Orto (In press), "Dietary proteins and nitrogen balance," in "Studies on Nutrition Problems" (E. Turchetto, editor), Arti Grafiche Emiliane, Bologna, Italy.

HYDROSTATIC PRESSURE EFFECTS ON MACROMOLECULES

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ASSISTED BY

WORK UNIT NO. NR 108-708 **CONTRACT** N00014-66-C0253-A02

OBJECTIVES

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

ABSTRACT

Polyvalyl-ribonuclease (PVRNase) made by reacting bovine pancreatic ribonuclease A with N-carboxy-anhydrides of valine has been chosen as the model system for studying the effects of hydrostatic pressure and temperature on protein-protein interactions. This choice was suggested by earlier observations in this laboratory that certain polyvalyl derivatives undergo thermal aggregation at moderate temperatures (about 30° - 40°C) compared to the unmodified enzyme (little aggregation seen even at 99°C). Studies of the PVRNase system in a high pressure optical cell had revealed that the aggregation has associated with it an unusually high volume of activation ($\Delta V^* = 250$ ml/mole). This was ascribed to the hydrophobic nature of the intermolecular side-chain interactions of the added valine peptides in PVRNase. In addition an increase in the ionic strength of the solution was found to have a significant lowering effect on the ΔV^*. This indicated that the alpha-amino termini of the added valine chains could not be neglected in considering the forces involved in these aggregation reactions.

In efforts to purify PVRNase for subsequent studies, gel filtration (Bio-Gel P-30, a polyacrylamide gel) was tried. Two fractions were obtained which corresponded to monomeric PVRNase and aggregates of PVRNase. This separation on the basis of molecular size however, did not rule out contamination of monomeric PVRNase by high molecular weight valine peptides. Hence, we resorted to column chromatography on phospho-cellulose following the general approach reported by Anfinsen et al., JBC 237, 1825 (1962).

A preparation of PVRNase (designated P5) was selected for studies at normal atmospheric pressure on the nature of the thermal aggregation. Amino acid analysis and dinitrophenylation indicated that P5 contained 9 added valine chains with an average length of 2.5 valines per chain. With yeast RNA as the substrate, enzymatic assay of P5 showed 63% of the activity of unmodified RNAse.

Thermal aggregation studies were performed in a Cary 14 Spectrophotometer with thermostated cuvette holders held at 39°C. The actual temperature of the solution inside the cuvette was monitored with a Y.S.I. thermistor thermometer which was coupled to a recorder to plot change of temperature with time. Aggregation was measured by a change in absorbance at 320 mu.

The reaction order, n, for the aggregation was found to be about 1.7 at pH 7.40 and 7.93, both at 39°C.

The rate of aggregation increased monotonically with ionic strength from zero to 0.6 M NaCl.

In a study of pH effects, no aggregation was observed from pH 3.5 to pH 6.2 in 0.05 M buffers. In phosphate buffers a wide peak in the aggregation rate was observed from pH 6.2 to 8.0 with a maximum rate noted at pH 7.1. It was further observed that in TRIS buffers the pH-rate profile was shifted to pH 7.2-8.3 with a new maximum at pH 7.7 or 7.8

A marked effect of specific polyvalent anion binding on the thermal aggregation of PVRNase has been noted. The interaction of PVRNase with these anions apparently affects the conformation of the added polyvalyl chains. Difference spectral techniques are being used to study the conformation of PVRNase relative to native RNase.

PLANS FOR FUTURE

(a) To elucidate the nature and magnitude of the anion-affected conformation of PVRNase (b) a systemic study of the response of PVRNase to high hydrostatic pressure (c) a systematic analysis of hydrophobic interactions and their behavior under hydrostatic pressure.

CURRENT REPORTS AND PUBLICATIONS

(a) A. H. Nishikawa, R. R. Becker and R. Y. Morita (1967), "Studies on the Thermal Aggregation of Polyvalyl-RNAse", submitted to Fed. Proc.

A STUDY OF ORGANIC MATERIALS FOUND IN THE UPPER 60 MICRONS OF THE SEA SURFACE AND THEIR RELATIONSHIPS TO SURFACE FILMS

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ASSISTED BY

WORK UNIT NO. NR 108-739 **CONTRACT** N 00014-67-A-0109-0006

OBJECTIVES

(a) To determine the kinds of particulate materials found in the 60 micron layer and to investigate their relationships with the adsorption of natural surfactants in fresh surface samples, (b) to observe the seasonal variations of populations of microorganisms and to relate these to ripple damping, surface tension, relative chlorophyll and protein concentrations, turbidity, wind velocity, salinity and light intensity, (c) to compare the 60 micron layers from Klamath Lake, Oregon, San Vicente Lake, Calif., and Concepcion Bay, Baja California, with that from La Jolla Bay, Calif.

ABSTRACT

Samples obtained by means of a newly devised surface collector indicate larger amounts of organic materials in the thin 60 micron surface layer of the sea than in the water at a depth of 10 cm. These organic materials include living nannoplankton organisms, structural components of disintegrated organisms, surface-active substances, chlorophyll and carotenoid pigments. Large populations of little known or undescribed microorganisms that fluctuate widely, apparently under seasonal influence, were found in this layer. Some of these organisms appear to be characteristic of the 60 micron layer. Similar organisms were found both in La Jolla Bay and Klamath Lake, Oregon. The sizes and kinds of population and presence of red tides and bioluminescence are correlated with turbidity, chlorophyll and tryptone content and time of day.

PLANS FOR FUTURE

(a) To identify and characterize the quantitately important microorganisms and the associated surface active materials of the 60 micron surface layer.

(b) To assess the role of microphysical oceanographic factors, light intensity and other seasonal conditions in the sea surface environment.

CURRENT REPORTS AND PUBLICATIONS

(a) G. W. Harvey (1965), "A Study of Plankton Organisms and Organic Materials in the Upper 60 Microns of the Sea Surface and their Relationships to Surface Films." Applied Oceanography Group Technical Report No. U-621, 25 pp.

(b) "Microlayer Collection from the Sea Surface: A New Method and Initial Results," Limnol. and Oceanog. , 11 (4): 608-613, 1966.

(c) G. W. Harvey (1966), " A Law Velocity Plankton Siphon." Applied Oceanography Group, Scripps Institution of Oceanography, 2 pp. Limnology and Oceanography, 11 (4).

(d) G. W. Harvey and M. B. Harvey, "A Rapid, Simplified Microfiltration Method for Nannoplankton," in preparation.

(e) G. W. Harvey, "A Ripple Tank for the Simultaneous Measurement of Surface Tension and Damping," in preparation.

(f) G. W. Harvey, "Neuston and Organic Films at the Sea Surface," in preparation.

(g) G. W. Harvey, " A Rapid, Simplified Method for the Fluorometric Estimation of Chlorophyll a," in preparation.

AN INVESTIGATION OF THE NUCLEIC ACIDS IN GRIFFITHSIA GLOBULIFERA

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WORK UNIT NO. NR 108-759 **CONTRACT** Nonr 3070(04)

OBJECTIVES

(a) To develop a method of isolating high molecular weight DNA from red algae and (b) to determine the constancy of the base ratios of DNA throughout the life cycle of an organism.

ABSTRACT

A method has been developed for the isolation of nuclei from red algae. A DNase has been described from Griffithsia globulifera. A modification of Marmurs method has been used to isolate the DNA from several species of algae. The base ratio of the DNA has been characterized by density gradient centrifugation and by the characteristics of its hyperchromic shift when heated.

PLANS FOR FUTURE

To try to grow Griffithsia in sterile culture so that the DNA can be labeled with isotopic markers. This will enable us to attempt a hybridization experiment in order to determine the similarities in the DNA throughout the life cycle.

CURRENT REPORTS AND PUBLICATIONS

(a) P.C. Maloney, A.E. Brooks, V.L. Biordano, R.L. Kornblith and M. Nasatir, (1965) "Determination of G-C content of DNA from Griffithsia globulifera", Biological Bulletin, Vol. 129, No. 2, 414-415

(b) A.E. Brooks, P.C. Maloney, R.L. Kornblith, V.L. Giordano and M. Nasatir, (1965) "Isolation of nuclei from red algae" Biological Bulletin, Vol. 129, No. 2, 400-401

(c) A.E. Brooks and M. Nasatir, (in press) "The DNA of a seven chromosome strain of Astrephomene gubernaculifera

(d) A.E. Brooks and M. Nasatir, (in press) "DNA of the marine red alga, Griffithsia globulifera

METABOLIC BIOCHEMISTRY

It is not enough to say, "Other agencies are supporting work in this field." The information gleaned from study of cancer may be applicable to the control of the proliferation of organisms responsible for fouling, and it can happen that busy investigators engaged in cancer research will not have the time nor interest to explore thoroughly aspects of new avenues of research which have specifically naval connotations. With this in mind, we must retain a certain amount of interest in studies which may appear superficially to be in the fields of public health or general science. It is also desirable to retain as much as possible of the on-going research, begun when the program was less specifically Naval in its objectives and now reaching the harvest time. The group of studies reported here are largely continuations, but we hold open the possibility of considering new research which could not possibly be called "Marine Biochemistry," but which promises to generate new information especially useful to the Navy.

THE ROLE OF ENERGY PRODUCTION AND ENERGY REQUIREMENTS IN THE PROCESS OF WOUND HEALING IN GRANULATION

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ASSISTED BY W. W. Nowinski and J. P. G. Williams

WORK UNIT NO. NR 108-198 **CONTRACT** N. ONR. 1598 (05)

OBJECTIVES

To investigate the role of glycogen and mechanism of its formation in epithelium, and its relation to the energy metabolism in wound epithelium.

ABSTRACT

Full thickness wounds are produced in rats and mice either by a simple incision or by the total removal of a small piece of skin, in mice the wounds are produced on the dorsal surface immediately posterior to the neck, the wounds in rats - being simple incisions, were made more posteriorly. After different time intervals the edge of the wound is removed and either quick frozen in freon cooled with liquid nitrogen or fixed in cold acetic acid - alcohol. Sections are prepared either from paraffin embedded material or in a cryostat. The sections are stained with the periodic-acid-schiff reaction using diastase as a control. Granular P.A.S. positive material which could be removed with diastase was identified as glycogen.

Glycogen could be detected from 12 hrs. postoperatively until the wound was fully healed and it was found in nearly all of the cells of the migrating portion of the epidermis. Contrary to others, glycogen has been observed in cells of the basal layer, however glycogen has never been found in cells undergoing mitosis:-- it is broken down prior to metaphase. Cell reproduction is not necessary for the migrations of the epithelium nor for the production of glycogen as both of these phenomenon are observed in animals which had been treated with 5-fluorouracil— an inhibitor of thymidylate synthetase. Epithelial glycogen is extremely stable and is found in large quantities in the wound epithelium after adrenalectomy and after the injection of Epinephrine. When the liver store of glycogen was depleted by starvation the glycogen accumulated in the regenerating epidermis was similar to that seen in the normally fed control animals.

Glycogen rapidly disappears from the epidermis after the wound has become completely covered with a continuous keratinised epithelial

sheet. Glycogen is not entirely restricted to the migrating epidermis covering the wound but after an injury can be demonstrated in a gradient extending approximately 1 mm into the 'old' epidermis. The edge of the old epidermis, -- identified by the proximity of hair follicles, the presence of keratin and granulation tissue, often shows cellular hypertrophy.

Since glycogen is found in cells of the hair follicles and sweat glands it might be suggested that these cells or their products contribute to the glycogen accumulation. It was of interest therefore to examine an essentially hairless mammal in this regard. The opportunity arose to examine several dolphins (Tursiops truncarta) shortly after death. These animals had small abrasions; these areas and pieces of normal skin were examined histochemically for the presence of glycogen. No glycogen was found in the normal epidermis but in the epithelium of the wound area glycogen was demonstrable. Since glycogen occurs in the wound epithelium of a mammal which has only one cell type in the epidermis, other types of cell cannot be implicated in the origin of this material.

Glycogen cannot be demonstrated in the normal uninjured epidermis by histochemical staining method, however after injury large amounts of this product can be observed in the migrating epithelium. It is clearly an anomaly that an energy storage material should be found in more active cells but not in their less active analogues. A few theories have been produced to account for this, they all depend on some unidentified breakdown in the normal carbohydrate pathway. We have constructed a new hypothesis which suggests that normal glycolysis energy production from glucose is not interfered with and that the accumulation of glycogen is of no direct significance to the cell or the organism but is bound to a common pathway with a mechanism that is essential for tissue repair. This hypothesis delineates the precise biochemical steps involved. The hypothesis accounts for all of the existing data but the crucial test can only be made when adequate techniques are developed. The necessary techniques, both ultramicrochemical and isotopic, are in process of being worked out. If the hypothesis is proved correct it is expected to have significance in several different areas, e.g. cell biology, biochemistry, pharmacology and physiology.

CURRENT REPORTS AND PUBLICATIONS

(a). Williams, J.P.G., Glycogen in the epidermis of the dolphin Tursiops truncarta, sent to Comparative Physiology and Biochemistry (ready for publication)

NUCLEIC ACIDS IN REGENERATING WOUND TISSUE

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ASSISTED BY T. C. Thachet

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

OBJECTIVES

(a) To study the metabolism of RNA in regenerating wound tissue; (b) to isolate the m-RNA in the nucleus of wound tissue cells which is involved in collagen monomer formation; (c) to investigate the conditions which affect the rate of protein synthesis in regenerating wound tissue.

ABSTRACT

Nuclear RNA found in wound tissue at various stages of formation has been separated into a large number of fractions (1). From wound tissue which has developed for 8 days (a time when fibroblasts are the most prevalent type of cell in this tissue (2) and, presumably, collagen synthesis is at or near a peak), we have isolated 12 RNA fractions from the cellular nuclei, nine of which are distinct on the basis of purine and pyrimidine composition. Further characterization of these RNA fractions has been carried out by studying their rate of formation.

The rate of synthesis and turnover of nuclear RNA has been followed in the wound tissue of rats on the 8th day after wounding by administration of tritiated-uridine and removal of the tissue at short intervals thereafter. The different s-RNA fractions are formed and leave the nucleus very rapidly. Since these fractions represent only a small part of the total nuclear RNA and are usually contaminated with some m-RNA, it is difficult to assess the fate of these RNA molecules with confidence.

Five distinct fractions of m-RNA can be identified on the basis of base composition. Undoubtedly, each of these fractions is a mixture. Two types of m-RNA fractions are identifiable according to the size of the molecule. The two smaller-sized m-RNA fractions are formed relatively slowly and turn over slowly. The m-RNA molecules in the other fractions are larger and are synthesized between 3-8 times as rapidly. The turnover of these heavier m-RNA molecules is also more rapid. One of these m-RNA fractions in particular is formed and turned over very considerably more rapidly than any other RNA fraction. It is postulated from

this and other evidence that the m-RNA involved in the synthesis of the monmeric precursor of collagen is probably to be found in this fraction.

Several fractions of essentially identical base composition have been isolated. Because these are thought to be the largest RNA molecules in the nuclei, it has been presumed that they consist mainly of ribosomal and polysomal RNA, contaminated with some amount of m-RNA. From the rate of synthesis of the RNA in these fractions, it has been deduced that one fraction is the principal one synthesized and that the others are derived from this one by some auxilliary process.

PLANS FOR FUTURE

(a) To study the effect of the various nuclear RNA fractions from wound tissue on the rate of incorporation of labeled amino acids with particular reference to the synthesis of collagenous and non-collagenous proteins

(b) To further purify and characterize the nuclear RNA fractions

CURRENT REPORTS AND PUBLICATIONS

(1) Williamson, M.B., and Thachet, T. C., Arch. de Biochim., 10, in press (1967)

(2) Williamson, M.B., and Beuret, L. J., unpublished data

PROPERTIES OF HYDROGENASE

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ASSISTED BY

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

OBJECTIVES

1. To investigate effect of wavelength on photochemical activation of CO inhibited hydrogenase.
2. To investigate the structure of the activated enzyme.
3. To investigate the relationships between structure and function in the deoxygenated enzyme inactivated by CO and not susceptible to subsequent photoactivation.
4. To investigate the fractionation of deuterium in metabolic processes.

ABSTRACT

None received.

THE DEVELOPMENT OF FUNCTIONAL CELL STRAINS

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

OBJECTIVES

(1) To study animal cell replication and intermediary metabolism,

(2) 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

Established animal cell strains, which can be sub-cultured indefinitely, propagate in suspension to population density maxima of about 3 to 4×10^6 cells per ml at doubling times of 24 to 36 hours under appropriate conditions.

Apparatus, embodying semi-automatic control of PH and nutrient supply, and capable of upscaling for mass production of cells, has been constructed. Two general types of suspension systems are in operation, one, the cytogenerator, the logarithimically increasing population is retained until harvested. In practice, an aliquot fraction of the volume, calculated from the doubling time, is harvested daily for use. In the other, a chemostat without conventional nutrient limitation, and under logarithmic growth, the progeny is harvested by overflow at the doubling rate. In the chemostat a true thermodynamic steady state is established under appropriate conditions. This steady-state is exquisitely sensitive to environmental alterations. Thus, a change in proliferative function in response to PH change or to the addition of a chemical compound is reflected in oscillations of measured parameters such as cell concentration or DNA or RNA synthetic capacity. Thus, the chemostat facilitates studies on metabolic and replicative mechanisms. Glycolysis, for example, has been shown to be a detoxication reaction countering excessive glucose flux.

The capacity to produce large quantities of viable cells opens up the possibility of establishing cell banks for therapeutic application, on the one hand, and the employment of mass cultures of living cells as functional reactors for the production of useful chemical products, species-specific substances especially, on the other. The production and study of mouse cells for these purposes, the essential preliminary to the production of human cells, is in progress. Thus, masses of L929 mouse fibroblasts, grown in suspension and harvested daily, are under experimental clinical trial as topical burn dressings. It is anticipated that antigenic sloughing of topically applied cells will not take place

until wound healing has commenced.

The efficacy of cultured mouse bone marrow cells which replicate in suspension after initial changes in cell type distribution to yield a preponderance of blast cells, is under study at Oak Ridge under Dr. Charles Congdon. Antigenic complications are not improbable, but already it appears that lymphocytes can be eliminated from the cultures by suitable manipulation of PH and oxygen tension. It has been observed that marrow explants from three-day old mice are not antigenic.

Preliminary studies on human cells for the several above purposes is in progress, but full scale operations must await the outcome of the experimental applications of the mouse systems.

PLANS FOR FUTURE

1. To continue studies in termediary metabolism of cells, as, for example, the mechanism of hormone action at cellular bulls.
2. To improve and upscale the culture systems.
3. To establish human epithelial cell banks.
4. To establish human bone marrow banks.
5. To produce human interferon.

PROPERTIES OF NUCLEIC ACIDS IN RELATION TO BRAIN FUNCTION

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WORK UNIT NO. NR 108-633 CONTRACT Nonr-908(20)

OBJECTIVES

The main objective is to establish whether ribonucleic acid (RNA) in brain fulfills a discrete function in memory and learning. To this end we have studied a) the isolation and characterization of the RNAs of rat brain, b) RNA synthesis in isolated brain nuclei, c) ribonucleoprotein particles from rat brain cortex and d) the kinetics of RNA and protein turnover in rat cortex.

ABSTRACT

a) About 80% of total rat brain RNA is ribosomal with two components showing $s^0_{20,w}$ = 17 and 28S, while 20% is soluble RNA with s = 4S. Base ratios A:U:G:C for these three fractions are 19.5:21.1:30.6:28.7; 18.6:18.7:31.3:31.4 and 18.1:17.5:33.3:31.1. They are therefore remarkably similar, and especially the two ribosomal species are closely complementary to rat DNA. RNA isolated from purified cortex nuclei on sucrose sedimentation shows peaks in the 4, 16, 28 and 45S regions with relative amounts of about 2:4:6:1 respectively. RNA from rat cortex pulsed with ^{14}C-orotate shows two specific activity peaks on MAK column chromatography, one in the region between soluble RNA and 18S ribosomal RNA, the other beyond the 28S ribosomal RNA.

b) Nuclei capable of catalyzing an active incorporation of radioactive nucleoside triphosphates into an acid-insoluble polymer have been isolated by a procedure modified from that of Widnell and Tata [Biochem. J. 92, 313 (1964)]. Two different incorporation systems can be distinguished depending on incubation conditions.

System A requires the simultaneous presence of all four nucleoside triphosphates and is inhibited by actinomycin D, pancreatic RNase, RNase T1 and DNase. It differs from most other DNA-dependent, polynucleotide synthesizing systems whether microbial or animal by its absolute requirements for spermidine, $MgCl_2$ ($MnCl_2$ is inhibitory), KCl (NaCl shows no activity) and the ATP-generating system. Incorporation is linear for 10 minutes and accounts for 2-4% of the total radioactivity added or approximately 2 μmoles. The product appears to be degraded to a heterogeneous mixture of various size classes when examined by density gradient centrifugation in sucrose; upon chromatography on MAK columns it is eluted in the "high salt" region.

System B incorporates CTP exclusively. It is stimulated only 20 per cent by the addition of KCl or spermidine. Rate and extent of incorporation is about half that of system A and is resistant to actinomycin D, pancreatic RNase, RNase T1 and DNase. The presence of any combination or permutation

of the remaining three bases has no effect on incorporation. Tentatively the product has been identified as a poly C homopolymer of low molecular weight.

c) Ribosomes were isolated by means of differential centrifugation of a deoxyclolate-treated post-mitochondria supernatant. The particle population obtained by this method consists mainly of 60S subunits and 80S monosomes with some dimeric and trimeric aggregates. All particles are highly active in catalyzing the incorporation of amino acids into polypeptide linkage.

Polyribosomes were isolated from rat cortex by a modification of the method described by Bont et al (Biochem. J. 95, 15c, 1965). The resulting population consists of polymeric aggregates to the extent of 80% or better. The $s^0_{20,w}$ values of the first six members determined in the analytical ultracentrifuge are 76, 118 148, 178 and 238S respectively. Sucrose density gradient analysis and electron microscopy support the assignment of s values. The particles are highly active in protein synthesis in vitro; in vivo pulses of labeled amino acids show incorporation of nascent protein predominantly into the largest members of the population. Therefore the system probably reflects the situation obtaining in cortex with relatively little degradation during isolation.

d) We have attempted to obtain a gross overall picture of macromolecular metabolism in rat cortex by following the uptake and loss of labelled RNA and protein precursors (^{14}C-orotate and ^{3}H-leucine) in several subcellular brain fractions. The labeled compounds were injected intraventricularly into a number of rats, which were then sacrificed after various lengths of time for determination of distribution of label in various subcellular fractions obtained by differential centrifugation. We draw the following inferences from the data obtained so far: (1) Synaptic vesicles have a long half-life, which implies either that they are transported to the nerve endings by slow axoplasmic flow or that they are reutilized after releasing transmitter at the synapses. (2) Mitochondria turn over at a much slower rate than any of the other subcellular structures, which fact is consistent with the theory that they reproduce semiautonomously and that their half-life approaches that of the cell as a whole. (3) Ribosomes decay as entities, since the ribosomal protein and RNA have the same half-life. (4) Myelin does not become labeled, indicating that myelin synthesis in adult rat brain is extremely slow. (5) Except for nuclei, which have a complicated labeling pattern, the RNA turnover rate is nearly the same for all the fractions - probably because we are detecting RNA that is predominantly ribosomal in all the fractions.

PLANS FOR FUTURE

The work on nuclear and messenger RNA synthesis in vivo and in vitro and the characterization of these species is to be continued, as are the turnover experiments. We also plan to explore the feasability of correlating RNA synthesis in neurons directly with brain function.

CURRENT REPORTS AND PUBLICATIONS

PAPERS PUBLISHED: a) Mahler, H.R., Moore, W.J., and Thompson, R.J., Isolation and Characterization of RNA from Cerebral Cortex of Rat, J. Biol. Chem. 241, 1283 (1966), b) Campbell, M.K., Mahler, H.R., Moore, W.J., and Tewari, S., Protein Synthesis Systems from Rat Brain, Biochemistry 5, 1174 (1966).

PAPERS SUBMITTED: a) Campagnoni, A. and Mahler, H.R., Isolation and Properties of Polyribosomes from Cerebral Cortex, Biochemistry.

STUDIES ON AMINE METABOLISM DURING ANAPHYLAXIS

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WORK UNIT NO. NR 108-716 **CONTRACT** N00014-67-A-0397-0001

OBJECTIVES

(a) To document the changes in amine content of guinea pig lung before and after antigenic challenge (b) to study amine substances of a peptide-like nature which cause contractions of guinea pig ileum (c) to determine the relationship of these materials to the "slow-reacting substance" of Brocklehurst.

ABSTRACT

A general method has been developed for the determination of amines in tissues. The method relies on the procedure, developed by other authors, of preparing fluorescent "dansyl" derivatives by reaction with dimethylaminonaphthalene sulfonyl chloride (DNS-Cl). Slices of lung from ovalbumin-sensitive guinea pigs were incubated with ovalbumin (1 mg/ml) in Tyrode solution for 10 minutes at 37°C. In addition to control slices (without ovalbumin) zero-time control lung was obtained by crushing freshly-excised lung between metal blocks chilled to the temperature of liquid nitrogen. All tissues were extracted by homogenization and deproteinization with zinc sulfate and sodium hydroxide.

Dansyl derivatives were extracted with chloroform from the supernatant before and after acidification to pH 2. The derivatives were separated both by chromatography and high-voltage electrophoresis and measured quantitatively on the paper by fluorometric scanning. The results showed that the intracellular concentration of histamine declined upon incubation and this decline was increased by ovalbumin.

Studies with 2-(ring ^{14}C) histamine indicated that about 20% of the histamine which disappeared had been methylated by the imidazole methyltransferase. The remainder appears to be lost by oxidation; this is inferred from the non-disappearance of histamine under anaerobic conditions. A dansyl-peptide which accumulated in the tissue after antigenic challenge was isolated by chromatography on columns of Sephadex G-25. This separation led the author to large scale fractionations of peptides from lung supernatants using 1 metre columns of Sephadex G-25. The individual peptide fractions were re-purified on columns of Biogel P-4 and lyophilized. A comparison of the peptides obtained from zero-time (frozen) lung and lung slices after 10 minutes challenge with ovalbumin indicated that certain peptide fractions decrease in weight while others increase. The possibility that there is enzymic interconversion of these peptides is being studied.

None of the peptides from the zero-time preparations caused contrac-

tions of guinea-pig ileum, but a peptide which accumulated in the 10-minute extract caused a slow sustained contraction apparently similar to the action of slow-reacting substance (SRS-A).

Amino acid analyses indicated that the peptide contained neither histamine nor histidine. Periodate estimations indicated 2-3% (w/w) of carbohydrate in glucose equivalents. After hydrolysis (1 hour at 100°C in 1 N HCl) chromatography for sugars indicated the presence of hexosamines and n-acetyl hexosamines. W. G. Smith (Nature 209, 1251, 1966) has reported that SRS-A contains carbohydrate. Attempts are being made to further purify the writer's peptide material in order to determine the effectiveness of the peptide and carbohydrate moieties in the bio-assay.

Of the peptides thus far investigated, none appear to contain significant amounts of histamine or histidine.

PLANS FOR FUTURE

(a) To ascertain whether amines from guinea pig lung can be converted by various enzyme preparations (esterases, proteolytic enzymes, etc.) into biologically active materials giving positive bio-assays on either the guinea pig ileum ("slow-reacting" contractions) or the skin (vaso-active response) (b) to perform further purification and chemical analysis on biologically active substances from guinea-pig lung (c) to investigate uptake of radioactive ^{14}C-histidine into subcellular fractions of guinea pig lung.

CURRENT REPORTS AND PUBLICATIONS

Callaghan, O. H.(1965), "Alterations in amine content, including Histamine, of Guinea Pig lung slices during antigenic stimulation; a possible histidine precursor." Fed. Proc. 24, 2475A.

IMMUNOCHEMICAL STUDIES ON MAMMALIAN HOMOTRANSPLANTS

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ASSISTED BY

WORK UNIT NO. NR 108-742 CONTRACT Nonr-233(86)

OBJECTIVES

(a) to study the effect of various thymus fractions on the maturation of lymphocytes in immature rats, (b) to investigate the histocompatibility antigens derived from thymus, spleen, skin, and liver, (c) to evaluate the effects of spleen, thymus and skin extracts on homograft tolerance.

ABSTRACT

Our previous investigations have indicated that strong histocompatibility antigens in the thymus are similar immunologically to those in the epidermis. The major effects of either thymus or epidermal antibody on neonatal rats is to decrease the growth rate, interfere with the development of the thymus and the maturation of the immunologic system generally, including the spleen, and to impair the growth and development of normal epidermis. Anti-epidermal antibody is nearly as potent as antithymus antibody in impairing the growth and normal development of the thymus. The specific spectrum of histocompatibility antigens shared by these two types of tissues are under intensive investigation, utilizing column gradient chromatography under a variety of conditions. As these fractions are isolated and characterized they will be utilized to determine their ability to induce the production of antibody which impairs the previously described biologic functions. Those fractions which show the highest degree of activity will be studied in an effort to decrease the homograft reaction and to determine the specificity of such decreases. Our studies in the past year have indicated that homologous cross absorbed antithymus and anti-epidermal antibody are promising in this respect. Antispleen antibody differs significantly from either of the two previously mentioned antibody preparations in that there is no diminution in growth rate in neonatal rats, nor obvious interference with the development of the thymus or epidermis. Thus it appears that the antibodies produced against the homologous spleen do not have the same spectrum or concentration of histocompatibility antigens

present in the previously described two tissues. It is of interest, however, that specific homologous antispleen antibody is more effective in prolonging homograft survival than either the anti-epidermis or antithymus antibody.

CURRENT REPORTS AND PUBLICATIONS

Ashley, Franklin L., Jones, Joyce, and Henderson, T.: Immunological Studies on the Histocompatibility Antigens of Homologous Rat Tissues. To be presented to the American Association of Plastic Surgeons, Toronto, May, 1967.

CHIMERA DRUG METABOLISM

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ASSISTED BY

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

OBJECTIVES

We propose to study the possibility of altered drug metabolism in mice that have been protected from lethal x-irradiation by the injection of foreign hemopoietic tissue (chimeras). The liver is the major site for drug detoxication. Recent experiments have indicated that x-irradiation suppresses the ability of the host to respond to a drug-mediated stimulation of microsomal drug-metabolizing enzymes. Hepatomegaly is a consistent finding in allogeneic and xenogeneic radiation chimeras. An important question to be resolved is whether the hepatomegaly reflects a return of the functional capacity of x-irradiated animals protected with foreign hemopoietic tissues. We would also like to investigate the effects of various antimitotic and antitumor agents on drug metabolizing enzymes of the microsomal fraction.

ABSTRACT

Contract has not been in force long enough for progress to be reported.

THE EFFECT OF LEUCOGENENOL ON ANIMALS SUBJECTED TO X-RAY RADIATION

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ASSISTED BY

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

OBJECTIVES

To study the effect of "Leucogenenol" on the production and distribution of blood cells in animals subjected to X-ray irradiation.

ABSTRACT

A compound that induces leucocytosis in animals has been isolated from the culture medium (Czapek-Dox) of P. gilmanii. It has been named "Leucogenenol". Preliminary experiments have shown that when Leucogenenol is injected i.v. into mice (0.4γ/mouse) 48 hours after irradiation with 400 rads, the mice show a fourfold increase in total white cell count over that of the controls at the end of approximately a week.

PLANS FOR FUTURE

To study the action of Leucogenenol on the production and distribution of white blood cells in irradiated mice as a function of the quantity, time or times of injection and the amount of irradiation.

CURRENT REPORTS AND PUBLICATIONS

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

I M M U N O C H E M I S T R Y

The description of interests given as a prelude to the sections on Marine Biochemistry and Metabolic Biochemistry is applicable throughout the program. The section of immunochemistry differs more in the tools used to serve those interests than in the reason for support. The phenomenon of serological specificity is a matter which may affect naval operations in a number of ways. Because vaccines are specific, a recruit must be given a great number of "shots" instead of one to protect him against whole classes of bacteria, against families of viruses or perhaps against a wide variety of toxins. Because of specificity, the technical capability for storing and applying blood and tissue banks for the treatment of injuries and disease must wait until the problems of rejection of alien proteinaceous material has been solved to be useful. Within the scope of comparative serology lies a great promise of rapid and accurate classification of organisms which might be used by the Navy in a variety of contexts. Hence, we have retained a program devoted to the establishment of the basic facts about one aspect of biochemistry--the nature and consequences of the immune response.

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

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

OBJECTIVES

(a) To study antibodies produced in two different animal species in response to the same antigen and to compare them; (b) to study particularly antibodies of the chicken and compare them with antibodies produced in the rabbit; and (c) to gain by these and other methods more insight into the mechanism of antibody formation.

ABSTRACT

Continuing last years experiments with doubly labelled BSA (bovine serum albumin) to which we coupled equivalent amounts of As(=azophenylarsonate) and R_4N(=azophenyl-N-trimethylammonium chloride), we isolated from the blood serum of rabbits injected with this antigen three types of antibodies (anti-As, anti-R_4N and anti-BSA). Starch gel electrophoresis of the reduced and alkylated antibodies revealed in all three instances the presence of 8-10 different L-chains and at least 2 different H-chains. However, the migration rate of these chains was very similar in all three types of antibodies. Similarly, we found that trypsin digests of anti-As and anti-R_4N gave almost identical peptide maps. We were not able to find any consistent difference between the finger-prints of anti-As and anti-R_4N, nor any significant difference in column chromatography of the tryptic digests (1,2). The lack of a clear difference between the maps of the two anti-haptens suggests that each of the combining sites might be heterogeneous and, therefore, does not show up in the peptide maps. The spots found in these maps are, evidently, caused by peptides from those parts of the L and H chains which are common to all anti-As or anti-R_4N molecules. Preliminary experiments in chicken gave similar results. We conclude from these results that complementariness of the combining site to the homologous hapten can be obtained by various amino acid sequences and that we have to abandon the idea of uniform, well-defined combining sites in an antibody directed against a chemically defined hapten. Two lectures on antibodies which the principal investigator delivered on invitation of the German Gesellschaft fuer physiologische Chemie have been published in English (3, 4), one delivered at the University of Marburg was published in German (5). Dr. G. Vidaver continued his work on the migration of glycine into pigeon red cells against a concentration gradient and described two models of carriers; each of these occurs in two alternate states which differ from each other either by allosteric transition or by hydrogen bonding (6).

PLANS FOR FUTURE

(a) Analysis of antibodies formed in chicken after the injection of the doubly labelled As-R_4N-BSA azoproteins used earlier for the injection into rabbits (see above) and comparison with rabbit antibodies formed against the same antigen; (b) Further examination of γ-globulins and antibodies in irradiated and splenectomized animals to find out whether some of the L chain bands are those of γ-globulins formed in the spleen; (c) Examination of the fragments I and II found in papain digests of most antibodies and γ-globulins, and determination of the ratio "fragment I: fragment II" in the antibodies directed against the azophenylarsonate anion and the azophenyl-N-trimethylammonium cation.

CURRENT REPORTS AND PUBLICATIONS

1. K. L. Knight, E. F. Gold, M. A. Lopez and F. Haurowitz: Heterogeneity of Hapten Specific Antibodies, Federation Proceedings, vol. 25, page 678, no. 2760.

2. Katherine L. Knight, Miguel Angel Lopez and F. Haurowitz: Comparison of Two Hapten-Specific Rabbit Antibodies, J. Biol. Chem., 241, 2286-2292, 1966.

3. F. Haurowitz: Structure and Formation of Antibodies (Mosbach Colloquium no. 15, held 1964, published 1966, p. 232-240).

4. F. Haurowitz: The Role of the Antigen in Antibody Formation (15th Mosbach Symposium on Biol. Chem., held 1964, published 1966, p. 240-254).

5. F. Haurowitz: Struktur und Bildung der Antikoerper (Behringwerke-Mitteilungen, 45, p. 37-55, 1966; Discussion p. 56-70).

6. George A. Vidaver: Inhibition of Parallel Flux and Augmentation of Counter Flux Shown by Transport Models Not Involving A Mobile Carrier, J. Theoret. Biol., 10, 301-306, 1966.

IMMUNOCHEMICAL CRITERIA OF PURITY OF PROTEINS AND POLYSACCHARIDES

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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

Work previously referred to as in press has been published (1 - 5). Studies are continuing on the isolation of oligosaccharides from A, B and H substances by the alkaline borohydride method. In addition an Le^a substance from human ovarian cyst fluid is being studied. Very few of the oligosaccharide fractions isolated appear to contain fucose. The A, B and H oligosaccharides with one fucose described in the last report and containing the unsaturated reduced residue, R, CH_2OH-CHOH-CH=CH-CHOH-CH_2OH have been subjected to a cis-hydroxylation which converts them to the hexitols. In each case as well as with the oligosaccharide GNAc-R gas chromatography showed the presence of glucitol, mannitol and iditol. R can exist in the erythro or D-threo forms and the cis-hydroxylation would convert the former to glucitol and the latter to a mixture of mannitol plus iditol as was established with authentic samples of these two compounds. The findings establish that each of the A, B and H oligosaccharides is a mixture, some molecules being terminated by the erythro and others by the D-threo form of R (6).

Purified human anti-A from single individuals has been obtained by absorption on insoluble polyleucyl-A substances and elution with N-acetyl-D-galactosamine. The anti-A consisted of a mixture of γG, γM and γA immunoglobulins. The γM and γG anti-A were separated by density gradient centrifugation. Inhibition studies with oligosaccharides with the γG anti-A showed the reduced A pentasaccharide to be the best inhibitor with an A trisaccharide next and N-acetyl-D-galactosamine poorest indicating the participation of all sugars in reaction with the combining site. With the γM anti-A, all three compounds were equally effective as inhibitors on a molar basis indicating that some γM antibodies may have combining sites complementary to units as small as a monosaccharide (7). Reviews on the nature of an antigenic determinant (8) and on structure and heterogeneity of antibodies (5) have been published.

PLANS FOR FUTURE

Studies on the amino acid composition of human antidextran from various individuals are under way. Efforts are continuing to study the various kinds of human anti-A and anti-B antibodies. The isolation of oligosaccharides continues and studies are in progress to develop a new method of degrading blood group substances which will not degrade those sequences of the molecules which are destroyed by sodium-hydroxide - sodium borohydride. Studies on the optical rotatory dispersion of oligosaccharides are continuing.

CURRENT REPORTS AND PUBLICATIONS

1. Beychok, S. and Kabat, E. A. (1965). Optical Activity and Conformation of Carbohydrates. I. Optical Rotatory Dispersion Studies on Immunochemically Reactive Amino Sugars and Their Glycosides, Milk Oligosaccharides, Oligosaccharides of Glucose and Blood Group Substances. Biochemistry, 4, 2565.
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3. Lloyd, K. O., Kabat, E. A., Layug, E. J. and Gruezo, F. (1966). Immunochemical Studies on Blood Groups. XXXIV. Structures of Some Oligosaccharides Produced by Alkaline Degradation of Blood Group A, B and H Substances. Biochemistry, 5, 1489.
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7. Kaplan, M. E. and Kabat, E. A. (1966). Studies on Human Antibodies. IV. Purification and properties of anti-A and anti-B obtained by absorption and elution from insoluble blood group substances. J. Exp. Med., 123, 1061.
8. Kabat, E. A. (1966). The Nature of an Antigenic Determinant. J. Immunol. 97, 1.

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 mechanism of destruction of mammalian cells by complement.

ABSTRACT

C'3c: We have developed a procedure for isolation of C'3c from guinea pig serum by isoelectric precipitation, chromatography on DEAE and CM cellulose and by zone electrophoresis on Pevikon. The overall yield is about 5 to 7%. The final product is pure as judged by DEAE and CM cellulose chromatography, as well as hydroxylapatite chromatography, immunoelectrophoresis and disc electrophoresis. The sedimentation coefficient is 7.5S, and the molecular weight, by sedimentation-equilibrium analysis, is 180,000.

The dose-response curve of its reaction with EAC'4,2a is concave to the abscissa. Kinetic data conform with the postulated enzymatic action of C'4,2a. Each hemolytically active C'2 molecule, as assayed by Borsos' method, produces at least eight hemolytically active C'3c sites. Various aromatic compounds, including N-acetyl-L-tyrosylethylester (ATEe), epinephrine and phloridzin, inhibit C'3c uptake by cells, but do not block C'3c destruction by C'4,2a. ATEe also inhibits the subsequent reactions with C'3b and C'3e.

C'3b: This factor has been partially purified by chromatography on DEAE and CM cellulose and by zone electrophoresis on Pevikon. Disc electrophoresis of the present product shows two minor bands in addition to the major band which carries C'3b activity.

C'2: We have continued studies of the cleavage of C'2 by C'1a in the fluid phase. Careful attempts to separate the cleavage products, C'2a and C'2i, by chromatography on Sephadex G100 have failed. We suspect this indicates that C'2 (MW = 130,000) is cleaved into two equal, or nearly equal, fragments. If so, our previous estimate of 81,000 for the molecular weight of C'2a would have to be revised to about 65,000.

C'4, C'2, C'3e, C'3f, C'3a and C'3d: Work on chromatographic separation of these factors from guinea pig serum is proceeding, but no significant results can be reported at this time.

PLANS FOR FUTURE

(a) Further work on the cleavage of C'2 by C'1a (b) studies of the chemical changes of C'3c produced by enzymatic action of C'4, 2a (c) initial exploration of chemical changes in C'3b during its reaction with C'4, 2a, 3c (d) further work on purification of C'4, C'2, C'3b, C'3e, C'3f, C'3a and C'3d.

CURRENT REPORTS AND PUBLICATIONS

(a) G. Sitomer, R. M. Stroud and M. M. Mayer (1966), "Reversible Adsorption of C'2 by EAC'4: Role of Mg^{++}, Enumeration of Competent SAC'4, Two-Step Nature of C'2a Fixation and Estimation of its Efficiency." Immunochemistry, 3, 57-69.

(b) R. M. Stroud, M. M. Mayer, J. A. Miller and A. T. McKenzie (1966), "$C'2a^d$, An Inactive Derivative of C'2 Released During Decay of EAC'4, 2a." Immunochemistry, 3, 163-176.

(c) H. S. Shin and M. M. Mayer (1967), "Guinea Pig C'3c: Purification, Reaction Mechanism and Effect of Inhibitors." Submitted to Federation Meetings, April.

THE MECHANISM OF ANTIBODY FORMATION

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

OBJECTIVES

(a) To attempt restoration of thymectomized mice by immunoglobulin G, (b) to study the dose-response for immunity and tolerance to pneumococcal polysaccharide in offspring of mice tolerant to the polysaccharide, (c) to devise techniques for the enumeration of cells producing antibodies to a protein antigen.

ABSTRACT

(a) Miller (Science, 144: 1544, 1964) reported that a humoral factor secreted by the thymus was able to prevent impairment of the immunologic functions and the wasting syndrome which are observed in neonatally thymectomized mice. On the basis of our previous work (J. Immunol., 89: 782, 1962; ibid., 89: 790, 1962; Am. J. Vet. Res., 25: 413, 1964) we advanced the hypothesis that the thymic humoral factor may be natural antibody globulin. Strain C57BL/6J mice which had been thymectomized at birth were treated with weekly injections of immunoglobulin G derived from rabbit thymus or serum or were left untreated. Although many of the immunologic effects of thymectomy failed to appear, approximately 50% of the untreated mice died by week 10 and were found to have depletion of splenic lymphocytes upon histologic examination. The treated mice all survived and were found to have normal splenic architecture. This finding supports the concept that the thymic humoral factor is antibody globulin.

(b) The model of antibody formation proposed by Eisen and Karush (Nature, 202: 677, 1964) postulates that immunologic tolerance results from the formation, in excess antigen, of trimolecular complexes of two molecules of antigen with one of the corresponding natural antibody. The trimolecular complexes would not be immunogenic and would be degraded and eliminated. Immunity would result when the ratio of the concentrations of antigen and specific natural antibody are such that bimolecular complexes are formed. Thus, the outcome of antigenic exposure, in terms of tolerance or immunity, would be regulated by the relative concentrations of antigen and antibody. On the basis of this model, we predicted that the doses of antigen required to induce tolerance and immunity in mice newly born of tolerant mothers should be smaller than the corresponding doses for normal newborn mice. This follows from the assumption that the concentration of natural antibody specific for the tolerated antigen should be smaller in the offspring of tolerant mothers than in normal newborn mice, since the former would not acquire maternal antibodies of

that specificity. The experimentally determined minimal doses required to induce tolerance to pneumococcal polysaccharide type III were the following: normal adult mice, 5 μg; normal newborn mice, 0.5 μg; newborn mice from tolerant mothers, 0.05 μg. Similarly, the following minimal doses were required to immunize: normal adult mice, 0.5 μg; normal newborn mice, 0.05 μg; newborn mice from tolerant mothers, 0.005 μg.

(c) Antibody plaques have been obtained with erythrocytic antigens and with polysaccharides and haptens coupled to erythrocytes. Plaque formation by cells producing antibodies to proteins has not been reported. Spleen cells from mice immunized with rabbit immunoglobulin G were plated in agar containing sheep erythrocytes sensitized with immunoglobulin G derived from commercial rabbit anti-sheep hemolysin. The sensitizing globulin was diluted so that addition of complement did not result in direct hemolysis. After incubation and addition of complement, hemolytic plaques appeared around those mouse spleen cells which released anti-rabbit globulin antibody.

PLANS FOR FUTURE

(a) Restoration of thymectomized mice by immunoglobulin G will be attempted using C_3H mice. Both neonatally thymectomized mice and thymectomized adult, x-irradiated mice will be used. Tests for immunologic competence will include the ability to reject allogeneic tumor transplants. Immunoglobulin G of rabbit and mouse origin will be used. (b) The effects of graded doses of passively administered anti-pneumococcal polysaccharide serum on the dose of antigen required to induce tolerance or immunity in mice will be studied. (c) The primary and secondary responses of mice to rabbit immunoglobulin G will be investigated using the plaque formation technique.

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BIOCHEMICAL AND PHYSIOLOGICAL FACTORS OF THE IMMUNE RESPONSE

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

OBJECTIVES

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

ABSTRACT

Our studies of the interrelationships of environmental stress and endocrine imbalance on the immune response have been continued during the past year.

The late spring and summer months were devoted primarily to further experiments on the effect of high altitude on the immune response. Groups of rabbits and guinea pigs were acclimatized at either 10,600 ft (Echo Lake Laboratory) or 14,150 ft (Summit Laboratory, Mt. Evans) and then immunized with bovine serum albumin. Multiple blood samples have been collected from these animals after primary, secondary and tertiary immunizations. Analysis of these serums for precipitating and hemagglutinating antibodies are in progress. 2-mercaptoethanol treatment of the serums is also being done to determine if qualitative changes in antibodies are present as a result of the stress situation.

Even though studies have been directed primarily toward the investigation of antibody formation under conditions of environmental stress and endocrine imbalance, we have not lost sight of our interest in the basic cellular events which occur during antibody synthesis. It is our hope that our work will not only have some possible practical application but also will provide information at a more fundamental level. To this end, we have re-examined some of our earlier experiments on the effect of pretreatment with homologous gamma-globulin on the subsequent production of antibody. The study of antibody formation in animals subjected to stress may shed light on the basic control of antibody synthesis including possible feedback mechanisms. Our preliminary experiments have shown that the level of precipitating antibody is decreased in animals immunized after pretreatment with homologous gamma-globulin, and that some animals produce antibody detectable only by techniques employing I^{131}-labeled antigen. Another set of experiments is underway to investigate further the role of feedback mechanisms in the control of antibody formation.

PLANS FOR FUTURE

The long range plans of this research are to investigate in detail the molecular basis of changes which occur in the immune response of animals subjected to stress. Increased emphasis is to be placed on studies of the distribution of antibodies among various fractions of serum obtained from stressed animals. It is anticipated that information will be obtained relating to differences in the immunological and physico-chemical properties of antibodies produced by stressed animals.

It is hoped that a clearer understanding of the immunochemical events at a molecular level will enable us to explain better the overall immune response. To this end, we have taken up again our experiments relating to the feedback control mechanisms of immunoglobulin synthesis. If it can be shown that the influence of stress and/or endocrines act on the immunological mechanism in a particular way, as evidenced by molecular changes which can be distinguished, perhaps it may be possible to circumvent or overcome in part an adverse situation and its effect on the host-parasite relationship.

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STRUCTURAL AND IMMUNOCHEMICAL STUDIES
ON MYOGLOBINS AND HEMOGLOBINS

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

OBJECTIVES

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

ABSTRACT

In a previous abstract we reported our results on the role of the amino groups and C-terminal of sperm whale myoglobin in the antigen-antibody reaction. These results have now been published (a). We have also reported our treatment on the significance of the amino acid composition of hemoglobins and myoglobins in relation to their structure, function and evolution. This work has appeared recently (b). Infrared spectral analysis of the apoproteins of some hemoglobins and myoglobins has also been published (c).

Our work on the periodate oxidation of proteins, and in particular of myoglobin, was continued in order to find the conditions under which the reagent acted with specificity. This was achieved by oxidation of apomyoglobin with a two molar excess of periodate (pH 5.0, 0°, 2 hr) which resulted in the modification of 1.66 residues of methionine. Metmyoglobin prepared from this modified apoprotein was identical with native metmyoglobin spectrally, electrophoretically and immunochemically. It was concluded that the methionines at positions 55 and 131 were not essential parts of antigenic sites in sperm whale metmyoglobin (d).

Our search for a new specific reagent for the modification of amino groups in proteins and for which the extent of modification could be determined by amino acid analysis has been successful. The extent of reaction of Mb with 3,3-tetramethyleneglutaric anhydride (TGA) was measured by determining the loss of lysine and the equivalent appearance of 3,3-tetramethyleneglutarimidolysine in the hydrolysate at various time-intervals. Of the 19 lysine residues in Mb, three did not react with TGA. Also, the N-terminal valine did not react. Reaction of the lysine residues in Mb with TGA will give rise to 3,3-tetramethyleneglutaryl-lysine which will cyclize in acid to 3,3-tetramethyleneglutarimiodolysine. The cyclization would account for the stability of the derivative to acid hydrolysis and for its appearance on the analyzer after phenylalanine. Slight spectral

changes were observed on modification of Mb. Electrophoretic changes were great since the derivative was negative throughout the range pH 6 - 8.8. The derivative did not react with antisera to native Mb. The loss of antigenic reactivity is probably due to the combined effect of conformational disturbance and of charge reversal of the amino groups (e).

We have recently developed a chemical method for the specific cleavage of tryptophyl peptide bonds. The procedure was applied to apomyoglobin. As a first step, the protein was oxidized with 60-70 molar excess of periodate for 7 hr at pH 5.0 and 0°. This was followed by mild acid hydrolysis (0.1 N-HCl, 25° for 24 hr). Fragments obtained by cleavage of the protein at the tryptophan sites were separated by gel filtration on appropriate grades of Sephadex and identified by amino acid and N-terminal analysis. With this procedure, 92-94% cleavage was obtained at tryptophan No. 7 and 17-22% cleavage at tryptophan No. 14 in sperm whale apomyoglobin (f).

The preparation and characterization of Mn, Fe, Co, Ni, Cu and Zn metalloporphyrins were carried out. Ferriheme was also esterified with 4-pyridine-propanol and the derivative characterized as the diester. Complexes of these various prophyrins, as well as protoporphyrin IX, with sperm whale apomyoglobin were formed and the resulting artificial myoglobins characterized. Very little complex-formation was obtained with Ni^{2+}, Co^{3+} and Mn^{3+} metalloporphyrins and apomyoglobin. Myoglobin prepared with Cu^{2+} metalloporphyrin was immunochemically identical with native Mb. All other artificial myoglobins were less reactive to varying degrees. The changes in antigen reactivities were attributed to conformational reorganization caused by the different coordination tendencies of the various metals or by the modification of the side chains of the heme group (g).

PLANS FOR FUTURE

(a) To continue our investigations on the correlation of structures of hemoglobin and of myoglobin with their antigenic properties. More specifically modified derivatives will be prepared, characterized, and their chemical and immunochemical properties investigated, and (b) to continue studies on the primary structure of finback whale myoglobin.

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BIOCHEMICAL PROPERTIES OF ANTIBODIES TO PURINE AND PYRMIDINE RIBONUCLEOTIDES

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ASSISTED BY

WORK UNIT NO. NR 106-646 **CONTRACT** Nonr 4259(11)

OBJECTIVES

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

ABSTRACT

During the past year, it has become feasible to prepare protein conjugates of the following dinucleoside phosphates:* CpC, CpA, CpU, ApC, ApA, ApU, ApG, UpA. The new antigens have yielded antibodies specific for the dinucleoside phosphate determinant groups. The antibodies precipitate with heat denatured DNA under conditions in which anti-mononucleoside antibody required DNA denatured in the presence of formaldehyde. Although precipitation with RNA has not been observed, the latter was able to inhibit the precipitin reaction between antibody and DNA.

Fluorescainated antinucleoside globulins were shown to react with the nuclei of L cells. The pattern of nuclear fluorescence was similar to the distribution of nuclear I \. Nuclear fluorescence occurred only in cells harvested during the period of maximum DNA synthesis as measured by the uptake of thymidine. This correlates with the previously demonstrated specificity of the antibodies for denatured DNA.

Attempts to study the effect of salt precipitated globulin preparations in various biochemical systems were complicated by the side reactions induced by salts and by impurities in these preparations. It was therefore decided to prepare specifically purified antibodies. This was accomplished by the specific precipitation of antibody with homologous antigen followed by solubilization with hapten. The antibody was then isolated from the supernatant solution by precipitation of the antigen using sodium sulfate and subsequent dialysis. The specifically purified antibodies are now being tested in a number of biochemical systems.

*Abbreviations: All dinucleoside phosphates are 3'--5'. A represents adenosine or adenylyl; C, cytidine or cytidylyl, etc. For example: ApC = adenylyl 3'--5' cytidine.

PLANS FOR FUTURE

(a) Study of biochemical properties specifically purified antinucleoside antibody, and (b) to study their effects on viable cells.

CURRENT REPORTS AND PUBLICATIONS

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(2) William J. Klein, Jr., Sam M. Beiser and Bernard F. Erlanger, "Nuclear Fluorescence Employing Antinucleoside Immunoglobulins," J. Exp. Med. 125, 61 (1967)

COMPARATIVE STUDIES ON THE IMMUNOGLOBULINS OF MARINE FISHES

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WORK UNIT NO. NR 106-665 **CONTRACT** Nonr-3656(24)

OBJECTIVES

(a) Elucidation of the structure of elasmobranch antibody polypeptide chains by physical chemical criteria, and selected N-terminal and C-terminal amino acid sequences, (b) to establish the evolution of the structural gene classes coding for immunoglobulin heavy chains in the teleost fishes, (c) to study possible homology of structure of invertebrate antibody-like proteins which have been shown to be active in agglutination of mammalian red blood cells and certain bacteria with vertebrate immunoglobulins.

ABSTRACT

Our studies have continued on structure of elasmobranch immunoglobulins. Antibodies have been produced in the leopard shark after prolonged immunization with a bacterial virus (T_2 phage of E. coli) and hemocyanin isolated from Megathura crenulata. The studies reported here summarize the structural evidence obtained at the present time that these proteins are immunoglobulins and further describe the structural basis for antibody activity in them. (1) The leopard shark (Triakis semifasciata) responded to continuing antigenic challenges by production of two size classes of immunoglobulins, an early appearing 17.4S macroglobulin which was supplemented by increasing amounts of a 6.7S globulin after 3 to 6 months of immunization. Both globulin fractions acted as neutralizing antibodies to a viral antigen (T_2 coli phage). However, the shark antibodies appeared at a much slower rate than similarly induced antibodies in the mouse. (2) The leopard shark was found to produce precipitating ant bodies, both 17S and 7S, to hemocyanin. This is the first report of precipitating antibodies in elasmobranch fishes. (3) Each immunoglobulin was purified by size (by gel filtration) and charge (DEAE cellulose chromatography) and verified for purity both by its migration as a single band in accrylamid gel electrophoresis and antigenic analysis in Ouchterlony tests and immunoelectrophoresis. (4) Microprecipitin tests using I^{125} labelled hemocyanin antigen produced precipitin curves for both immunoglobulins. The two curves were of the same shape and showed the same zone of equivalence. However, when compared on a weight basis, the 17S immunoglobulin was 3 to 4 times effective as the 7S globulin in precipitating the I^{125} labelled hemocyanin. The amount of antigen precipitated was of the same order of magnitude as a rabbit antiserum against hemocyanin. However, the general amount of antibody activity was between 3 and 10 fold less than the rabbit antibody on a weight basis. (5) By available chemical and antigenic criteria, the 17S immunoglobulin appears to be a pentamer of the 7S protein. Complete fusion of bands in Ouchter-

lony diffusion plates was always found. The isolated heavy chains were also identical in Ouchterlony analysis. The apparent identity of heavy chains from the two immunoglobulin fractions has been noted for other elasmobranchs by Clem and Small, and Marchalonis and Edelman.

PLANS FOR FUTURE

We propose to continue and expand our studies on immunoglobulins of lower vertebrates in the following ways: (1) To establish the relationship between the heavy polypeptide chains of the 17S and 7S immunoglobulins of Triakis semifasciata by investigation of the N-terminal and C-terminal amino acid sequences and selected peptide sequences. We will also compare peptide elution profiles from an ion exchange resin. (2) Continue a search in the teleost fishes for the first appearance of antigenicly different heavy chain classes and when found, the degree of structural similarity in the heavy chains will be studied. (3) Investigation will proceed on certain invertebrate proteins which are known to participate in antibody-like reactions. In particular we have a 7S globulin from the keyhole limpet (Megathura crenulata) and structural studies are being performed to decide whether this protein resembles vertebrate immunoglobulins in any way. From these studies we hope to derive sufficient data to help us decide between various alternatives on current theories of the evolution of immunoglobulin polypeptides and to compare the structural information with the accumulating information on mammalian immunoglobulins.

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COMPARATIVE IMMUNOCHEMISTRY

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WORK UNIT NO. NR 106-712 **CONTRACT** N00014-67-A-0308-0001

OBJECTIVES

To study immunochemical properties of homologous proteins.

ABSTRACT

This project employs (a) homologous proteins from mammalian and fish blood sera, mammalian milks, and avian egg whites and (b) chemically modified or derived preparations from these proteins. The project, presently only in the introductory phases, concerns the effects of variation in structure of these protein antigens on the physical chemical parameters of the antigen-antibody interactions.

Proteins initially studied on this project are one avian (domestic chicken) egg white lysozyme, avian (domestic chicken and Adelie penguin) egg white ovomucoids and a trypsin inhibitor from lima beans. Rabbit antibodies against these four antigens have been prepared. Preliminary results with lysozyme indicates an antigenic valency of approximately 2.5, as compared to values of 2 and 4 in the literature. This indicates a molecular weight for the complex of approximately 500,000 g. Attempts are now being made to determine the molecular weight of such a complex by conventional physical methods.

We have shown that the ovomucoids lose their inhibitory capacity against proteolytic enzymes after scission with mercaptoethanol and carboxymethylation, but that they retain their inhibitory capacities after scission of the two methionines with cyanogen bromide. Chemically modified preparations of both chicken and penguin ovomucoids are being prepared for the studies with the ovomucoids.

PLANS FOR FUTURE

To study:

(a) chicken lysozyme and chicken and Adelie penguin ovomucoids and chemically modified derivatives thereof.

(b) chicken and penguin ovotransferrins and human serum transferrin

(c) trypsin inhibitor from lima beans.

CURRENT REPORTS AND PUBLICATIONS

(These on other projects. This project only just begun)

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STUDIES ON THE RATES AND TYPES OF PROTEINS SYNTHESIZED IN BIOLOGICAL SYSTEMS

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WORK UNIT NO. NR 106-758 CONTRACT Nonr-035(05)

OBJECTIVES

To study mechanisms that control the rates and types of proteins synthesized in biological systems; (a) The phylogeny of the immune system, (b) the role of basic proteins on the rates of protein synthesis in cell-free systems.

ABSTRACT

Workers in other labs have shown that: (1) Hela cell metaphase ribosomes, (2) sea urchin egg polysomes and (3) Ascaris fertilized egg polysomes are incapable of supporting in vitro protein synthesis. Treatment of the preceeding preparations with trypsin enabled them to support protein synthesis.

As trypsin selectively attacks lysine and arginine linkages, which are in high concentration in histones, it was suggested that histone was attached to the polysomes thereby inhibiting protein synthesis at the translational level. This suggestion was the first speculation that histones might act at the translational level in addition to their readily observed effects on transcription.

We have recently shown that histones, ribosomal proteins and lysozyme (all soluble in .1N HCl) inhibit a rat liver cell-free protein synthesizing system. The site of inhibition appears to be RNA. S-RNA charging is inhibited to the following extent (dependent on basic protein concentration): histone - 80%, ribosomal protein-50%, lysozyme - 25%. Histone is 4-5 times more effective in inhibition than the other two basic proteins which are about the same.

The inhibition by basic proteins on polysomes (again dependent on protein concentration) is as follows: histone - 35%, ribosomal protein - 48%, lysozyme - 47%. Histone is about three times more effective than the other two.

Basic protein inhibited polysomes cannot be reactivated with trypsin at concentrations considerably lower than that used by other investigators. The trypsin concentration used, 5 micrograms/ml., caused considerable damage to the polysome (60-70% inhibition) without apparently attacking the basic protein.

Thus, our observations of translational inhibition by basic proteins is apparently not the same phenomenon as that postulated for Hela metaphase cells, urchin eggs and Ascaris zygotes.

The antibody forming system is not common to all forms of life. There is no definitive evidence in invertebrates on the hagfish, a primitive vertebrate, of an immune response comparable to that of other vertebrates. All other vertebrates exhibit a strong response to exposure to antigen in the form of specific antibody production. Evidence is beginning to accumulate indicating that antibody specificity resides primarily in unique amino acid sequences of different antibody molecules. If this is true, then a very large number of genes must be involved in the immune system. It seems very unlikely that such complex machinery should have arisen de-novo so abruptly during evolution. It seems more reasonable to postulate that the immune system arose through the modification of existing systems. If this were true then one might hope to find some indications of the nature of phylogenetic precursor molecules in the antibodies of existing forms. For example, if the precursors were a family of enzymes, then one might find catalytic properties still remaining in antibody molecules.

This possibility has been tested by examining the antibodies of hyperimmune sera for a large number of enzymic activities on acrylamides gels using histochemical methods of characterization. Antisera and antigens were electrophoresed before and after combination. A disappearance of enzyme bands found on the gamma-globulin region of antisera after antigen-antibody precipitation would indicate the presence of molecules with both antibody and enzymic activity. Loss of enzyme activity was observed in the antibody-precipitated serum only when assayed for alpha naphthol acetate esterase activity.

Decomplementation of the antisera resulted in the loss of the same band of esterase activity which was lost when antibody was treated with antigen. Thus it was apparent that the esterase enzyme activity lost upon precipitation with antigen resided in complement rather than antibody. In summary it was not possible to associate enzyme activity with the antibodies used in the study.

IMMUNOLOGICAL FUNCTION IN SEVERE THERMAL INJURY

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WORK UNIT NO. NR 106-733 **CONTRACT** Nonr 1503(00)

OBJECTIVES

(a) To evaluate specific changes in the immune state of experimental animals and human subjects following severe thermal injury, with particular reference to the ability of the burned host to develop and exhibit delayed hypersensitivity and autoimmune-type responses.

ABSTRACT

Previous studies in this laboratory indicate that thermal injury induces a marked inhibition in the ability of tuberculin sensitive guinea pigs to respond to intradermal challenge with tuberculin. This effect is most marked during the first and second weeks, but persists as long as five weeks after burning. In addition, it has been determined that thoracic duct lymph obtained from severely burned rats contains a hemagglutinin specific for rat erythrocytes. This hemagglutinating factor has been identified as a gamma globulin, and may constitute an immune response to altered autologous tissues. Such studies have currently been extended to burned guinea pigs. The working hypothesis has been that the release of altered autologous tissue constituents from the burn site might play a role in the development of a variety of alterations in immunological responsiveness. Previous reports by Milgrom and Witebsky that rabbits immunized with denatured autologous gamma globulin produce antibodies against gamma globulins of some foreign species were particularly suggestive in this regard. The present communication describes a heterophile-like and an antiglobulin antibody response in guinea pigs after repeated exposure to thermal injury.

In the course of this study, 39 guinea pigs received 20% body surface area burns by application of a thermal-regulated metal surface maintained at 250°C. After the first injury, 27 of these animals received five additional full-thickness skin burns of one square centimeter area, applied at weekly intervals. Baseline and serial sera were obtained from these animals and from control donors. They were tested against erythrocytes of various species (man, ox, sheep, pig, rabbit, guinea pig, rat and mouse). In addition, an attempt was made to detect heterophile antibody (anti-human gamma globulin) by the latex slide and sensitized human cell hemagglutination tests. Twenty-two of 27 animals had rising serum titers of anti-rat erythrocyte agglutinins. Ten guinea pigs also showed significant anti-human

gamma globulin antibody responses. The heterophile agent was sensitive to Mercaptoethanol, while the antiglobulin factor was resistant to Mercaptoenthanol. Both factors are presumably antibodies, and may constitute a response to repeated challenge of the host with denatured autologous tissue antigens released from the burned sites. These observations lend further support to the probability that thermal injury is associated with sensitization of the host to a variety of altered autologous tissue constituents.

PLANS FOR FUTURE

(A) Continued study of mechanisms whereby cellular immunological responses are inhibited in the course of thermal injury.
(B) Characterization of the heterophile anti-rat erythrocyte and anti-human gamma globulin antibody responses demonstrated after thermal injury in the guinea pig.
(C) Search for similar immunological responses in other mammalian species, including man.

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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

Sera obtained at various intervals after open heart surgery have been collected in 56 individuals. Three had antibodies directed to γ-globulin in the control specimen. 26 had anti-γ-globulin antibodies. These were identified as specific for one or another of the known Gm factors in 19 instances. In the remaining 9 patients the specificity was not directed toward any known Gm factor, but presumably defines new hereditary antigenic determinants. One such factor, Gm(20), has been delineated and shown to be genetically determined by appropriate family studies. Studies are underway to define the additional seven factors. One of these factors, tentatively termed "San Francisco 1" is of special interest since it seems to be inherited independently of the known Gm factors. In view of the much more frequent antigenicity of the Gm(a) factor as compared to other Gm factors, studies of the biochemical basis for Gm(a) molecules as opposed to "non-a" appeared warranted. Peptide mapping studies were performed of Fc fragments of γ2 myeloma proteins. All Gm(a+) myelomas contained one peptide that was absent in all Gm(a-) myelomas. However, all Gm(a-) myelomas contained a different peptide spot which was absent in the Gm(a+) myelomas. Elution of the relevant peptide spot from the peptide maps and amino acid analysis of the peptides showed that the Gm(a) peptide and the "non-a" peptide each contained five amino acids, three of them common to both (lysine, threonine, and glutamic acid). However, the non-a peptide is characterized by a methionine and an additional glutamic acid; the Gm(a) peptide is characterized by a residue of aspartic acid and one of leucine, both missing in the Gm(a+) myelomas.

Studies were also continued on the biologic "antigenicity" of the Gm(a) factor. Infusion of large amounts (1000 ml) of Gm(a-) plasma into a hemophiliac with high titer anti-Gm(a) caused no biologic effects. Infusion of the same amount of Gm(a+) plasma into the same donor on the next day produced an immediate fall in titer (128-0 within 1/2 hr) and produced severe adverse biologic reactions consisting of generalized urticaria and pruritis, tachycardia, abdominal pain, headache, tachypnea, nausea, and

emesis. (Absence of demonstrable antibodies to red cell, platelet, and leucocyte antigens in the patient studied does indeed strongly suggest that the observed biologic effects were due to anti-Gm reactions with homologous antigen. The incidence of anti-Gm antibodies in a control hospital population with repeated multiple transfusions was 4%.

Studies dealing with genetic aberration in human γ-globulin have been proceeding well. Addition of tetanus toxoid to sensitized lymphocytes from normal subjects and patients with "secondary" acquired agammaglobulinemia resulted in an increased incorporation of tritiated uridine into RNA, and this increase was sustained for 48 to 72 hrs in vitro. In contrast, the quantity of H^3-uridine incorporated into the RNA of lymphocytes from patients with "primary" acquired agammaglobulinemia resulted in a decrease after 48 hrs of exposure to specific antigen. Lymphocytes from patients with primary agammaglobulinemia were also distinguished by their subnormal and poorly sustained response to phytohemagglutinin and to rabbit antiserum to lymphocytes. These data suggest that the defect in primary agammaglobulinemia involved, either as a primary phenomenon or as a secondary event, an abnormality of quantitative RNA synthesis or of RNA stability in the circulating lymphocyte.

The α_2M content of normal, pathological and cord sera was estimated by an antibody-agar radial diffusion procedure (employing pure α_2M standards) which gives highly reproducible results (100 $\pm$ 2.2%). A significant difference (P 0.05) was observed between the serum α_2M levels of normal Caucasian men (265 $\pm$ 55 mg/100 ml) and women (335 $\pm$ 57 mg/100 ml). The serum concentrations of α_2M was greatly increased in agammaglobulinemia, ataxia telangiectasia, and in the newborn (who had normal levels of γ-globulin but who is synthesizing very little). Values were significantly decreased in multiple myeloma and in macroglobulinemia. The data suggest that α_2M may play an important part in regulating γ-globulin synthesis. Further evaluation on the interaction of human γ-globulin molecules with complement was carried out. Human γ2a, γ2b, and γA myeloma proteins and γG, γA, and γM globulins from normal individuals and from Waldenstrom macroglobulinemia were aggregated by coupling with bis-diazotized benzidine, and both untreated and aggregated proteins were studied for complement fixing properties and skin reactivity in normal guinea pigs. Neither untreated nor aggregated γA-globulins have either activity. The other immunoglobulins, e.g., γ2a, γ2b, γ2c, and γM induced C'-fixing properties upon aggregation. The results were confirmed by C'-fixation tests. The aggregated γ2b and γ2c globulins induced increased permeability of guinea pig skin capillaries, whereas aggregated γ2a and γM globulins did not. These activities of aggregated γ-globulins were independent of types of light chains and of the presence of Gm factors (a),(b), and (f). The results indicated that the heavy chain structure which is essential for C'-fixation is different from those involved in fixation of the γ-globulin molecules to guinea pig tissues for passive sensitization.

PLANS FOR FUTURE

Continuation of broad studies of the biochemical basis of genetic differences in γ-globulins and antibodies from one individual to another, investigation of all facets of γ-globulin, namely, chemical structure, genetically determined antigenic properties, regulation of quantitative aspects of its synthesis and its aberrations in various disease states, and conditions characterized by abnormal γ-globulin and abnormal antibodies. We also plan to continue to utilize the fingerprinting technique

to explain the serologic heterogeneity of the group of Gm(b) factors and to study differences in the primary structure of the γ-globulins of Caucasians and non-Caucasians. We hope further to document the biologic implication of Gm-anti-Gm interaction by further studies of these genetic factors in families of infants with "transitional" hypogammaglobulinemia, and to document the biologic role of Gm-anti-Gm interaction in producing plasma transfusion reactions. We will continue our attempts to produce precipitating reagents for Gm typing by immunization of primates, thus circumventing the laborious inhibition of agglutination method in current use for typing γ-globulins for their various genetic factors. Further family studies in patients with various diseases of immunologic aberration and their first degree relatives are being continued.

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THE THYMIC INFLUENCE ON THE IMMUNE RESPONSE

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

OBJECTIVES

The objective is to determine the relative contribution of thymocytes, thymic humoral factor and thymus subcellular particles to the initiation and restoration of immune competence. (a) Is subcellular material derived from thymic lymphocytes able and necessary to reconstitute the immunologic reponse in the neonatally thymectomized mouse? (b) What is the immunologic competence and what is the anatomical status of lymphoid organs of animals thymectomized in utero at different times before birth? (c) What are the effects of various experimental replacements in animals thymectomized before or after birth?

ABSTRACT

At present, our first aim is to develop a refined quantitative assay system for thymic function. Our preliminary results in developing such an assay are promising but too premature to be detailed here after only four months of work.

A surgical technique has been developed in which the thymus of an intra-uterine rabbit fetus in the 23-24th day of gestation is removed. The fetus is returned to the uterus where it continues intra-uterine life until normal birth takes place at the 30-31st day. Although the thymus is lymphoid at day 24, there are no recognizable lymphoid elements in the peripheral tissues. With more experience we hope to be able to remove the thymus before it becomes lymphoid. At present, too few animals have been carried through this procedure for any reportable results.

T O X I C O L O G Y

New research capabilities are opening avenues of investigation of toxicological problems with specific naval interest. In the following group of studies, some are dictated by the stated needs of other agencies; others reflect the need for more studies on the toxicological effects of materials under the special circumstances created by present and future naval technology. Of particular interest to us at this time are the biochemical aspects of subclinical, chronic toxic actions and means of recognizing and evaluating the consequences of such exposure. The peculiar results of factors such as pressure, exotic atmospheres and absorption through the skin which may be expected to operate on an increasing number of naval personnel must also be evaluated in order to establish the proper methods of treatment and protection as the navy moves into more intimate association with the sea.

ADVISORY CENTER ON TOXICOLOGY

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

ASSISTED BY

Director and Staff

TASK NO. NR 303-366 CONTRACT Nonr 2300(29)

OBJECTIVES

(a) To provide information and toxicological evaluations on problems as requested by the sponsors: Army, Navy, Air Force, Atomic Energy Commission, National Aeronautics and Space Administration, U.S. Coast Guard, and Air Pollution Division of the U.S. Public Health Service; (b) to compile and code for retrieval published and unpublished toxicity data as a basis for replies by the staff to questions and in support of the activities of the Committee on Toxicology; (c) to assist in the dissemination and coordination of information on toxicology research projects.

ABSTRACT

(a) The number of requests for toxicity reports has continued to increase this year. In addition to problems involving air contaminants in confined spaces there have been many questions concerning the ingestion of materials as water contaminants. Replies to these inquiries have been submitted to the requesting organizations. Those problems involving acceptable concentrations for human exposures or emergency exposure limits have been referred to the Committee on Toxicology along with supporting data from the Center; (b) the compilation of toxicity data is being continued and expanded; (c) the efforts to keep track of toxicological research projects being conducted in federal laboratories have met with some success and will be continued. Personal contact with the investigators appears to be an effective procedure in this regard.

PLANS FOR FUTURE

The Advisory Center on Toxicology intends to (a) collect, interpret, and maintain a comprehensive file of toxicological data from published and unpublished sources; (b) to provide information and advice on problems involving toxicology which are referred to the Center by the Sponsoring Agencies; (c) to assist in the coordination of research projects in the field of toxicology; (d) to provide assistance to the NAS-NRC Committee on Toxicology in connection with their recommendations for exposure levels and policy matters.

ADVISORY CENTER ON TOXICOLOGY

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

ASSISTED BY

Director and Staff

TASK NO. NR 303-450 **CONTRACT** Nonr-2300 (15)

OBJECTIVES

To evaluate the toxicity of materials in submarine atmospheres.

ABSTRACT

Numerous materials have been evaluated by the Center for their potential health hazard when used aboard ships or in submarines. The Committee on Toxicology was assisted in its recent review and revision of this list of recommended concentrations of air contaminants for nuclear submarines.

PLANS FOR FUTURE

To continue to assist the Naval Ship Engineering Center by evaluating materials of construction and operation.

DETECTION OF TOXIC AGENTS IN THE ATMOSPHERE BY NEGATIVE ION ANALYSIS

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ASSISTED BY Robert C. Kindel and Roger F. Wernlund

WORK UNIT NO. NR 303-710 **CONTRACT** Nonr-4977(00)

OBJECTIVES

(a) To design and construct pulsed ion drift chamber, and associated electronics, to measure and analyze negative ion currents in air resulting from objectionable contaminants, (b) to test the feasibility of the technique for detecting certain candidate contaminants, including Sarin, and (c) to improve the sensitivity of the instrument by employing more sophisticated electronic signal detection equipment.

ABSTRACT

As mentioned in the abstract for 1965, we had developed and successfully calibrated an instrument which should provide a novel method for detecting the presence of certain contaminants at low concentrations in atmospheric air. Shortly thereafter, two candidate chemicals (dimethyl hydrogen phosphite, and trimethyl phosphite) were suggested by NRL as an aid in ascertaining the feasibility of the method. Both could be detected at concentrations of less than 1 part in 10^6.

Mr. George Fielding of NRL personally transported a sample of highly diluted Sarin to our facilities for examination. Within two days, adaptors had been installed to allow a continuous flow of Sarin mixed with air at 1 part in 10^6 into our drift chamber. A negative ion peak was clearly observable when Sarin was added at the above concentration. To avoid an error due to the presence of O_2 in air, the same test was made using pure N_2 as the diluting medium. The results were essentially the same.

Following acceptance of the above evidence, Phase II of the program was initiated. This requires that feasibility be established for reducing the equipment to a portable instrument. Thus far, we have successfully employed electronic circuits to produce a repetitive pulse of ultraviolet light for generating attaching electrons in the drift chamber. Synchronous integration techniques, synchronized to the ultraviolet pulse are now being used to increase the signal-to-noise ratio. Satisfactory completion of this effort should result in a significant increase in the detection sensitivity of the instrument.

PLANS FOR FUTURE

(a) To verify sensitivity improvement achieved by electronic pulsing and synchronous detection techniques, (b) to test equipment with candidate contaminants examined previously, and (c) to prepare the design of a portable instrument based on the above information.

CURRENT REPORTS AND PUBLICATIONS

Robert W. Crowe, "Investigation of Electron Attachment Characteristics of Specific Candidate Materials in Atmospheric Air," Phase I Technical Summary Report - Contract Nonr-4977(00), August 15, 1966.

STUDIES RELATED TO AN EARLY DETECTION OF TOXIC EFFECTS OF ORGANIC COMPOUNDS

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ASSISTED BY Hasmukh Shah and Susan Minor

WORK UNIT NO. NR 303-723 **CONTRACT** N00014-66-C0006

OBJECTIVES

To investigate some biochemical or pharmacological effects which are produced by the chronic inhalation of certain organic chemical compounds prior to the occurence of any gross pathological damage. Methylchloroform was selected for the initial study because of its use as a solvent and degreaser in the Naval instillations.

ABSTRACT

Mice and rats of specified strain were exposed to methylchloroform air mixtures in a continuous flow inhalation chamber. After the exposure for various lengths of time, they were placed in their home cages for a specified period before testing. The exposed as well as the control animals were divided into two groups. One group was tested for the loss of the righting reflex after an injection of hexobarbital. The other group was sacrificed by decapitation to dissect out the livers for biochemical investigation. The supernatant fraction containing microsomes and soluble constituents of the livers was incubated with the appropriate substrates to measure its enzymatic activity in vitro.

Continuous inhalation of methylchloroform for 24 hr. in mice or 36 hr. in rats reduced the duration of barbiturate hypnosis significantly (38-64% reduction in 24 hr. exposed mice, $P < 0.005$). Discontinuous exposure (4 hr. daily) for the same number of total hours, however, was ineffective. Both male and female animals were susceptible. The effect was optimum at 24 hr. but could still be detected even at 48 hr. after the exposure was discontinued. The effect was always reproducible when 2000 ppm of methylchloroform were used; lower concentrations produced variable results. The exposure in the same chamber to air not containing methylchloroform was ineffective. Post exposure study of the body weight and organ pathology did not reveal any difference from the controls.

In a search for a biochemical basis of the methylchloroform-induced changes in the pharmacological response of the animals, drug metabolizing ability of their microsomal enzymes was measured. Oxidation of hexobarbital, N-demethylation of aminopyrine and reduction of p-nitrobenzoic acid by the microsomal preparations from the methylchloroform-exposed animals did not differ from those of the controls.

From the studies, it was concluded that the reduction of barbiturate hypnosis in the methylchloroform exposed animals was not due to the altered ability of their microsomal enzymes to metabolize drugs.

PLANS FOR FUTURE

The future experiments are planned to study (1) the effect of discontinuous exposures to methylchloroform on barbiturate hypnosis, (2) sensitivity of methychloroform exposed animals to drugs other than barbiturates, (3) effect of methylchloroform inhalation on the convulsive threshold, and (4) biochemical mechanisms other than microsomal involvement to account for the altered pharmacological responses of the exposed animals.

CURRENT REPORTS AND PUBLICATIONS

H. Lal and H. Shah (1967), "Effects of prolonged methylchloroform inhalation on the drug induced CNS activity." Paper presented at the scientific meeting of the Society of Toxicology, 1967. Abstract in Toxicol. Appl. Pharmacol., (in press).

ENVIRONMENTAL EFFECTS OF TERMITE CONTROL INSECTICIDES

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Hazleton Laboratories, Inc.
Falls Church, Virginia

ASSISTED BY H. J. Paulin

WORK UNIT NO. NR 303-729 **CONTRACT** N00014-67-C-0224

OBJECTIVES

To determine the residues of certain termite control insecticides in the environment of a dependent's housing development at a naval installation.

ABSTRACT

This project is an expansion of an earlier program in which relatively limited sampling under winter conditions was conducted. The current program was initiated to obtain samples of the environment during a warm season, so that an increased array of flora, fauna, soils, and water can be collected and analyzed for insecticide residues.

PLANS FOR FUTURE

Insecticide residue analyses, now in progress, will be completed and results will be presented in a Phase II final report.

CURRENT REPORTS AND PUBLICATIONS

J. M. Barnes and D. G. Shaheen (1966), "A study of the environmental effects of termite control insecticides." Final Report, Phase I, ONR Contract No. Nonr-5156(00), 36 pages.

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 program was commenced in August 1966 and much time has been spent in the "tooling up" phase as well as developing methodology. Initial investigative efforts have been in the direction of the effects of helium and helium/oxygen mixtures at ambient pressure. In this regard, the effects of the gas mixtures on microsomal drug metabolizing systems has been screened using hexobarbital sleeping time in mice as the indicator. Preliminary results have indicated that there is no significant difference in the sleeping time when compared in atmospheres of air, 100% oxygen, helium/oxygen, 79%/21% and helium/oxygen, 70%/30%. These results must be substantiated by further experimentation using the above technique and using in vitro microsomal enzyme systems.

The effects of helium on lysosomal membrane stability are also being studied. Hepatic lysosomes of the rat are studied using a centrifugal fraction which comes down between 650-g x 20 minutes and 15,000-g x 20 minutes from 0.25 M sucrose. This fraction was incubated at 37° in unbuffered sucrose in atmospheres of air or 100% helium at ambient pressure. There was no statistically significant difference between the two atmospheres when the kinetics of acid phosphatase release were studied between 30 and 180 minutes. This was also true when total accessibility of acid phosphatase was studied. These results were interesting in view of the fact that, in vivo, hypoxia results in increased lability of lysosomes.

PLANS FOR FUTURE

(a) Effects of pressure on microsomal enzyme systems (b) the absorption, distribution and biological half-life of isotopically labeled drugs (c) effects of steroids on stability of lysosomal membranes under pressure (d) effects of anti-inflammatory and antihistaminic agents on capillary permeability under pressure.

ACUTE INTRAVENOUS STUDY OF OTTO FUEL II IN RHESUS MONKEYS

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ASSISTED BY D. Butts and K. Woolbert

WORK UNIT NO. NR 303-787 **CONTRACT** N00014-67-C-0306

OBJECTIVES

To study the effects on visually discriminated avoidance behavior in rhesus monkeys produced by various intravenous dosage levels of OTTO Fuel II.

ABSTRACT

Two or four rhesus monkeys per dosage level, previously trained in a visual discrimination under shock avoidance contingencies, were tested for from two to four hours postinjection of OTTO Fuel II as well as for one hour preinjection and, on the previous day, for four hours following equal volumes of normal saline administered intravenously. Observations of pharmacotoxic signs were made on these monkeys while restrained in plastic primate chairs and on two other monkeys while in their cages at each of the three highest dosage levels tested and at 0.41 ml/kg.

The results with OTTO Fuel II (behavioral testing) were as follows:

Dosage Level ml/kg	N	Effect
0.004	4	3-No effect; 1 slight effect (6 missed avoidance responses out of 100-trials)
0.007	4	4-No effect
0.041	2	2-No effect
0.13	2	2-Marked effect followed by complete incapacitation within four hours
0.153	2	2-Complete incapacitation within two hours (one death)

Complete incapacitation was indicated by five successive failures to terminate the five second escape shocks.

The four animals at the two highest levels were treated therapeutically with 0.5 ml/kg of a 4% solution of methylene blue soon after complete incapacitation was observed. Three animals recovered, one died immediately thereafter.

Two caged monkeys were treated with the antidote after 0.153 ml/kg of OTTO Fuel II had produced prostration at four hours, and both recovered. Two animals given 0.41 ml/kg of the OTTO Fuel died, one within eight minutes, the other after 2-3/4 hours. Neither were given the antidote.

In general, the signs produced by OTTO Fuel II were dose related and followed the pattern of nitrate poisoning: Mydriasis accompanied by occasional momentary miosis, eyelid ptosis, decreased motor activity, ataxia, decreased sensitivity to touch and pain stimuli, depressed pupillary light reflex, appearance of blindness, prostration, vasodilation, salivation, protruding tongue, irregular breathing, retching, and occasional tremors and sub-convulsive movements. The signs seen at 0.041 ml/kg were mydriasis, retching, decreased activity, and vasoconstriction lasting less than one hour; all the signs listed were seen at 0.153 ml/kg in at least one monkey. On the basis of the degree of effects observed at this dose, including one death out of four monkeys tested, it is estimated that the LD_{50} is approximately 0.15 ml/kg; however, since the antidote was successfully administered to three of these four monkeys, it is possible that the LD_{50} is somewhat lower than this.

PLANS FOR FUTURE

To determine the effects of a 90-day continuous exposure to vaporized OTTO Fuel II (at a concentration to be selected) on the visual discrimination performance of rhesus monkeys.

T O X I N O L O G Y

The distinction between toxicology and toxinology is of fairly recent origin, and is a very logical one. What has been said about Marine Biochemistry becomes of critical importance when the materials elaborated by the normal living elements in the sea are harmful or even deadly. A study of the toxins produced by a wide variety of organisms, from microbes to mammals, may range from the applied research likely to be needed for survival manuals to the very basic studies which may be the soil in which new ideas for medication and for the understanding of physiological processes may develop.

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

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ASSISTED BY Joseph T. Tomita, E. Sanz, Denis J. Prager, & Bruce Reitz

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

OBJECTIVES

(a) To characterize the peripherally active principle in tetanus toxin, (b) to continue studies on its mode of action, and (c) to continue studies on the properties and mode of action of other "releasers": antigen-antibody complexes, sea urchin toxin, streptolysin. (d) To work on sensitization with anti-ragweed and with certain modified protein antigens.

ABSTRACT

TOXINS. Tetanus toxin: Previous work had shown that treatment of crude tetanus toxin by combined ammonium sulfate fractionation and cerebroside-ganglioside adsorption sequences resulted in a product which had little lethal toxicity and a great deal of peripheral electrophysiological potency. Concurrent immunochemical and ultracentrifugal characterization showed that this preparation had a Svedberg coefficient of 4S and that it gave 7 lines in immunoelectrophoresis as compared with over 20 lines in the starting material. In order to show that lethal activity in turn could be concentrated independently of Mepp activity, a second set of fractionations was made by repeated precipitation of the parent material with methanol at low temperatures. That product had over 6 times the lethal potency of the crude toxin but nearly 100,000 times less Mepp potency. Immunochemical and ultracentrifugal analysis showed that this preparation consisted of entirely 7S molecules and that it gave only a single line in immunoelectrophoresis and therefore that it was a single antigen. This demonstrated that Mepp activity and lethal activity were owed to different molecules which could be separated by selective fractionation procedures. Isolation of the Mepp activity as a single antigenic determinant was effected by chromatography of the most potent Mepp preparation on Sephadex G-200. Bioassay of this component showed that it possessed only Mepp stimulating activity but was entirely lacking in lethal activity.

Streptolysin: Purified preparation of groups A and C streptolysin O in the reduced state cause changes in the mechanical and electrical behavior of the isolated atria of the guinea pig. The active agent has been identified as streptolysin O by experiments which show the material to be heat denaturable, blocked by anti-streptolysin O and by cholesterol, and inactivated by oxidation and treatment with acid. Groups A and C appear to have equivalent cardiotoxic effects as related to their hemolytic potencies. In the spontaneously beating, isolated atria, challenge with streptolysin O is followed by a decrease in rate and strength of contraction as well as an increase in the rate of repolarization of the atrial action potential. The degree of response and the lag time is dependent upon the dose

of toxin. Electrophysiological, pharmacological, and kinetic evidence suggest that these changes are the effect of release of acetylcholine from the atria after challenge with streptolysin O.

Sea Urchin Toxin: Studies on the pharmacological effects of sea urchin toxin show that the toxicity to isolated tissues is owed to the release of histamine (identified chemically as such) and also to the formation of certain non-histamine-like materials, similar to bradykinin. Enzymological experiments show that the incubation of plasma with toxin produces a dialyzable material which chemically and pharmacologically is distinct from histamine. substrates--A study of various substrates indicated that γ-globulin, and serum albumin were ineffective but that Cohn fractions III-O and IV-I, which contain a high proportion of α_2-globulins were effective in the reaction. enzymes--Previous pharmacological studies showed that the fraction precipitated with 65% saturated ammonium sulfate was the most potent. This was also found to be the case in experiments with substrates.

ANAPHYLAXIS. Ragweed: Immediate in vitro sensitization was achieved by perfusing isolated guinea pig hearts and incubating guinea pig guts with rabbit anti-ragweed. Tests with the dialyzable product of the hydrolyzate of the ragweed antigen showed that it could produce an inhibition of the ragweed-anti-ragweed anaphylactic response as a function of the concentration of the inhibitor present before the sensitized tissue was challenged with the whole antigen. Thus far it has not proved possible to sensitize guinea pig tissue with anti-ragweed of human origin.

Cardiac Anaphylaxis: Present studies deal with the kinetics of anaphylactic histamine release from guinea pig atria rendered sensitive to ovalbumin by active immunization, passive in vivo transfer, and passive in vitro sensitization with specific rabbit antibody. Experiments with tissues from both actively and passively sensitized animals indicate that the effective temperature range for the anaphylactic release of histamine is from 24 to 44°C with an optimum temperature of 39.6°C. The time-course of histamine release was determined for actively sensitized atria at 27 and 31°C. In both cases, histamine output reached a maximum value 1.5 minutes after challenge and was 90% complete within 4 minutes. Total output at the higher temperature was about four times greater than at the lower one. Estimations of total tissue histamine content reveal that the right atrium contains 40% more histamine per gram of dry tissue than does the left atrium.

PLANS FOR FUTURE

Toxins: (a) To produce sufficient quantities of the purified Mepp-stimulating factor for detailed physical-chemical characterization. (b) To continue isolation of sea urchin toxin by column chromatography. (c) To continue work on the mode of action of streptolysin by pharmacological and active transport experiments. (d) To identify reaction products of toxin-substrate reactions.

In Vitro Sensitization: (a) To study the inhibitors of sensitization. (b) To study conditions affecting the life span of the enzyme responsible for histamine release. (c) To study sensitization and anaphylaxis with chemically modified protein antigens.

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BIOPHYSICAL AND BIOCHEMICAL STUDIES ON ELECTRICAL ORGANS AND MUSCLE MEMBRANES

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ASSISTED BY

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

OBJECTIVES

(a) To purify the toxins from uncellular red tide organisms, (b) to study their mode of action on cellular tissue, (c) to elucidate composition and metabolic origins of these compounds.

ABSTRACT

Studies have been made on the toxins from three organisms, all of which bloom to produce high mortalities of fish. The main studies during this year have been made on the Chrysomonad Prymnesum parvum, which is a small flagellate. It lives over a range of salinities from fresh up to sea water but is toxic only at the lower salinity ranges. The toxins studied are intracellular and are isolated by spinning cells off from a culture, lyophilising and purifying the precipitate.

The toxins have both hemolytic and neurotoxic actions and our efforts have been directed towards separating the two components and showing that these actions are due to separate substances. A variety of techniques have been used, the most successful being the use of Sephadex columns and paper chromatography. The test for hemolysis has been rabbit red blood cells, while for neurotoxic action two tests have been employed. One is the death time for small fish and the other is the block of the frog rectus abdominus muscle. A contracture is induced by the toxin, and after this effect has been washed away, the acetyl-choline response remains inhibited. It has been shown with a Sephadex column that all hemolytic activity is eluted early in the sequence whereas no significant neurotoxic activity appears until much later. Further studies on the hemolytic fractions have failed to demonstrate more than one component separable on a paper chromatograph with a solvent mixture: The spot shows UV fluorescence and a positive ninhydrin reaction.

Both factors, hemolytic and neurotoxic are inactivated by light, and attempts are continuing to characterize these properties. Measurements at various temperatures and in the presence of nitrogen or oxygen indicate that heat inactivation of the hemolytic factor in solution is significant at room temperature, but that the neurotoxic factor is more stable. There is no marked peak of absorption in the visible range although UV absorption shows several peaks. No reasonable explanation of the inactivation by visible light has been obtained.

The action of the neurotoxic factor on muscles has been further

studied. Its action on the frog rectus is two fold. The first action is a contracture and our results throw doubt on earlier studies on this toxin. Our results show that the toxin has a double effect on many muscles but that the two actions are not similar. In the frog heart, the plateau is eliminated before the action potential is blocked and it can be inhibited by atropine. In intestinal muscle, the early contracture also can be blocked by atropine but the block to acetylcholine can not be reduced. A contracture also develops in the frog rectus muscle but we have been unable to interfere with this effect except by repeated washes.

Experiments on the toxin from the Red Tide organism, Gymnodinium breve and on the Lyophylised extracts from a blue-green alga which caused mass mortality of fishes in areas of New Hampshire this summer, have been directed to locating the site of action of the toxins. The evidence accumulated suggests that the dinoflagelate toxin acts by depolarizing the endplate region. When appliesd to a vertebrate nerve muscle preparation, intense fibrillation occured and miniature endplate potentials increased at first and then disappeared. Nerve-muscle block occured well before blockage of either nerve or muscle. The most dramatic result was on the small fibers in the cockroach abdominal nerve cord. Immediately on application of the toxin the small fibers discharged about 200 impulses per second continuously although discharges of the large fibers were unaffected. The preliminary measurements on the blue-green alga show that this toxin is very potent and blocks nerve and muscle conduction rapidly without depolarization.

Studies begun under this contract on the biophysical properties of excitable membranes have continued without financial support. Measurement of the heat production during action potentials in the rabbit vagus nerve at low temperatures have shown that heat is liberated on the depolarizing phase of the action potential and that heat is reabsorbed during repolarization. Some further studies are being carried out on this nerve, but it appears that much more information will be obtained from the olfactory nerve of the gar fish. Gasser showed that this nerve is very suitable for heat measurements. The action potential lasts nearly 30 msec at 20° C and propagation is slow. A suitable thermopile has been constructed for heat measurements and experiments are in progress.

PLANS FOR FUTURE

The toxins from three toxic organisms are grown in pure culture and are harvested at suitable periods of growth. Studies will be made of their mode of action on excitable membranes using especially the sucrose gap method for voltage clamping. Concurrent studies will be made on hemolytic actions of the toxins and separation studies will be carried out.

CURRENT REPORTS AND PUBLICATIONS

In preparation:

1) The mode of action of the toxin from Gymnodinum breve

2) Light inactivation of the toxic fractions in Prymnesium parvum.

BIOCHEMISTRY OF PHYSIOLOGICALLY ACTIVE PRINCIPLES IN ECHINODERMS

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

OBJECTIVES

(a) To elucidate the chemical structures of the components of holothurin, (b) to correlate its various pharmacological and surfactant properties with the particular chemical and configurational features of the molecule.

ABSTRACT

Holothurin, a family of physiologically active saponins, isolated from the sea-cucumber Actinopyga agassizi, are triterpenoid steroids combined with a carbohydrate moiety of 2-4 sugars and generally exist as sulfate esters. Four groups A, B, C, D (chromatographically homogeneous) have been separated, analyzed chemically, and examined for their pharmacological and physiological effects. The groups are distinguished from each other by differences in the number and nature of their sugar components, and in the presence or absence of a sulfate moiety. The individual members within the group differ with respect to their steroid components.

Holothurin A, the principal component (65-75 %), $C_{54}H_{84}O_{27}SNa$ (av.emp. form.), on strong acid hydrolysis yields one mol. of sulfuric acid, 4 monosaccharides, xylose, glucose, 3-O-methylglucose, and quinovose, and a water-insoluble mixture of holothurinogenins (described below). Enzymatic hydrolysis yielded 3-O-methylglucosylglucose and quinovosyl-3-O-methylglucose (?). Periodate oxidation resulted in the destruction of quinovose only. These results established the sequence (starting with glucose attached to the steroid) glucose-3-O-methylglucose, and possibly quinovose, as the last member of the chain. Mild non-aqueous acid hydrolysis gave one mole of sulfuric acid and a glycoside with the 4 sugars. The latter oxidized with periodic acid and then hydrolyzed gave neither xylose nor quinovose. The sulfate moiety is therefore attached to the xylose.

Holothurin D (15 %) contains no sulfate and gives on acid hydrolysis the same 4 sugars and holothurinogenin mixture as holothurin A. Holothurin D is desulfated holothurin A.

Holothurin B (5-10 %) contains xylose, quinovose, and sulfuric acid. Its aglycones correspond to those from holothurin A, but differ in their relative proportions.

Holothurin C (5 %) contains xylose, quinovose, and glucose.

Two of the principal genins, holothurinogenins I and II, were separated and their structures elucidated completely. Holothurinogenin I, $C_{30}H_{44}O_5$, is the tetracyclic triterpene 3β,17α,20ξ-trihydroxy-22,25-oxido-5α-lanosta-7:8, 9:11-diene-18-carboxylic acid lactone (18-20). Holothurinogenin II is the 17-deoxy derivative of holothurinogenin I. Of the remaining mixture of holothurinogenins (ca.6) at least one-half were shown to contain the same annular portion as holothurinogenin I, but differed as to the nature of their side-chains.

Holothurin A, B, C, and D do not contain the conjugated diene system, all holothurinogenins do. Mild non-aqueous acid hydrolysis of holothurin A cleaved the sugars and sulfate moiety and furnished the unconjugated aglycone precursors of the holothurinogenins. A comparison by N.M.R. and I.R. spectra of holothurin A, the holothurinogenins and the latter's immediate precursors suggests that significant modifications of the aglycone moiety of intact holothurin ensue after strong acid hydrolysis.

The neurotoxicity of holothurin was studied in collaboration with Dr. S.L. Friess of the National Naval Medical Center, Bethesda, Md. Certain of its features are unique: the action resembles that of cholinesterase, but is in contrast irreversible and an all-or-none effect. Its irreversible inhibition of twitch response may be largely negated by preincubation with small concentrations of representative anticholinesterases. Comparative experiments employing the purified subfractions holothurin A, B, C, D, and desulfated holothurin have pointed up the stringent structural requirements of synaptic receptors, susceptible to holothurin attack. The hemolytic action of holothurin is 20-25 times stronger than that of the strongest hemolytic saponin of plant origin. Holothurin kills a fifty pound shark in dilution of 1:500,000 within ca. one hour.

PLANS FOR FUTURE

To extend our biochemical studies to related, uncharacterised, physiologically active principles in echinoderms.

CURRENT REPORTS AND PUBLICATIONS

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AFLATOXINS IN MAMMALIAN CELL SYSTEMS

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WORK UNIT NO. NR 305-779 **CONTRACT** N00014-67-C-0252

OBJECTIVES

The proposed investigation aims at obtaining information on the influence of aflatoxins on:

1. The RNA and protein synthesis in different cellular organelles and on the time-course of DNA, RNA and protein synthesis, in a few selected mammalian cells differing in their amino acid metabolic patterns;

2. The three RNA components, (28S, 18S and 4-6S).

ABSTRACT

Aflatoxins in Mammalian Cell Systems

Aflatoxins are metabolites of some strains of Aspergillus flavus. They comprise a group of structurally related coumarin derivatives which have been discovered as contaminants of certain lots of animal feed ingredients by virtue of their hepatoxic properties. Aspergillus flavus commonly invades grains and seeds, stored with moisture contents in equilibrium with relative humidities of 85-89% producing compounds toxic to animals. Not only might aflatoxins cause great loss among domestic animals and marine animals but they might present a potential public health hazard which might arise from contamination of the food supply. Furthermore, aflatoxins have exhibited potential carcinogenic properties in rats and in rainbow trouts. Although some of the biochemical alterations caused by aflatoxins have been studied in a number of biological systems, comparatively little is known about the primary biological effects of aflatoxins. The biological effects on cell growth, cell morphology, mitotic division and DNA synthesis in cultured human embryonic lung cells have been reviewed recently. Within a few hours DNA synthesis and mitosis were suppressed. In addition, it has been found that low concentrations of various groundnut samples destroyed cultured calf-kidney cells and that the toxicant inhibited the incorporation of C-14 leucine into protein in various liver preparations. The interpretation of these biological effects is limited and therefore it is proposed in this study to investigate the biological effects of aflatoxin on sub-cellular metabolism in mammalian cells on established cell lines. A recent study of the amino acid metabolism of sixteen mammalian cells of specific genotype in our laboratories has resulted in the finding that the level of C-14 (from glucose) incorporation into amino acids by these cells differ from cell strain to cell strain. These cells, therefore, would provide varied in vitro biological systems to elaborate on the sequence of events resulting from the presence of aflatoxins.

These studies would provide important implications for prevention or at least moderation of the action of aflatoxin.

PLANS FOR FUTURE

To obtain information on the influence of aflatoxins on (1) the RNA and protein synthesis in different cellular organelles and on the time-course of DNA, RNA and protein synthesis, in a few selected mammalian cells differing in their amino acid metabolic patterns, and (2) the three RNA components (28S, 18S, and 4-6S).

CURRENT REPORTS AND PUBLICATIONS

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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-0220-0001

OBJECTIVES

(a) To isolate the toxic principles of sea snake venom, (b) to determine whether toxic principles are enzymatic or non-enzymatic, (c) to determine chemical properties of toxic principles, and (d) to neutralize toxicity with chemical compounds.

ABSTRACT

Venoms of sea snakes, Laticauda colubrina and L. semifasciata, collected in Formosa, were tested for their possible proteolytic activities. In contrast to venoms of land snakes, Crotalidae and Viperidae, sea snake (Hydrophiidae) venoms did not hydrolyze the synthetic substrates, p-toluenesulfonyl-L-arginine methyl ester and N-beuzoyl-L-arginine ethyl ester. This was quite similar to Elapidae venoms which also did not hydrolyze these substrates. Specific substrates for chymotrypsin such as N-acetyl-L-tyrosine ethyl ester and N-benzoyl-L-tyrosine ethyl ester were not hydrolyzed by venoms of any terrestrial snakes or by sea snake venoms. The venom of L. colubrina coagulated fibrinogen to fibrin-like material; however, L. semifasciata venom had fibrinolytic action

PLANS FOR FUTURE

Sea snake venoms will be collected in Southeast Asia and the Far East and the chemical properties associated with the toxic principles will be investigated.

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