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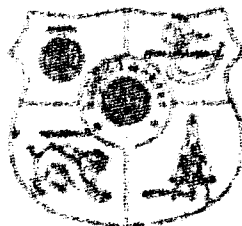
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PROGRESS REPORT ABSTRACTS



BIOCHEMISTRY BRANCH

Office of Naval Research



JANUARY 1968

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FOREWORD

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

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

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

Robert K. Jennings

ROBERT K. JENNINGS, Ph.D.
Head, Biochemistry Branch
Office of Naval Research

CONTENTS

INTRODUCTION	1
MARINE BIOCHEMISTRY: EXPLANATION	3
STUDIES OF PROTEOLYTIC ENZYMES	4
H. Neurath	
EXPERIMENTAL STUDIES ON THE BIOCHEMISTRY AND BIOPHYSICS OF ENZYME MOLECULES	6
T. P. Singer	
BIOCHEMISTRY OF MARINE ORGANISMS	9
T. E. King	
FREE AMINO ACIDS AND RELATED SUBSTANCES IN MARINE ORGANISMS	10
E. Roberts	
BIOCHEMISTRY OF THE LUMINESCENCE OF MARINE ORGANISMS	12
J. W. Hastings	
INVESTIGATIONS ON THE CARBOHYDRATE BIOCHEMISTRY OF MARINE ALGAE	14
B. J. D. Meuse	
COMPARATIVE CARBOHYDRATE METABOLISM OF MARINE MOLLUSCS	16
H. I. Nakada	
LYSOSOMES AND INTRACELLULAR DIGESTION IN SEA STARS	18
G. S. Araki	
HYDROSTATIC PRESSURE EFFECTS ON MACROMOLECULES	20
R. Y. Morita	
AN INVESTIGATION OF THE NUCLEIC ACIDS IN <u>GRIFFITHSIA</u> <u>GLOBULIFERA</u>	22
M. Nasatir	
METABOLIC BIOCHEMISTRY: EXPLANATION	24
PROPERTIES OF HYDROGENASE	25
D. Rittenberg	
NUCLEIC ACIDS IN REGENERATING WOUND TISSUE	26
M. B. Williamson	
THE DEVELOPMENT OF FUNCTIONAL CELL STRAINS	27
C. Graff	
PROPERTIES OF NUCLEIC ACIDS IN RELATION TO BRAIN FUNCTION ..	29
H. R. Mahler and W. J. Moore	

STUDIES ON PHOTOSYNTHESIS AND PHOTOTROPHISM	31
H. Gaffron	
APPLICATION OF MICROWAVE ABSORPTION AT 3000 MC FOR THE DETERMINATION OF ELECTROLYTE CONTENT OF SOLUTIONS	32
A. A. Albanese	
STUDIES ON AMINE METABOLISM DURING ANAPHYLAXIS	34
O. H. Callaghan	
DRUG METABOLISM IN RADIATION CHIMERAS	36
E. M. Uyeki	
BIOCHEMISTRY AND PHARMACOLOGICAL PROPERTIES OF LEUCOGENENOL	38
F. A. H. Rice	
LOCALIZATION OF A METABOLIC BLOCK IN THE GRANULATION TISSUE AT PROGRESSIVE STAGES OF WOUND HEALING	40
W. W. Nowinski	
LUMINESCENCE OF BIOPOLYMERS AND MICROORGANISMS	41
R. F. Steiner	
IMMUNOCHEMISTRY: EXPLANATION	43
MECHANISM OF ANTIGEN-ANTIBODY REACTIONS AND SIMILAR NON- SPECIFIC REACTIONS BETWEEN PROTEINS AND OTHER MACROMOLECULES	44
F. Harewitz	
IMMUNOCHEMICAL CRITERIA OF PURITY OF PROTEINS AND POLYSACCHARIDES	46
E. A. Kabat	
NATURE OF CYTOTOXIC REACTIONS MEDIATED BY ANTIBODY AND COMPLEMENT AND RELATED PHENOMENA	48
M. M. Mayer	
MECHANISM OF ANTIBODY FORMATION	50
D. Segre	
BIOCHEMICAL AND PHYSIOLOGICAL FACTORS OF THE IMMUNE RESPONSE	52
I. L. Trapani	
STRUCTURAL AND IMMUNOCHEMICAL STUDIES ON MYOGLOBINS AND HEMOGLOBINS	54
M. Z. Atassi	
BIOCHEMICAL PROPERTIES OF ANTIBODIES TO PURINE AND PYRIMIDINE RIBONUCLEOTIDES	57
B. F. Erlanger	

COMPARATIVE STUDIES ON THE IMMUNOGLOBULINS OF MARINE FISHES	59
B. W. Papermaster	
COMPARATIVE IMMUNOCHEMISTRY	60
R. E. Feeney	
IMMUNOLOGICAL FUNCTION IN SEVERE THERMAL INJURY	62
J. M. Converse and F. T. Rapaport	
STUDIES ON THE HEREDITARY GAMMA GLOBULIN (Gm) GROUPS	64
H. H. Fudenberg	
SWITCHING MECHANISMS FOR TYROSINE METABOLISM IN DEVELOPING CELL SYSTEMS. THE EFFECT OF POLYSOMAL PROTEIN ON IMMUNOGLOBULIN SYNTHESIS	67
E. L. Triplett	
THE ROLE OF THE THYMUS IN THE IMMUNE RESPONSE	69
W. A. Kisker	
THE ANTIGENICITY OF CONJUGATED PEPTIDES	71
D. D. Watt	
IMMUNO-CHEMICAL STUDIES ON THE K ANTIGEN OF <u>VIBRIO</u> <u>PARAHAEMOLYTICUS</u>	72
G. Omori	
TOXICOLOGY: EXPLANATION	73
ADVISORY CENTER ON TOXICOLOGY	74
National Academy of Sciences	
ADVISORY CENTER ON TOXICOLOGY	75
National Academy of Sciences	
CELLULAR ACTION OF PHARMACODYNAMIC AGENTS UNDER HYPERBARIC CONDITIONS	76
S. Schwartz	
OXYGEN METABOLISM: METABOLIC IMPLICATIONS IN HYPERBARIC SYSTEMS	78
A. H. Soloway and R. L. Stern	
EARLY DETECTION OF TOXIC EFFECTS OF ORGANIC CHEMICALS	80
H. Lal and G. Fuller	
TOXINOLOGY: EXPLANATION	82
A STUDY OF IMMEDIATE SENSITIZATION OF TISSUES BY ANTIGENS AND ANTIBODIES, <u>IN VITRO</u>	83
G. A. Feigen	

BIOPHYSICAL AND BIOCHEMICAL STUDIES OF MARINE TOXINS	86
B. C. Abbott	
BIOCHEMISTRY OF PHYSIOLOGICALLY ACTIVE PRINCIPLES IN ECHINODERMS	88
J. D. Chanley	
STUDIES ON THE COAGULANT ENZYMES OF FRESH SNAKE VENOMS	90
A. L. Copley and B. W. Luchini	
AFLATOXINS IN MAMMALIAN CELL SYSTEMS	92
R. A. Chang	
TOXIC PRINCIPLES OF SEA SNAKE VENOMS FROM SOUTHEAST ASIA AND THE FAR EAST	94
A. T. Tu	
STINGRAY VENOM	96
F. E. Russell	
SCULPIN TOXIN	97
F. E. Russell	
THE CHEMISTRY OF COELENTERATE TOXINS AND THEIR MODE OF ACTION	98
B. I. Shapiro	
AUTHOR INDEX	99
INDEX OF CONTRACTOR INSTITUTIONS	100

INTRODUCTION

The abstract reports in this volume show what research was carried out with support from the Biochemistry Branch of the Office of Naval Research in the latter half of Fiscal Year 1967 and the first half of Fiscal Year 1968. They do not show why these particular tasks were chosen for support, what the Biochemistry Branch wished to accomplish, nor yet what it is likely to sponsor in the future. The abstracts are the statements of scientists reviewing their recent accomplishments as scientists and not as naval personnel.

Typically, the scientist wishes to know what lies in the uncharted seas to the West. If he finds that out, he has done well, even if it proves not to be a new trade route to India. Nor has he failed if the true value of his discovery is realized only after additional years of exploration and use. Had Columbus been successful, his research would have made a worthwhile saving to shipping costs. What he did produce, in his failure, was of far greater value, and in contexts not imagined when the research project was planned. Isabella's jewels were not wasted, although a quid pro quo accounting might have made it seem otherwise.

The reader who asks, "How will knowledge of the molecular structure of such and such an enzyme help the Navy?", will not find the answer here. The question itself implies thinking in terms of a search for preconceived values--a trade-route to India, whereas ONR alone in the Navy structure is charged with exploration of the unknown. But the question must be asked. Such information is essential to the operation of the valves controlling wise and equitable distribution of government support for research.

The true answer is that such knowledge is the first step toward control of life processes, toward anticipation of their possible impact on naval operations and toward the possible imitation of the principles in accomplishing desirable aims. It may provide essential information needed to establish a new vaccine, and a new method of breathing under water, and a new method of obtaining food or power; but if any or all of these technological advances develop, it is unlikely to be identifiable with any one of them. Yet it is all the more valuable because of its potential for use in many contexts.

Inevitably, such contributions will serve other mission than those peculiar to the Navy. Why not let one of the other potential beneficiaries foot the bill?

Readers who have access to the Abstract Reports of previous years will find that there is indeed a trend toward less support of work which promise knowledge of very wide, general utility to the state of the art, in favor of more research which may be expected to prove especially valuable in a particular field. We seek to identify unexplored areas of science ripe for biochemical exploration. We then seek to assess the probable naval utility of information likely to be found in such areas.

The final step is to choose the best available scientific talent for un-directed research to find out what does indeed lie to the West. If it can be foreseen that this is likely to be a trade route, that is all the more reason for its support. But it must be our responsibility and not that of the authors of these abstracts to determine the naval utility of what they do find. Therefore, this volume is not suited for the purpose of forming a judgment as to the return to the Navy on the investment in Biochemistry.

STUDIES OF MARINE BIOCHEMISTRY

Some factors typical of the marine environment which may constitute barriers to the use of the waters by man can be essential to the life processes of the organisms normally found in the sea. Pressure (Morita), oxygen availability (Singer), pH and ionic strength (Neurath), and temperature are among these factors. Perhaps knowledge of how the metabolism of the native cells differs from those of land-based animals may suggest ways to overcome our limitations or to anticipate limitations not now suspected.

The wide variety of life forms to be found in the sea offers an opportunity for comparative studies giving new insight into the functioning of cells and tissues at higher levels on the evolutionary scale. Unusual biological activities (Hastings, Roberts, Nakada) can be investigated to advantage and since the findings will be of special importance to the understanding of life in the sea, the eventual use of the knowledge is certain to assist the navy.

It was pointed out last year that the sea is a living tissue, and has a metabolism worthy of study. This may be on the level of biochemical ecology (Meeuse) or may take other forms as interest on the part of biochemists and opportunity provided by advancing naval technology permits us to increase the emphasis on this part of the Branch program.

STUDIES OF PROTEOLYTIC ENZYMES

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ASSISTED BY K. A. Walsh, W. P. Winter, P. H. Petra, R. L. Stevens,
L. H. Ericsson, A. G. Lacko, G. R. Reeck
WORK UNIT NO. NR 108-152 CONTRACT NONR 477 (35)

OBJECTIVES

To determine the relation between chemical structure, enzymatic function and phylogeny of proteolytic enzymes of various species.

ABSTRACT

This program deals with a comparison of proteases of higher animals, e.g. bovine, and analogous enzymes obtained from marine organisms. The purpose of these investigations is to establish relationships between the structure and function of enzymes and the environment in which they act. The following projects have been carried out to this end during the period covered by this report:

1. Chemical structure of bovine pancreatic carboxypeptidase A. The sequence of the first 103 amino acid residues has been established and, except for some minor variations, fitted into the structure proposed by W. F. Lipscomb and coworkers. The remainder of the sequence is being elucidated. Several allotypic variants have been discovered and some of them separated and isolated by chromatographic procedures.
2. Carboxypeptidases A and B of the Spiny Pacific Dogfish have been isolated, characterized and subjected to chemical modifications by procedures similar to those described by Vallee and coworkers for the bovine carboxypeptidase A. The response to acetylation and nitration is similar but not entirely analogous to the bovine enzyme. The activation of monomeric dogfish pro-carboxypeptidase A has been investigated in detail.
3. Proteolytic enzymes analogous in function to those of bovine pancreatic origin have been found in the starfish and African lungfish. These include trypsin, chymotrypsin and carboxypeptidases A and B. Some of these are being isolated and characterized by chemical and enzymatic procedures.

PLANS FOR FUTURE

Future work will be directed toward a detailed comparison of proteolytic enzymes of marine organisms with those of higher animals, including their chemical structure, enzymatic function, and where possible, their formation from their respective zymogens.

CURRENT REPORTS AND PUBLICATIONS

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(b) T. M. Radhakrishnan, K. A. Walsh and H. Neurath (1967), "Relief by Modification of Carboxylate Groups of the Calcium Requirement for the Activation of Trypsinogen", *J. Am. Chem. Soc.* 89, 3059.

(c) H. Neurath (1967), "Procarboxypeptidase: Structure, Mechanism of Activation and Phylogenetics", *Seventh Int. Congr. Biochem. Symp.* III-1,4.

(d) James H. Freisheim, K. A. Walsh and Hans Neurath (1967), "The Activation of Bovine Procarboxypeptidase A. I. Isolation and Properties of the Succinylated Enzyme Precursor", *Biochemistry* 6, 3010.

(e) James H. Freisheim, K. A. Walsh and H. Neurath (1967), "The Activation of Bovine Procarboxypeptidase A. II. Mechanism of Activation of the Succinylated Enzyme Precursor", *Biochemistry* 6, 3020.

(f) H. Neurath, K. A. Walsh and W. P. Winter (in press 1967), "Evolution of Structure and Function of Proteases", *Science*.

(g) J. G. Beeley and H. Neurath (to be submitted 1967), "Reaction of Trypsin with Bromoacetone", *Biochemistry*.

EXPERIMENTAL STUDIES ON THE BIOCHEMISTRY AND BIOPHYSICS OF ENZYME MOLECULES

Thomas P. Singer
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San Francisco, California

ASSISTED BY R. Biggs, D. J. Horgan, J. Salach, H. Tisdale, and P. Turini

WORK UNIT NO. NR 108-337

CONTRACT Nonr-5024(00)

OBJECTIVES

(a) To establish the relation of DPNH-CoQ reductase to DPNH-cytochrome c reductase, (b) to define the site and mechanism of action of rotenone, barbiturates and piericidin A in the respiratory chain, (c) to establish whether the iron or copper in fumarate reductase is catalytically active, and (d) to define the pathway of cysteinesulfinate synthesis in animal tissues.

ABSTRACT

(a) The DPNH dehydrogenase of the respiratory chain, upon exposure to alcohol & acid pH at 43°, under the conditions used in the extraction of Pharo and Sanadi's "DPNH-ubiquinone reductase" is quantitatively degraded to an 80,000 m.w. fragment with the same activity toward CoQ₆ and CoQ₁₀ and same sensitivity to amytal, rotenone, and piericidin A as authentic DPNH ubiquinone reductase. If Mahler's DPNH-cytochrome c reductase is isolated from strictly fresh particles but from the same source and by the same procedure as used by Mahler et al. (except for the omission of lyophilization), it is indistinguishable from DPNH-ubiquinone reductase in any of its catalytic activities, in sensitivity to inhibitors, flavin content, molecular weight, etc. The only differences between the two preparations noted were in migration on polyacrylamide and in elution from hydroxylapatite. While rotenone and piericidin are very strongly bound at the site near the flavoprotein in intact preparations, in these soluble samples only loose binding occurs and at a new site, which is not evident in intact particles. These observations suggest that soluble DPNH-ubiquinone reductase is a grossly modified fragmentation product of DPNH dehydrogenase, which does not retain the original reaction site of CoQ but is very closely related to the other 80,000 m.w. fragments, such as cytochrome reductase.

(b) With the acid of rotenone-¹⁴C it has been shown that binding of the latter to the mammalian respiratory chain is far from specific. Besides a "specific" site between DPNH dehydrogenase and CoQ, several unspecific binding sites occur, one of which is in the cytochrome b-c₁ region. This conclusion has been confirmed with EPR studies and with dual wavelength spectrophotometry. Rotenone, piericidin A, and various barbiturates compete for the same "specific" site. The binding of the first two appears to involve strong hydrophobic interactions with lipid and protein. DPNH dehydrogenase, itself, does not bind rotenone significantly.

(c) Studies in collaboration with Dr. H. Beinert have shown that cytoplasmic fumarate reductase contains a non-heme iron distinct from the "g = 1.94" type of iron of succinate dehydrogenase, which is not associated with labile sulfide but undergoes

valency change during catalysis. On the other hand the copper present in all preparations does not appear to cycle during catalytic activity.

(d) L-cysteine, in the presence of DPNH or TPNH, Fe^{++} , and O_2 is oxygenated to cysteinesulfinate (CSA) in liver cytoplasm. The reaction requires a heat stable cofactor of m.w. ~ 750 . Studies with cysteine - ^{14}C suggest the possible involvement of an intermediate, formed nonenzymatically, between cysteine, cofactor, and Fe^{++} .

PLANS FOR FUTURE

Studies on problem b) are continued with ^{14}C -piericidin; on problem (c) the kinetics of EPR change are going to be quantitated, while on (d) the nature of the intermediate(s) and the mechanism of the oxygenation will be explored.

CURRENT REPORTS AND PUBLICATIONS

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(2) Studies on the Respiratory Chain-Linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase. XI. Transformation of the Dehydrogenase to NADH-Coenzyme Q Reductase, James Salach, Thomas P. Singer, and Peter Bader, J. Biol. Chem., **242**, 4555 (1967).

(3) Studies on Succinate Dehydrogenase, XIII. Reversible Activation of the Mammalian Enzyme, Tokuji Kimura, Joseph Hauber and Thomas P. Singer, J. Biol. Chem., **242**, 4987 (1967).

(4) The Coenzyme Q Reductase Activity of DPNH-Cytochrome c Reductase, D.R. Biggs, Joseph Hauber and Thomas P. Singer, Biochem. Biophys. Res. Commun., **27**, 632 (1967).

(5) Inhibitors of NADH-Ubiquinone Reductase from Mitochondria, J. Salach, H. Tisdale, T. P. Singer and P. Bader, Biochem. Biophys. Acta, **143**, 281 (1967).

(6) Studies on the Respiratory Chain-Linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase. XII. Interrelations of NADH-Cytochrome c Reductase and NADH-Coenzyme Q Reductase, D.R. Biggs, J. Hauber and Thomas P. Singer J. Biol. Chem., **242**, 20 (1967).

(7) Studies on Succinate Dehydrogenase, XIV. Intracellular Distribution, Catalytic Properties, and Regulation of Fumarate Reductase in Yeast, J. Hauber, and Thomas P. Singer, Europ. J. Biochem., **3**, 107 (1967).

(8) Studies on Succinate Dehydrogenase, XV. Isolation, Molecular Properties, and Isoenzymes of Fumarate Reductase, Howard Tisdale, J. Hauber, Paola Turini, G. Prager and Thomas P. Singer, Europ. J. Biochem., submitted.

(9) Characteristics of the Binding of Rotenone in the Respiratory Chain and the Inhibition sites of Amytal and Piericidin A, Douglas J. Horgan and Thomas P. Singer, Biochem. Journal, 104, 50 C (1967).

(10) Conversion of NADH Dehydrogenase to NADH-Ubiquinone Reductase, J. Salach, T. P. Singer and P. Bader, Biochem. Jour., 104, 22C (1967).

(11) Studies on the Respiratory Chain-Linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase, XIII. Binding sites of Rotenone, Piericidin A, and Amytal in the Respiratory Chain, D. J. Horgan, T. P. Singer and J. E. Casida, J. Biol. Chem., (1967). 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

None received.

FREE AMINO ACIDS AND RELATED SUBSTANCES
IN MARINE ORGANISMS

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City of Hope Medical Center
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ASSISTED BY M. Horiguchi

WORK UNIT NO. NN 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, and (c) the investigation of the biosynthesis and metabolism of compounds containing covalent C-P bonds.

ABSTRACT

(a) Metabolism

It has been shown that microorganisms can utilize the phosphorus of aminoalkylphosphonic acids, however, there is no experimental evidence concerning their degradation. Transamination is the likely route, therefore, we have utilized α -ketoglutarate-1- ^{14}C as the acceptor and glutamate decarboxylase to release ^{14}CO to examine this possibility in homogenates of a bacteria, E. coli, a ciliate, Tetrahymena, a sea anemone, A. elegans, and mouse liver (Swiss). We have demonstrated an active transaminase for 2-aminoethylphosphonic acid (2-AEP) in the ciliate and in the anemone but not in other tissues. Phosphonoalanine was transaminated by all four tissues. The products of transamination, phosphonoacetaldehyde and phosphonopyruvic acid, may have a more labile C-P bond (the aldehyde decomposes). Thus, transamination is probably the initial step in catabolism of aminoalkylphosphonic acid.

(b) Active transport

Dr. J. T. Holden of this laboratory demonstrated accumulation of phosphonoalanine, 1-AEP, 1-amino-2-methylphosphonic acid and 2-amino-4-phosphonobutyric acid by Lactobacillus plantarum which was dependent on glucose metabolism and attained gradients in excess of 10-fold. Competition experiments indicated that the amino acid transport systems are utilized.

(c) Biosynthesis

Following the observation of incorporation of ^{14}C from glucose into 2-AEP we utilized specifically labeled precursors. It was found that 2- ^{14}C -pyruvic acid does not yield labeled 2-AEP, but that with 3- ^{14}C -pyruvate 0.9% of the label incorporated is found in 2-AEP. Similarly, 1- ^{14}C -acetic acid did not yield labeled 2-AEP, but 2- ^{14}C -acetate yielded 0.6% in 2-AEP. Also, 1,4- ^{14}C -succinate does not yield label, but 2,3- ^{14}C -succinate yields 0.6% label and 1,4- ^{14}C -fumarate yields no label while 2,3- ^{14}C -fumarate yields 0.9% of the total label in 2-AEP. The 2-AEP synthesized was degraded and the labeling in each case was evenly distributed in C1 and 2 of the 2-AEP.

The results suggest that an active glyoxylate cycle in Tetrahymena provides the precursor for the C-P bond formation and that fumaric acid is a likely candidate. Since Tetrahymena is known to have an active pyruvate carboxylase, the failure of 2-¹⁴C-pyruvate to yield label eliminates the likelihood that phosphoenolpyruvate, which was recently proposed, is the direct precursor.

(d) Time of synthesis

Synchronized cultures of Tetrahymena were utilized to elucidate the period of 2-AEP synthesis. It was found that during the synchronization the specific activity of the 2-AEP (ten minute label) dropped to 10% of that of log phase cells. Seventy-five minutes post shock a division index of 85% was obtained and this coincided with the peak in specific activity of the 2-AEP. These results indicate that the synthesis of 2-AEP is closely associated with cell division.

(e) Lipids containing N-methyl derivatives of 2-AEP

Following the isolation and characterization of the N-methyl derivatives of 2-AEP, we fractionated the lipids of ³²PO₄ labeled sea anemones (A. elegantissima) utilizing Sephadex and DEAE chromatography and TLC. This has led to the detection of two lipids containing 2-AEP and one containing monomethyl-2-AEP (MM) and one with trimethyl-2-AEP (TM). Also, a lipid yielding phosphonoacetic acid (Pac) was detected. The relative amounts were: 2-AEP, 18.6%; MM, 6.4%; TM, 5.0%; and Pac, 16.0%.

PLANS FOR FUTURE

- (a) Characterization of the "initial C-P compound."
- (b) Isolation and characterization of the lipid yielding phosphonoacetic acid.
- (c) Examination of the role of aminophosphonic acids in the phosphorus cycle and marine productivity.
- (d) Examination of the distribution of phosphonic acids in subcellular fractions in an attempt to elucidate their role in the cell.

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(b) M. Horiguchi (1967), "C-P compounds." Tanpakushitsu-kakusan-koso, 20, 315-323 (A review.)

(c) M. Horiguchi (in preparation), "Synthesis of 2-aminoethylphosphonic acid in growing and heat synchronized Tetrahymena pyriformis."

(d) E. Roberts, D. G. Simonsen, M. Horiguchi and J. S. Kittredge (Accepted for publication in Science), "Transamination of aminoalkylphosphonic acid with α -ketoglutarate."

Papers presented at the Seventh International Congress of Biochemistry, 1967: (a) J. S. Kittredge, "N-Methyl derivatives of 2-aminoethylphosphonic acid in the lipids of the sea anemone;" (b) M. Horiguchi and J. S. Kittredge, "The incorporation of ¹⁴C-labeled intermediates into 2-aminoethylphosphonic acid by Tetrahymena pyriformis;" (c) D. G. Simonsen, M. Horiguchi and J. S. Kittredge, "Transamination of aminoalkylphosphonic acid with α -ketoglutarate;" (d) J. T. Holden, J. N. A. Van Balgooy, and J. S. Kittredge, "Transport of aminophosphonic acids in Lactobacillus plantarum and Streptococcus faecalis."

**BIOCHEMISTRY OF THE LUMINESCENCE
OF MARINE ORGANISMS**

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ASSISTED BY M. Coen, W. Duane, M. Fogel, J. Friedland, S. Grossman,
S. Kruskall, T. Platt, L. Short, and M. Vergin
WORK UNIT NO. NR 108-495 **CONTRACT** N00014-67-A-0298-0001

OBJECTIVES

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

ABSTRACT

The studies in our laboratory are concerned with the biochemical mechanisms in bioluminescent reactions in systems extracted from two marine organisms, bacteria and dinoflagellates.

The work with the bacterium *Photobacterium fischeri* during the past year has been concerned with the properties of the pure enzyme, luciferase. Treatment with 8 M urea or 5 M guanidine-HCl leads to a rapid loss of activity. However, on dilution into phosphate buffer (pH 7) full activity can be recovered. The reconstituted protein appears identical to native luciferase. The protein in 5 M guanidine is not sensitive to heat denaturation. However, the activity of heat denatured luciferase can be fully recovered by dissolving the inactive precipitate in guanidine or urea followed by dilution into phosphate buffer as described.

The rate of recovery is dependent upon protein concentration, which suggested that the enzyme is composed of subunits whose reassembly is rate limiting. These subunits have now been directly demonstrated by virtue of the fact that there are two non-identical ones. With pure enzyme only a single band of protein occurs upon acrylamide gel electrophoresis; the same electrophoresis in 8 M urea results in two bands. The two subunits have been separated in quantity by DEAE-chromatography in the presence of 8 M urea. The subunits are similar in molecular weight, but are different in several interesting ways.

Studies are also continuing with the photo-excited luminescence of this enzyme. It has been shown that oxygen is required, but that it need not be present during the actual irradiation. The removal of a factor required for this photo-excited luminescence has now been achieved, using chromatography in the presence of guanidine. Its characterization is in progress.

Studies with the dinoflagellates have been directed towards the purification and properties of the enzyme and substrate(s) involved in the reaction. The enzyme activity has been found to involve a somewhat complex system. At least two species (M.W. 150,000 and M.W. 35,000) are

involved, and these differ in their pH/activity profile. The substrate, luciferin, has a low M.W., about 400. It is currently being accumulated in quantity for characterization. Both of these components have now been found to be associated with the previously described cell organelle, the scintillon, which is believed to be the structure responsible for the in vivo flashing of these dinoflagellates. Gonyaulax polyedra are among the organisms which one sees as the "phosphorescence" of the ocean at night, and which are responsible for the "red tides" especially off the West Coast of the United States.

PLANS FOR FUTURE

(a) To determine the amino acid sequence and x-ray structure of bacterial luciferase. In addition, the mechanism of the reaction and the emitting species involved will be studied (b) The electron microscopy of scintillons and their chemical analysis will be continued.

CURRENT REPORTS AND PUBLICATIONS

(a) J. W. Hastings and Q. H. Gibson (1967), "The role of oxygen in light-induced bioluminescence." J. Biol. Chem., 242, 720-726

(b) J. M. Friedland and J. W. Hastings (1967), "The reversible denaturation of bacterial luciferase." Fed. Proc., 26, 826

(c) J. M. Friedland and J. W. Hastings (1967), "The reversibility of the denaturation of bacterial luciferase." Biochemistry, 6, 2893-2900

(d) G. T. Reynolds, A. Liuzzi, J. W. Hastings, B. L. Strunk, and T. Reynolds (1967), "Studies of radiation effects and lifetime characteristics of the light output of scintillation liquid light standards." Biol. Bull., 133, 480-481

(e) J. M. Friedland and J. W. Hastings (1967), "The non-identical subunits of bacterial luciferase: their isolation and recombination to form active enzyme." Proc. Natl. Acad. Sci. U.S. (in press)

(f) R. DeSa and J. W. Hastings (1968), "The characterization of scintillons: bioluminescent particles from the marine dinoflagellate, Gonyaulax polyedra." J. Gen. Physiol. (in press)

INVESTIGATIONS ON THE CARBOHYDRATE BIOCHEMISTRY OF MARINE ALGAE

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WORK UNIT NO. NR 108-535

CONTRACT Nonr-477 (42)

OBJECTIVES

(a) To study, by means of biochemical and enzymatic methods, the carbohydrate reserve material of the Dinoflagellate Thecadinium, (b) to investigate possible pathways for the biosynthesis of floridean starch, (c) to purify the alpha amylase of the giant marine chiton, Cryptochiton stelleri, in order to study its action pattern.

ABSTRACT

Methods for the culture of Thecadinium on at least a modest scale have been worked out. The reserve material of this organism, which appears in the cells in granular form and stains blue with iodine, has been isolated by extraction after solubilization, but also in the native condition with the aid of mechanical methods. The "finger print" of the native product was obtained by X-ray diffraction; the pattern corresponded with that of cereal starches, in contrast to that of floridean starch (from red algae) which agrees closely with the pattern of tuber starches (potato). Studies on the solubility and the blue value of the reserve product, combined with the results of enzymatic methods (such as the breakdown by beta amylase) indicate that the Dinophycean reserve material is indeed a member of the starch family, composed of amylopectin with a fair proportion of amylose. Studies carried out with the aid of p32-labeled phosphate revealed that the Thecadinium cells are rich in starch phosphorylase, an enzyme still believed by many authors to be implicated in the synthesis of higher plant starch. No evidence could so far be obtained for the presence of ADPG-starch synthetase, another enzyme often invoked in this connection (T. Akazawa). In the flagellate Polytomella coeca and the arum lily Sauromatum, synthetase tests were strongly positive, so that the failure in Thecadinium cannot too easily be ascribed to errors in technique.

Possible pathways for the synthesis of floridean starch were investigated in the red algae Constantinea subulifera, Antithamnion spec., and Lophosiphonia spec. Thus far, no conclusive evidence has been obtained to postulate the presence of a starch phosphorylase or a particle-bound ADPG- (or UDPG)-starch synthetase. In collaboration with T. Akazawa's laboratory in Nagoya (Japan), a beginning has been made with the separation and identification of the various nucleotides in red algae by means of column chromatography.

Further improvement was sought in the methods for purification of Cryptochiton alpha amylase. The method developed by Marten Snel in our

laboratory, essentially based on fractionated precipitation with ice-cold acetone and ammonium sulfate and leading to an amylase devoid of laminarase and maltase activity, was found to give very low yields, so that it can hardly be considered to be the method of choice in efforts to arrive at crystalline Cryptochiton amylase. Present attempts are centered around glycogen/enzyme adsorption procedures.

PLANS FOR FUTURE

(a) To complete the investigation of Dinophycean starch, (b) to continue the search for ADPG-starch synthetase and starch phosphorylase in the red algae with the aid of C^{14} -labeled ADPG and P^{32} -labeled inorganic phosphate, (c) to include in our investigations the carbohydrate reserve product of the alga Cyanidium caldarium, whose affinities are very uncertain and controversial; this not only in the hope of shedding some light on the position of Cyanidium in the Plant Kingdom, but also in order to extend the list of previously mysterious algal carbohydrates whose nature we have helped to elucidate (paramylon, floridean starch, Cryptophycean starch, and leucosin or chryslaminarin).

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 pathway, (b) to learn whether or not unique metabolic sequences play an important role in the life of these animals.

ABSTRACT

The extent of carbohydrate oxidation by intact tissues of two species of marine molluscs, Mytilus californianus and Haliotus rufescens, has been studied. The conversion of ^{14}C labeled sugars, glucose, fructose and galactose, to $^{14}\text{CO}_2$ and glycogen by M. californianus and H. rufescens suggests the presence of enzyme systems capable of utilizing these compounds at a slow but easily demonstrable rate to give classically expected results. Positionally labeled glucose produced results that suggest the operation of the pentose phosphate pathway. The presence of all the enzymes leading from glucose to glycerophosphate and lactic acid have been demonstrated including key enzymes of the pentose phosphate pathway. Notable differences when compared to mammalian tissues lies in the metabolism of trioses. A pattern was obtained that indicated a lowered rate of triose metabolism when compared to mammalian tissues or yeast. Coupling our results with those obtained by other workers indicates that invertebrate carbohydrate metabolism may differ considerably from other types of organisms. This raises the possibility that control mechanisms, energy production or hitherto unknown pathways may exist in invertebrates. Indications of such differences are the reports of the importance of the α -glycerophosphate system in insect flight muscle and the presence of succinate as a major product of anaerobic glucose metabolism in molluscs.

We are presently attempting to confirm the production of succinate in two species of marine molluscs and will attempt to elucidate the pathway leading to the anaerobic formation of succinate. In addition, the effect of compounds such as AMP, ADP, ATP, 3', 5' AMP and adrenaline on the activity of enzymes such as phosphorylase and phosphofructokinase are being examined to determine whether control mechanisms that apply to higher animals are operative in molluscs.

PLANS FOR FUTURE

(a) Investigate the mechanism for formation of L-galactose (b) Assay for enzymes of galactogen metabolism (c) examine the ways by which such organisms control their metabolic rate (d) examine pathways for anaerobic succinate formation.

CURRENT REPORTS AND PUBLICATIONS

(a) R. Bennett, Jr. and H. I. Nakada (accepted for publication in "Comparative Biochemistry and Physiology"), "Carbohydrate Metabolism in Marine Molluscs I. The Intermediary Metabolism of Mytilus californianus and Haliotis rufescens."

LYSOSOMES AND INTRACELLULAR DIGESTION IN SEA STARS

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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

Histochemical studies have indicated the presence of lysosomes in large numbers in the columnar epithelial cells of the pyloric and intestinal caeca and an absence of lysosomes in the cardiac stomach. The main enzymes detected were acid phosphatase and acid N-acetyl-beta-D-glucosaminidase. At the histological level, the activity is concentrated at the apical third of these unusually tall cells. At the cytological level, these enzymes are localized within vacuoles measuring about 1-2 microns in diameter. Another group of vacuoles, clustered together to form what appear to be secretory granules, is distributed throughout the epithelium and shows acid esterase activity.

Biochemical studies indicate that the acid phosphatase and acid N-acetyl-beta-D-glucosaminidase activities are structure-linked, that is, they display latency. Some acid beta-galactosidase and acid beta-glycosidase activities have also been demonstrated. Osmotic and membrane disrupting techniques were used in demonstrating structure-linkage of these enzymes.

The combined histochemical and biochemical evidence indicates that particles present in the pyloric and intestinal caecal cells are lysosomal in nature.

PLANS FOR FUTURE

(a) To isolate and identify the various vacuoles of the intracellular digestive system including phagosomes, heterolysosomes, and residual bodies by use of labeling techniques in conjunction with density gradient centrifugation (b) to follow protein or other substrate digestion in isolated heterolysosomes (c) to sort out and characterize the variety of vacuoles which appear in the pyloric caeca and to determine the physiological relationship among the different vacuoles.

HYDROSTATIC PRESSURE EFFECTS ON MACROMOLECULES

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ASSISTED BY

WORK UNIT NO. NR 108-708

CONTRACT N00014-66-C0253-A02

OBJECTIVES

(a) Ultimately to 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

The model system of polyvalyl ribonuclease (PVRNase) containing 23 moles of valine added per mole of RNase was studied further at normal atmospheric pressure. The response of the kinetics of thermal aggregation of PVRNase to various salts and nonelectrolytes was examined. Sodium chloride influence the aggregation in two distinct ways. At low concentrations up to 0.1-0.15 M the log of the aggregation rate changed linearly with the square root of the ionic strength (Bronsted-Bjerrum behavior). This analysis yielded a strong support of the idea that the intermolecular interactions of the PVRNase molecules took place at the site of the added valine peptides. At concentrations higher than 0.15 M NaCl, the log of the aggregation rate increased linearly with salt concentration. This result could be best interpreted as an acceleration by the salt of a salting-out of the protein derivative. Several of the strong salts exhibited the Bronsted effect of a stimulation of the aggregation up to about 0.15 M. Above this concentration the salt effects varied according to the nature of the salt. Tetramethylammonium chloride salted-in at intermediate concentrations and salted-out at higher concentrations. Tetra-n-butylammonium bromide and guanidine hydrochloride (GuCl) strongly salted-in at rather low concentrations. The correlation of the salt effects above 0.15 M on the aggregation rates to the chemical nature of the salts was consistent the idea that the thermal aggregation of PVRNase was a good model for hydrophobic interactions in protein structure.

The effect of urea and ethylene glycol on PVRNase aggregation was a more gradual function of solute concentration compared to the effects of electrolytes. Both of these agents diminished the aggregation at high concentrations.

The difference in the effects of urea and GuCl on PVRNase aggregation has been used to propose a general protein denaturation

mechanism which asserts that these two agents act on proteins in different ways.

A major concern of this project has been to find conditions of thermal equilibrium for the PVRNase aggregation system. This has been necessitated by the requirements of obtaining meaningful data under conditions of high hydrostatic pressure. To this end efforts were made earlier this year to study the aggregation phenomena by difference spectral techniques. But the neutral pH's and the native RNase references used yielded no useful result. More recent attempts to examine our system by difference spectra at low pH's and a variety of PVRNase derivatives seem more promising. Presently there appears to be a correlation between the added valine content and the T_m (melting temperature) of the protein derivative. These results suggest a more feasible approach to obtaining thermodynamic data for the PVRNase system under high hydrostatic pressure. The high pressure optical cell has been therefore adapted for use in the Cary 11, which would facilitate this.

An alternative approach to studying the reversibility of the thermal aggregation system has been to examine the aggregation effects on the enzymatic activity of the protein derivative. At 39° there appears to be a direct relation of the enzyme activity to its being in the nonaggregated state.

PLANS FOR FUTURE

(a) To establish equilibrium conditions for studying the PVRNase aggregation. (b) To determine thermodynamic and kinetic parameters of the thermal aggregation at normal atmosphere and at elevated hydrostatic pressures.

CURRENT REPORTS AND PUBLICATIONS

(a) A. H. Nishikawa, R. Y. Morita and R. R. Becker (1967)
"Effect of the solvent medium on polyvalyl RNase aggregation".
(submitted to Biochemistry)

(b) Morita, R. Y. (1967), "Hydrostatic pressure and temperature as environmental parameters in geobiochemistry. VII. International Congress of Biochemistry. Col VII-6, p. 493-494.

AN INVESTIGATION OF THE NUCLEIC ACIDS IN
GRIFFITHSIA GLOBULIFERA

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dano

WORK UNIT NO. NR 108-759

CONTRACT Nonr 3070(04)

OBJECTIVES

Some additional objectives have been formulated for this research program: (a) To find conditions which will yield a reasonable rate of growth of this alga in culture, and (b) to determine if the multinucleate condition of the cells reflects an alternative method to that proposed by Dr. Donald Brown of providing multiple doses of genes.

ABSTRACT

During the last year, attempts were made to culture Griffithsia globulifera. These attempts were hampered somewhat by the peculiar weather conditions which prevailed on Cape Cod in the summer of 1967. Water temperatures were 10° below normal until the middle of July. This delayed the collecting of specimens until that time. The first attempts at culture were designed simply to find out if Griffithsia globulifera could be grown in still culture. For this purpose a soil-sea water medium was prepared. The plants were grown on a 12 hour day length under "Gro Lux" lights. Temperature was maintained by running sea water. Under these conditions isolates of male, female and tetra-spore plants survived. The plants also grew, but at a rate far slower than the rate in their normal habitat.

PLANS FOR FUTURE

Experiments are continuing for the purpose of finding conditions of culture which will support rates of growth approximating those of under natural conditions. Such an intermediate step is necessary in order to prepare radioactive DNA from Griffithsia. With suitably labelled DNA it will be possible to determine precisely the proportion of homologous sequences of bases in diploid and haploid stages in the life cycle of the plants. In addition, if suitable culture conditions can be found a new system will have been developed in which the normally multinuclear cell can be studied. This is of significance since Dr. Donald Brown has recently proposed that multiple duplication of a single gene is a mechanism for controlling the quantity of gene product present. The question raised here is whether or not multinuclearity represents an alternate control mechanism.

CURRENT REPORTS AND PUBLICATIONS

(a) A. E. Brooks and Maimon Nasatir (1966), "DNA of a seven-chromosome strain of Astrephomene gubernaculifera." *Journal of Phycology* Vol. 2, No. 4: 140-144.

(b) Maimon Nasatir and A. E. Brooks (1966), "DNA of the marine red alga Griffithsia globulifera." *Journal of Phycology*. Vol. 2 No. 4: 144-147.

STUDIES OF METABOLIC BIOCHEMISTRY

It is difficult to find a truly descriptive name for the interest which the Navy must retain in those aspects of biochemistry which do not deal directly with life processes associated with marine environments. This may extend beyond the medical aspects of metabolism, yet cannot justifiably be described in terms which imply support for the development of the science per se. Certain technics or tools (Graff, Albanese, Steiner) may have potential usefulness in strictly naval contexts which are not apparent in the work of those directly associated with their genesis, and might be lost or delayed if the work was sponsored elsewhere. Some association with medical progress in fields of importance to the Navy is desirable even though the importance is not unique to naval operations; contractors who were making excellent contributions before the recent revision of the program (Williamson, Callaghan, Nowinski) remain a part of the team, though little or no new work of a similar nature is being accepted. In general, the area we have designated "Metabolic Biochemistry" is intended to make provision for such aspects of life processes (Uyeki) as may have naval significance meriting support, but do not fit into the other categories of interest provided for in the program.

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.

NUCLEIC ACIDS IN REGENERATING WOUND TISSUE

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Loyola University
Chicago, Illinois

ASSISTED BY

WORK UNIT NO. NR 108-315

CONTRACT Nonr 3502(02)

OBJECTIVES

(a) Clarification of the metabolic events associated with wound healing.

ABSTRACT

None submitted due to delays in research associated with moving to new quarters and to lack of qualified assistants during the current report period.

THE DEVELOPMENT OF FUNCTIONAL CELL STRAINS

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ASSISTED BY Robert Kassel, Ph.D., Olga Kastner, and Ada M. Graff

WORK UNIT NO. NR 108-517

CONTRACT Nonr-266(76)

OBJECTIVES

(a) To develop methods for the propagation of animal cells in vitro and (b) to develop methods for the production of useful products from these cells.

ABSTRACT

Methods have been developed for the cultivation of continuous animal cells under the controlled conditions which are necessary for their utilization as graft replacements or for production of their metabolic products. The production of interferon is emphasized. Mouse interferon has been prepared in high activity and the conditions for long-time stability of this important species specific anti-viral protein. Therapeutic trials are in progress. The laboratory's facilities are now under conversion to the production of human interferon.

PLANS FOR FUTURE

(a) To upscale and improve the cell production operation, (b) to increase the yields of interferon from these cells, (c) to purify and characterize interferon, (d) to employ live cells as dressings in burn therapy, (e) to develop methods for the cultivation of human bone marrow suitable for post radiation replacement.

PROPERTIES OF NUCLEIC ACIDS IN RELATION TO BRAIN FUNCTION

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ASSISTED BY M. Burnel, M. Soller, G. Dutton, K. von Hungen and A.
Campagnoni

WORK UNIT NO. NR 108-633

CONTRACT Nonr-908(20)

OBJECTIVES

(a) Isolation, characterization, properties and mode of biosynthesis of the RNA of rat brain; (b) isolation, characterization, properties and mode of biosynthesis of ribonucleoprotein particles from the same source; (c) RNA synthesis in isolated brain nuclei; (d) kinetics of RNA and protein turnover in isolated, characterized sub-cellular fractions of rat cortex, and (e) synthesis of RNA and protein as related to functional activity of neurons.

ABSTRACT

The kinetics of incorporation of ^3H -uridine in vivo into various sub-cellular fractions of rat cerebral cortex and into their isolated RNA, obtained by sedimentation analysis in sucrose density gradients, has been investigated. The fractions studied include nuclei, free ribosomes and polysomes, isolated without the use of detergents, polysomes attached to endoplasmic reticulum and the cell sap. Nuclei acquire label extremely rapidly (≤ 5 min) into RNA extremely polydisperse as to sedimentation coefficients. With time (≥ 2 hrs) label begins to be concentrated in the ribosomal, 18S and 28S, regions. There is no evidence for the accumulation or even occurrence of a large (45S) precursor of ribosomal RNA either on the basis of these studies or in ones designed to follow the rate of maturation of ribosomal RNA by means of transmethylation with ^{14}C -methylmethionine as the donor. Label enters the cytoplasmic RNA-containing fractions (constituted almost exclusively of free and reticular polysomes) with a delay of 40 min. There is some evidence that for about one hr. subsequent to this the only species of RNA entering the cytoplasm from the nucleus is of the messenger type. Later ($t \geq 2$ hrs) ribosomal RNAs begin to make their appearance with no indication that 18S precedes 28S r-RNA. The kinetic and sedimentation properties of rapidly labeled polysomal and reticular RNA appear to be different: previously published data can be accounted for in terms of a mixture of components from the two compartments.

The incorporation of various ^3H -labeled nucleoside triphosphates (NTP's) into polymeric form by morphologically intact nuclei, isolated from rat brain cortex, has been studied. One system, measured by means of ^3H -GTP, requires the simultaneous presence of the other three NTP's and appropriate cofactors and is virtually completely inhibited by RNase, DNase and Actinomycin D. Another system is operative only with ^3H -CTP, does not require the other NTP's and is insensitive to the inhibitors mentioned. The product of system 1 has been characterized as RNA and by means of appropriate double-labeling techniques as closely resembling the type(s) of RNA synthesized in vivo.

The time course of appearance and decay of radioactive label in the protein and RNA of carefully separated and characterized subcellular fractions from rat cerebral cortex following intraventricular injection of ^{14}C -orotate and ^3H -leucine has been followed from 4 hr to 70 days. The half life (τ) for RNA was the same in all fractions (except nuclei where there is evidence for one or more rapidly decaying components) with a value of 12 $\pm$ 1d. The ratio of $^{14}\text{C}/^3\text{H}$ activities in the ribosomal fraction remained constant throughout. The results for τ (protein) were as follows: cell body mitochondria 19d; synaptosomal mitochondria 21d; whole synaptosomes 19d; ribosomes 12.5d; the microsomal fraction showed heterogeneity in τ 's with both a 8d and a >14 d component. Synaptosome appeared to acquire label with a definite delay during the first few days providing support for the concept of transport of macromolecules from soma to synaptic regions. The τ for the proteins of synaptic vesicles equalled 19d. This value supports a model in which vesicles are used repeatedly during the firing of synapses rather than just once for each firing.

These studies are being extended to provide comparisons between sensorily deprived and sensorily enriched rats, as well as those reared in the light and in the dark. Studies are also under way to measure protein and RNA synthesis in the visual system of Limulus as a function of illumination by means of radioautographic techniques at the electron micrograph level.

PLANS FOR FUTURE

The studies just described will be continued and extended to include characterization of the kinetics and mole of biosynthesis for individual species of RNA (if any) and protein molecules, in reticulum, synaptic membranes, synaptic mitochondria and synaptic vesicles. Increased emphasis will be placed on a comparison between components studied in or isolated from sensorially or behaviorally stimulated individuals and appropriate controls.

CURRENT REPORTS AND PUBLICATIONS

Campagnoni, A. & Mahler, H.R., Isolation and Properties of Polyribosomes from Cerebral Cortex, Biochemistry 6, 956 (1967).

von Hungen, K., Mahler, H.R. & Moore, W.J., Protein and RNA Turnover in Synaptic Subcellular Fractions from Rat Brain (Accepted by J. Biol. Chem.)

Brown, Bette & Mahler, H.R., Protein Synthesis by Cerebral Cortex Polyribosomes. Characterization of the System (Accepted by the Arch. Biochem. Biophys.)

Dutton, G.R. & Mahler, H.R., In vitro RNA Synthesis by Intact Rat Brain Nuclei (Accepted by J. Neurochem.)

STUDIES ON PHOTOSYNTHESIS AND PHOTOTROPISM

H. Gaffron
Florida State University

ASSISTED BY G. H. Schmid, W. Kowallik and P. H. Homann

WORK UNIT NO. NR 108-668

CONTRACT NONR 4641 (00)

OBJECTIVES

Continued use of instruments acquired under NONR 988 (10), now terminated, for the measurement of photochemical responses in pigment-sensitized artificial and natural systems.

ABSTRACT

With the aid of the Biospect and the Isco Spectroradiometer, the latest items of apparatus bought under our expired contract No. NONR 988 (10) we have followed the spectral changes in naturally chlorophyll-deficient leaves. Such leaves lose some of their photosynthetic capacity while they increase their chlorophyll content when left in the dark or under very dim illumination. This work is part of the studies on the photosynthetic capacity of aurea mutants which grow vigorously in strong light with only 1/20 of the normal chlorophyll content.

Another piece of work concerns a newly discovered metabolism of algae induced specifically by blue light. It appears to be the metabolic reaction which initiates a number of blue light effects in plants known under the term "phototropism". The saturation curve, as well as the action spectrum for this metabolism point to traces of a flavin as the light absorbing pigment.

While we found that the visible microstructure of the chloroplasts changes with the specific nature of the partial photosynthetic reaction prominent in the different plant tissues studied, the site of the presumed flavin pigments is unknown, for, as Kowallik discovered, the blue light effect in algae persists even in complete absence of chlorophyll and lamellae.

CURRENT REPORTS AND PUBLICATIONS

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(b) P. H. Homann (1967). Studies on the Manganese of the Chloroplast. *Plant Physiol.* 42 997-1007.

(c) W. Kowallik and H. Gaffron (1967). Enhancement of Respiration and Fermentation in Algae by Blue Light. *Nature* 215 1038-1040.

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

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ASSISTED BY L. A. Orto, E. H. Wein, and M. E. Rosenquest

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

Intensive efforts during the past year have resulted in the development of a total electrolyte analyzer based on the microwave absorption principle previously described which reduced the size of the apparatus from approximately 14 cubic feet to less than 1 cubic foot in size. The new unit is portable and can be 110V AC line or 24V DC battery operated. It has proved to be remarkably stable and permits the use of samples approximately one-third the previous requirement of 18 cc. without loss of reproducibility. Calibration curves with various electrolyte solutions are coincident with those obtained previously with the larger instrument.

These results indicate that the new principle and instrument will permit estimation of water density in terms of rapid and convenient measurements of total electrolyte content rather than the conventional and more laborious determination of chlorinity. Measurements of water density have been found to have many naval applications, e.g., understanding of movements of ocean and coastal currents; and calculation of safe channel depths, siltation and submarine diving operations (J. W. Chanslor: Water Density and its Applications, Science and the Sea, U. S. Naval Oceanographic Office, Washington, D. C., pp. 69-75, 1967).

During the past summer, the new apparatus was employed to determine the osmotic fragility of human erythrocytes. These measurements demonstrated that the results so obtained were comparable to those reported by other investigators employing more complicated and difficult techniques.

Applications to other aspects of human biochemistry are under way, namely, determination of electrolyte excretion in various states of disease and dietary restriction.

PLANS FOR FUTURE

(a) Further exploration of applications of the new total electrolyte analyzer to problems of marine biology, e.g., determination of osmotic characteristics of various marine organisms; (b) continuation of studies on the migration of electrolytes in human whole blood and red cells subjected to various freezing temperatures for various periods of time; (c) continuation of studies on the effect of various dietary regimens on electrolyte excretion.

CURRENT REPORTS AND PUBLICATIONS

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(b) A. A. Albanese (1967), "Nutritional and metabolic effects of physical exercise." Food and Nutrition News, 38, Nos. 8 and 9, May and June.

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STUDIES ON AMINE METABOLISM DURING ANAPHYLAXIS

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WORK UNIT NO. NR 108-716

CONTRACT N00014-67-A-C397-0001

OBJECTIVES

(a) To study amine substances containing carbohydrate which can be extracted from guinea pig lung after anaphylaxis and which cause contractions of guinea pig ileum (b) to determine the relationship of these materials to the "slow reacting substance" of Brocklehurst (c) to determine the metabolic origin of these materials.

ABSTRACT

Ovalbumin-sensitive guinea pigs were sacrificed in groups of six and one lung from each animal was crushed between metal blocks chilled to the temperature of liquid nitrogen. The frozen pooled lungs served as controls to the other lungs which were sliced and incubated with ovalbumin (1 mg/ml) in Tyrode-bicarbonate solution for 10 minutes at 37°C with O₂/CO₂ (95/5). The tissues were extracted by homogenization and deproteinization with zinc sulfate and sodium hydroxide. Extracts were concentrated and fractionated on 1 meter columns of Sephadex G-25 with distilled water. A series of peaks absorbing at 280 mμ from these columns were re-purified on columns of Biogel P-4 and lyophilized. Bio-assay showed that none of the fractions from quick frozen lung caused a contraction which was not due to occasional traces of histamine (determined by a fluorometric method). In the fractions from the lung slices after a 10-minute challenge with ovalbumin, one fraction could be separated which produced the contractions characteristic of "slow reacting substance" (SRS) and satisfied the following criteria: (a) the contraction was delayed and prolonged as compared with the response due to histamine; (b) the fraction potentiated the response to subsequently-administered histamine; (c) 10⁻⁸M pyribenzamine failed to abolish the response due to the fraction although the response to histamine was abolished by the drug; (d) ethanol extracts of the fraction (which were dried by a stream of nitrogen gas and re-dissolved in Tyrode) elicited contraction of ileum.

In order to determine whether SRS could be released from inert substances, portions of all fractions were incubated in appropriate buffers with the following enzymes: papain, α-chymotrypsin, trypsin, hyaluronidase, neuraminidase, and pronase. Bio-assay indicated that papain, α-chymotrypsin and trypsin were effective in producing a small amount of active material from previously-inactive fractions. Neuraminidase was without effect on the active fraction.

Carbohydrates are believed to be present in SRS (W.G. Smith, Nature 209, 1251, 1966). Accordingly, lyophilized samples (3-10 mg) of all column fractions were hydrolysed in 0.1 N H₂SO₄ at 80°C for 2 hours, and

the neutralized hydrolysates were chromatographed on thin-layer plates of silica gel using propanol: H₂O (7:3) or propanol:ethyl acetate:H₂O:25% ammonia (60:10:30:10) as solvents.

Different plates were sprayed with p-anisidine resorcinol (Svennerholm, Biochem. Biophys. Acta 24, 604, 1957) or the spray sequence of ninhydrin-Dragendorff reagent-ammonium molybdate.

The hydrolysates gave a variety of sugar and amine spots; the SRS hydrolysate showed a sugar amine, most closely resembling galactosamine and other unidentified carbohydrates.

Because of the very small quantities of material obtained from the lung a series of experiments has been initiated in which guinea pigs are given a pulse injection of radioactive sugar and the lungs are fractionated as above. Injection of 200 μ C/Kg of N-acetyl-³H-mannosamine into 5 guinea pigs one hour prior to sacrifice has revealed a small radioactive peak coincident with the active fraction. Liquid column samples were counted using Triton-X-100 in the scintillation fluid.

PLANS FOR FUTURE

(a) To study the metabolic fate of a variety of radioactive sugars in guinea pig lung as a means of ascertaining the composition of SRS, (b) to further study the effect of enzymes on the transformation of inert substances into SRS, (c) to study the uptake of ¹⁴C-histidine into extractable materials and also into subcellular organelles of guinea pig lung.

CURRENT REPORTS AND PUBLICATIONS

Manuscripts are in preparation.

DRUG METABOLISM IN RADIATION CHIMERAS

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WORK UNIT NO. NR 108-745

CONTRACT N00014-67-C-0166

OBJECTIVES

A. To study whether radiation and/or drugs can inhibit the stimulation of microsomal enzymes brought about by phenobarbital injection.

B. To study drug metabolism in radiation chimeras, gamma-irradiated animals, and normal animals.

ABSTRACT

A. Radiation and drug effects on hexobarbital sleep time:

In weanling rats, whole body gamma irradiation (700-900 rads) does not bring about a significant difference in sleep time as compared with non-irradiated controls; nor, did irradiation significantly alter the shortening of sleep time brought about by 3 daily injections of phenobarbital. Similarly, groups of rats and mice receiving anti-tumor drugs were studied for drug effects on hexobarbital sleep-time. The anti-tumor drugs used for these studies were: busulfan, cyclophosphamide, methotrexate, vinblastine and 6-mercaptopurine. The animals were pretreated 3-4 days with anti-tumor drugs prior to assessment of hexobarbital sleep time. Generally, the anti-tumor drugs increased sleep time significantly over control animals; the possibility that this effect may be due to inanition cannot be excluded since animals generally lost weight during the course of drug treatment. Sleep time of animals in group B (anti-tumor drug plus phenobarbital) were not significantly different from group D animals (phenobarbital alone). These results indicated that pretreatment with anti-tumor drugs did not significantly alter the ability of phenobarbital to stimulate the microsomal drug metabolizing enzymes.

B. Drug metabolizing enzymes in livers of normal, radiated and chimeric animals:

To study the effects of the three states on a microsomal drug metabolizing system, livers from three groups of weanling male Sprague Dawley rats were analyzed for their ability to detoxify EPN (O-ethyl o-nitrophenyl phosphonothioate) to paranitrophenol (PNP). One PNP unit is defined as 0.01 micromole of paranitrophenol formed from EPN per 20 mg of liver homogenate per 30 minute incubation at 37°C. The results indicated that the ability of livers from normal animals to detoxify EPN to PNP rose from 15 to 25 PNP units at 5 weeks post-treatment. At the same time

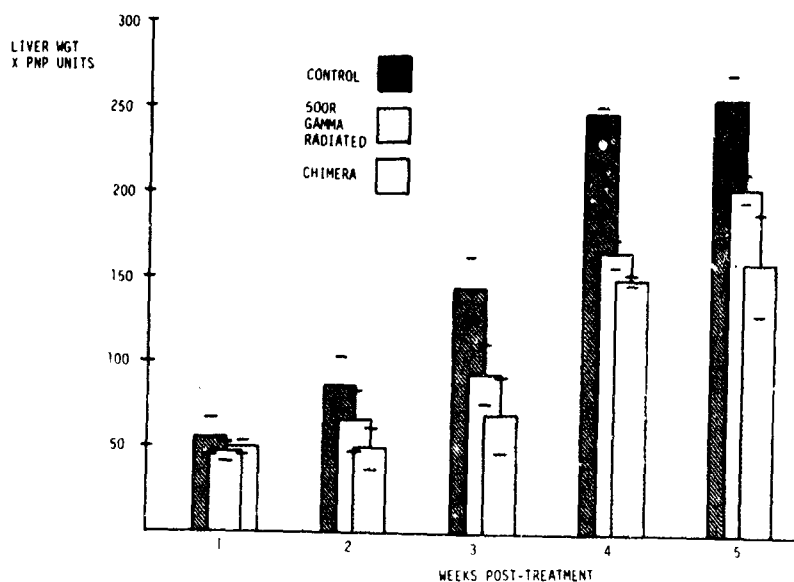


Fig. The total PNP units (liver weight x PNP units) of control, 500 rad radiated, and chimeric animals. Each bar represents the average of values obtained from at least 4 rats. Distance between lines represents two standard errors of the mean.

livers from 500 rad whole body gamma-irradiated animals rose from 14 to 20 PNP units; animals given 500 rads of gamma radiation and injected with 1×10^7 bone marrow and spleen cells (chimeras), showed a gradual rise in the enzymes detoxifying EPN (15 to 22) that were intermediate between control and 500 rad gamma-irradiated animals. In the figure the ability of the animals in the three groups to detoxify EPN is expressed as liver weight x PNP units, i.e., total PNP units. The control animals showed a stepwise increase from 50 to 250 total PNP units, whereas radiated animals showed an increase from 50 to 160 total PNP units during the five weeks of observation. Chimeric animals showed an increase from 50 to 210 total PNP units, indicating values intermediate between control and radiated animals. These results suggested that the increased liver weight in chimeric animals resulting from foreign bone marrow transplantation does not bring about a complete return of drug metabolizing enzymes during the 5-week period of observation.

PLANS FOR FUTURE

1. To examine the effects of the chimeric state on diet-inducible enzymes of the liver, i.e., ornithine transaminase and serine and threonine dehydrase.
2. To determine the effects of phenobarbital-injected animals and chimeric animals to alter the metabolism of cyclophosphamide. Tissue and serum extracts of animals injected with cyclophosphamide will be tested in short-term spleen cultures to assess the levels of cytotoxically active metabolites of cyclophosphamide.

BIOCHEMISTRY AND PHARMACOLOGICAL PROPERTIES OF LEUCOGENENOL

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WORK UNIT NO. NR 108-772

CONTRACT N00014-67-C-0275

OBJECTIVES

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

ABSTRACT

Leucogenenol, on injection into rabbits, dogs, monkeys and mice, induced first an increase in the number of circulating neutrophils and later an increase in the number of circulating lymphocytes. Leucogenenol showed no toxicity when quantities up to 200 ug/kilo (the largest amount investigated) were repeatedly injected i.v. into dogs, mice, rabbits and monkeys. Injection of leucogenenol stimulated the rate of production and/or the rate of maturation of the progenitor cells of the neutrophil and of the lymphocyte.

Mice that were sublethally treated with 400 rads of X-ray radiation and 48 hours later injected with leucogenenol recovered more rapidly than uninjected controls. The rate of recovery was indicated by a comparison of femur sections, bone marrow differential counts, spleen sections, spleen differential counts and the general peripheral leucocyte picture.

PLANS FOR FUTURE

(a) Study possible action of leucogenenol on antibody production due to its ability to stimulate primitive types of cells. (b) Action of leucogenenol on the concentration of enzymes such as lysozyme and alkaline phosphatase in the blood. (c) Continued studies on the action of leucogenenol in the irradiated animal.

CURRENT REPORTS AND PUBLICATIONS

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

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WORK UNIT NO. NR 108-810

CONTRACT

N00014-68-A-0105-
0001

OBJECTIVES

In our previous work we demonstrated in earlier granulation tissue (6th and 10th day after operation) the existence of a metabolic "block" in the Embden-Meyerhof pathway at the height of fructophosphokinase. Because the granulation tissue is composed of three different types (whose relative amounts vary directly with the progress of wound healing) it was of importance to determine which type--inflammation (aseptic), fibroblasts or connective tissue--is responsible for the "block".

ABSTRACT

In our studies of tissue slices of granulation tissue we measured the formation of lactic acid from various substrates of the Embden-Meyerhof pathway, after anaerobic incubation at 38°C in Warburg apparatus. For the present study we are working out techniques which will enable us to isolate the three types of structures from 20 μ sections and to incubate anaerobically each part separately in the presence of substrates as used in the previous experiments (glucose; fructose-6-phosphate; fructose-1,6-diphosphate, etc.) After an adequate time, the reaction is stopped by addition of perchloric acid, the medium neutralized and lactic acid determined with ultramicrochemical methods. Amounts from 10-50 μ g dry weight will be analyzed spectrophotometrically, those below 10 μ g dry weight will be estimated by a fluorometric technique worked out in this laboratory. A special incubation cell for amounts of tissue of 1 μ g - 10 μ g has been devised and used in our preliminary experiments.

PLANS FOR FUTURE

We intend to try to correlate our results with the rate of wound healing and see whether the latter can be influenced by biochemical means. We should like also to investigate the sources of substrates for the Krebs Cycle, if the pyruvic acid formation (as in our case) is too low to be of any importance in the synthesis of ATP.

LUMINESCENCE OF BIOPOLYMERS AND MICROORGANISMS

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WORK UNIT NO. NR 108-815

CONTRACT

OBJECTIVES

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

ABSTRACT

The luminescence of a series of model peptide compounds containing tryptophan, phenylalanine, or tyrosine has been examined. These three chromophores are responsible for the luminescence of proteins. The fluorescence quantum yields and excited lifetimes of tryptophan derivatives have been found to be very sensitive to any modification of the α -amino or α -carboxyl groups. A dominant factor in many cases is the quenching of fluorescence by transfer of a proton from the α -amino to the excited indole group of tryptophan. The efficiency of this process is enhanced by conversion of the α -carboxyl to an amide or ester or by formation of a peptide bond at this position. The most probable explanation is in terms of removal or reduction of the retarding electrostatic potential of the ionized α -carboxyl. At liquid nitrogen temperatures proton transfer is inhibited and the above tryptophan derivatives have fluorescence yields similar to those of tryptophan itself. The efficiency of intersystem crossing, as reflected by the ratio of fluorescence to phosphorescence at liquid nitrogen temperatures, is relatively insensitive to the nature of the nearest neighbor substituent of tryptophan in a dipeptide, as is the phosphorescence lifetime.

The luminescence of a series of tyrosine oligomers, i.e., (Tyr)₂, (Tyr)₃, (Tyr)₄, and (Tyr)₆ has been studied as a function of their state of ionization. In each case the ionization of a single tyrosine confers the luminescence properties of ionized tyrosine. This indicates an important degree of transfer of excitation energy from un-ionized to ionized tyrosine, probably at the singlet level.

Studies have been initiated upon the luminescence of α -chymotrypsin. Although virtually all of the luminescence arises from tryptophan, its emission properties differ considerably from those of the free amino acid, indicating a major influence of the protein structure.

The luminescence of tobacco mosaic virus and of T-2 bacteriophage has been examined. Major differences in properties have been observed.

PLANS FOR FUTURE

To extend the studies of protein luminescence, focusing attention upon chymotrypsin and examining the effects of selective modification, and to extend studies to other viruses.

STUDIES OF IMMUNOCHEMISTRY

The field of immunochemistry ranges from considerations of the formation of protein antibodies endowed with specificity to the nature of the phenomenon we call "resistance", as this is affected by exposure, stress and so on. It similarly ranges from interests verging on direct contributions to the practice of naval medicine (Kisken, Converse, Fudenberg, Mayer), to establishment of underlying principles of specificity determination (Haurowitz, Kabat), the structure-function relationships of protein molecules involved in the immune process (Atassi, Triplett, Papermaster, Segre) the employment of the properties of specificity in useful contexts (Erlanger, Watt, Feeney) and other biological and medical considerations.

Inevitably, some of the work supported as marine or as metabolic biochemistry would qualify also as immunochemistry, and some studies listed here as immunochemistry might equally well be defended as biochemistry. Nature, of course, makes no such distinctions.

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

ABSTRACT

Continuing work on rabbit and chicken antibodies directed against the phenylarsonate anion (As) and the phenyl-N-trimethylammonium cation (R_4N), we compared antibodies formed in rabbits against these two haptens with antibodies formed in chickens against the same chemical groups. Peptide maps of the antibodies of these two animal species are quite different (1). These results indicate that complementary fit of the antibody molecule to the two haptens can be accomplished by different amino acid sequences. -Both antibodies, those from rabbit and from the chicken, are highly heterogeneous, as shown by multiple bands in starch gel electrophoresis of the reduced and alkylated peptide chains of the hapten-specific antibody molecules (1). Further heterogeneity is indicated by the fact that both rabbit antibodies, anti-As and anti- R_4N , yield on papain digestion and subsequent passage through carboxymethyl-cellulose columns the slightly acidic fragment I and the less acidic fragment II described by Porter. The ratio I/II was always higher in anti- R_4N than in anti-As (2,3). -The multiplicity of the 8-10 bands of the light chains of immunoglobulins has been attributed by S. Cohen to their origin in different types of cells. We investigated, therefore, the possibility that the multiplicity of light chains might reflect their formation in different organs. In these experiments one group of rabbits was splenectomized; another was exposed to 500 or more roentgen units of x-rays; in the latter group the spleen was protected by a metal shield. Since the halflife of the immunoglobulins in rabbits is approximately 5-6 days, elimination of the spleen or its protection in the irradiated animals should have affected considerably the band pattern in starch gel electrophoresis. This was not observed (4); we conclude that the multiple bands do not reflect origin in different organs, but rather origin in different types of cells, each of these types occurring in the spleen as well as in other sites of immunoglobulin formation.

PLANS FOR FUTURE

- (a) Comparative amino acid and peptide analyses of various antibodies;
- (b) Injection of isotopically labelled antigens into animals and determination of their distribution in subcellular fractions. Similar analyses had been made in our laboratory at a time when nothing was yet known on the important role of ribosomes in protein biosynthesis. It will be important to know whether the ribosomal fraction contains the labelled antigens or their determinant fragments.

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3. K. L. Knight, F. Haurowitz: Ratio of Fragment I/II in γ -Globulins and Rabbit Antibodies, Federation Proceedings, 26, p. 339, no. 473, 1967 (Abstract).
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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). A study of the optical rotatory dispersion and circular dichroism spectra of monofucosyl and difucosyl oligosaccharides from blood group A, B and H substances has been published (2).

Earlier findings showing that a fucosyl residue on the N-acetyl-D-glucosamine residue of an oligosaccharide makes a relatively high contribution to the ORD and circular dichroism spectra have been confirmed by studies of a series of mono- and difucosyl oligosaccharides from blood group A, B and H substances, the second fucose being substituted on the N-acetyl-D-glucosamine. The linkage of the fucose to the N-acetyl-D-glucosamine has been established by methylation studies to be (1→3). An analysis of the invariant residues of human K and L and mouse K Bence Jones protein from sequences published by others pointed out the large number of hydrophobic residues alanine, valine and leucine as well as of histidine in the constant region while the variable region contained many invariant glycines. The latter were thought to give the flexibility needed to accommodate substitutions in the variable positions while the former could provide the non-covalent bonds for heavy and light chain interactions. The evolutionary significance of these structural relations was pointed out.

PLANS FOR FUTURE

Many oligosaccharides have been isolated from blood group A, B, H and Le^a substances and their structures are being investigated. They are also being used to study the combining sites of purified human anti-A of the γ M and γ G classes. A new antigenic determinant, a terminal α -2-acetamido-2-deoxy-D-glucopyranosyl residue has been found in hog blood group substances. Antibodies to this determinant exist in the sera of humans immunized with hog blood group substances.

CURRENT REPORTS AND PUBLICATIONS

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2. Lloyd, K. O., Beychok, S. and Kabat, E. A. (1967). Immunochemical Studies on Blood Groups. XXXVII. The structures of difucosyl and other oligosaccharides produced by alkaline degradation of blood group A, B and H substances. Optical rotatory dispersion and circular dichroism spectra of these oligosaccharides. Biochemistry, 6, 1440.
3. Kabat, E. A. (1967). A Comparison of Invariant Residues in the Variable and Constant Regions of Human K, Human L, and Mouse K Bence-Jones Proteins. Proc. Nat. Acad. Sci., 58, 229.

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

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WORK UNIT NO. NR 106-104

CONTRACT N00014-67-A-0163-0003

OBJECTIVES

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

ABSTRACT

Comment on Nomenclature: Agreement has been reached by investigators of complement that the components of guinea pig complement will be henceforth designated by the same numerical system as that in use for human complement, i. e., C'3 will replace C'3c, C'5 will replace C'3b, C'6 will replace C'3e, C'7 will replace C'3f, C'8 will replace C'3a and C'9 will replace C'3d.

Studies of C'2: We have continued studies of the cleavage of C'2 by C'1a in the fluid phase. Ultracentrifugation of the reaction products in a sucrose density gradient, followed by assay of the fractions with anti-C'2, has demonstrated an inactive C'2 derivative of sedimentation coefficient 4.2 (native C'2 has a sedimentation coefficient of 5.5). According to our cleavage hypothesis, this reaction mixture should contain C'2a and C'2i, and the sedimentation coefficient of 4.2 would represent an average value. C'2a and C'2i were not resolved in the density gradient, but the C'2a/i distribution curve was spread wider than that of the human serum albumin used as a marker. Similarly, analyses of C'2a/i reaction mixtures on Sephadex G-150 have shown evidence of heterogeneity.

A method has been developed for isolation of pure C'2, based on the capacity of cell-bound C'4 to adsorb C'2. By shifting pH, temperature and ionic composition of the medium, C'2 can be eluted from its complex with C'4. Such preparations of C'2 have yielded a single arc in immunoelectrophoresis tests.

The availability of pure C'2 has enabled us to initiate chemical studies of its destruction by C'1a. Analysis of the reaction products by disc electrophoresis on polyacrylamide gel has revealed three bands. Thus, the action of C'1a on C'2 appears to be more complex than the

cleavage into C'2a and C'2i proposed by us. At the present time, we do not know the relationship between these three bands and the postulated fragments C'2a and C'2i.

Studies of C'3: It has been found that EAC'4, 2a cleaves the C'3 molecule into several fragments which can be detected and distinguished by immunoelectrophoresis. Two of these fragments have been isolated from the reaction mixture and their molecular weights have been found to be 120,000 and 60,000, respectively. (The molecular weight of native C'3 is 180,000).

Kinetic studies have shown that the action of EAC'4, 2a on C'3 resembles an enzymatic process in the sense that the initial velocity of C'3 destruction is directly proportional to the concentration of the C'4, 2a complex, the presumed enzyme, while the relation between the initial velocity of destruction and the concentration of C'3 (substrate) is curvilinear.

Studies of C'5: C'5 has been obtained in partially purified form. Its molecular weight is about 200,000. The intermediate complex EAC'4, 2a, 3 alters C'5 and combines with it. From the reaction product, EAC'4, 2a, 3, 5a, activated C'5 can be dissociated and transferred to EAC'4, 2a, 3.

Studies of C'6: It has been found that cell-bound C'2a plays an essential role in the reaction between EAC'4, 2a, 3, 5a and C'6.

Significance of C' Fragments: The study of fragmentation of complement components is important not only from a chemical viewpoint, but also in relation to the diverse biologic activities of the complement system. There are indications that some inflammatory phenomena evoked by complement may be attributed to certain fragments. Hence, the isolation and characterization of the reaction products of the C' sequence has now become of great interest to students of immunopathology.

PLANS FOR FUTURE

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

CURRENT REPORTS AND PUBLICATIONS

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THE MECHANISM OF ANTIBODY FORMATION

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WORK UNIT NO. NR 106-509

CONTRACT Nonr-1834(37)

OBJECTIVES

(a) Attempt passive transfer of Freund's adjuvant action with immunoglobulin G from rabbits treated with the adjuvant, (b) to follow development of natural antibodies in mice as a function of age by means of the dose response to pneumococcal polysaccharide, (c) to develop a localized hemolysis in gel technique for enumeration of cell producing antibodies against protein antigens.

ABSTRACT

(a) The natural selection theory proposed by Jerne (Proc. Nat. Acad. Sci., 41: 849, 1955) holds that preformed natural antibody is the immunologic recognition system. We proposed, on the basis of the natural selection theory, that the antibody-enhancing action of Freund's complete adjuvant might be brought about by an increase in concentration of natural antibody. In support of this hypothesis it has previously been reported from this laboratory (Science, 148: 1345, 1965) that part of the action of the adjuvant could be passively transferred with serum from adjuvant-treated rabbits. It was also found that the ability of serum from adjuvant-treated rabbits to passively transfer adjuvant action was directly related to the concentration of gamma globulin in the serum. In the present studies the concentration of immunoglobulin G (IgG) in the serum from rabbits treated with Freund's adjuvant was measured using radial immunodiffusion and monospecific antisera. The increase in the concentration of IgG closely followed the previously reported increase in gamma globulin and amounted to about a 230% increase by 7 weeks after injection of the adjuvant. IgG prepared from the serum of adjuvant-treated rabbits enhanced the antibody response of recipient rabbits to diphtheria toxoid and bovine serum albumin. The enhancement appeared to be directly related to the quantity of IgG used. These findings support the role of natural antibody in the antibody-enhancing action of Freund's complete adjuvant.

(b) Previous work in this laboratory has supported the concept that induction of tolerance and immunity is regulated by the ratio of the concentration of antigen to that of natural antibody. On the basis of this work, the prediction was made that offspring of tolerant mothers should be more susceptible than normal newborn mice to the induction of both tolerance and immunity. This prediction was verified experimentally using pneumococcal polysaccharide type III. (Science, 155: 1514, 1967). It was found that both the immunizing and tolerance inducing doses of polysaccharide were ten times lower in offspring of tolerant mice than

in normal newborn mice. The minimal immunizing and tolerance inducing doses of polysaccharide for various ages of mice from tolerant and normal mothers have now been determined. The marked difference previously observed between the two groups using newborn mice appears to persist through the third week of life, but not the fourth. Minimal immunizing dose: 3 and 4-week-old normal mice, 0.5 μ g; 3-week-old mice from tolerant mothers, 0.05 μ g; 4-week-old mice from tolerant mothers, 0.5 μ g; normal adult mice, 0.5 μ g. Minimal tolerance inducing dose: 3 and 4-week-old normal mice, 5.0 μ g; 3-week-old mice from tolerant mothers, 0.4 μ g; 4-week-old mice from tolerant mothers, 5.0 μ g; normal adult mice, 5.0 μ g. The concentration of natural antibody specific for the polysaccharide appears to reach adult levels in the mouse by the fourth week of life.

(c) The technique of localized hemolysis in gel (LHG) as described by Jerne and co-workers (*Science*, 140: 405, 1963) is applicable only to cells producing antibodies against erythrocytes, some bacterial polysaccharides and certain haptens. An adaptation of this technique to allow detection of cells producing antibodies against protein antigens has been devised in this laboratory during the past year. The technique involves plating spleen cells from mice immunized with rabbit IgG in agar containing sheep erythrocytes sensitized with a non-hemolytic dose of the IgG fraction of commercial hemolysin. After incubation and addition of complement, areas of hemolysis appear around spleen cells that release anti-rabbit IgG antibodies. The technique has also been successfully employed to measure anti-ovalbumin antibodies by conjugating ovalbumin to the IgG fraction of commercial hemolysin by means of glutaraldehyde.

PLANS FOR FUTURE

(a) The ability of normal and immune serum and globulin preparations to influence the immunologic response of mice born of tolerant mothers will be studied. Attempts will be made to apply the LHG technique to the pneumococcal polysaccharide. (b) The development of the LHG technique for antibodies against IgG will be exploited in a study of immunologic tolerance to this protein. The effects of preformed antibodies on the immune response and on abrogation of tolerance will also be investigated.

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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 immunoglobulins produced by animals subjected to stress.

ABSTRACT

During the past year our efforts have concentrated on studies of the immune response of animals adapted to high altitude. Our prime interest was to distinguish, if possible, a qualitative alteration in immunoglobulin synthesis.

Eighty AKR mice were maintained at each of three laboratories: Denver (altitude = 5280 ft), Echo Lake (altitude = 10,600 ft) and Mt. Evans Summit Laboratory (altitude = 14,200 ft). At the end of a 30-day adaptation to the environment, each mouse was immunized intraperitoneally with 0.2 ml of 10% sheep red blood cells (SRBC). Eight mice from each group were sacrificed on each of the 10 days following immunization. The number of plaque forming cells (PFC) in the spleen of each mouse was determined by the Jerne method. All serum pools were titered for SRBC agglutinins before and after treatment with 2-mercaptoethanol (2-ME).

Maximum levels of PFC were attained on day 4 in all groups. Those animals adapted to 14,200 ft altitude had PFC levels only half as high as those of the Denver or Echo Lake groups. Peak titers of circulating sheep red cell agglutinins were reached on day 5. Total circulating antibody in the Denver and Echo Lake groups were quantitatively similar, and higher than in the mice kept at 14,200 ft. However, the level of 2-ME-resistant antibody was the same in all groups. This indicates that the animals at 14,200 ft produced little or no detectable 2-ME-sensitive antibody after 5 days postimmunization.

In order to correlate 2-ME sensitivity with the synthesis of immunoglobulins of various density, all serum pools were subjected to sucrose density gradient (10-40%) fractionation. The titer of 2-ME-resistant and 2-ME-sensitive agglutinins was determined on each 4-drop fraction.

Our preliminary analysis of the data suggests that not only is there a quantitative difference in immunoglobulin production in mice adapted to 14,200 ft and immunized with SRBC, but a qualitative difference as well. Additional work is in progress to substantiate and extend this interesting finding.

PLANS FOR FUTURE

Our long range plans are to investigate in detail the molecular basis of changes which occur in the immune response of animals subjected to stress. It is hoped that a clearer understanding of immunochemical events at a molecular level will enable us to relate synthesis of various immunoglobulins to certain host-parasite relationships.

CURRENT REPORTS AND PUBLICATIONS

Four manuscripts in final preparation.

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

ABSTRACT

Work previously reported has now been published (1-4). The reactions of β -propiolactone with amino acids have been investigated under various conditions of pH and temperature to find those under which the reagent acted with specificity. At pH 9.0 and 22°, after 15 min. of reaction, each amino acid has reacted at least 85%, with methionine and cystine being the most reactive. At pH 7.0 and 22° the majority of amino acids reacted; methionine, cystine and histidine reacted, almost entirely and proline and lysine to a significantly smaller extent. At pH 3.0 and 22° further specificity was obtained; methionine and cystine were the only reactive amino acids. Reaction at pH 3.0 and 0° was specific for methionine, since it was the only amino acid modified even after 145 hr. of reaction (5).

A new procedure for the specific and permanent modification of carboxyl groups in peptides and proteins has been developed. This involves the reduction of the trifluoroacetate salt of the peptide or protein with diborane. The reduced amino acids are converted to their corresponding alcohols and the extent of modification can be determined by amino acid analysis of an acid hydrolysate of the reduced sample (6,7).

Apomyoglobin was reacted with 2-hydroxy-5-nitrobenzyl bromide under two different conditions. A derivative modified at tryptophan-7 and another modified at tryptophans 7 and 14 were obtained and characterized chemically and physicochemically. Modification at tryptophan -7 did not alter the electrophoretic behaviour of the protein and only slightly altered the spectral behaviour. Also, upon modification of this tryptophan residue, the Stokes radius and molar frictional coefficient of the molecule remained unchanged. Modification of both tryptophans resulted in drastic changes in all these physical properties and especially, there was an almost twofold increase in the values of the Stokes radius and the molar frictional coefficient. The antigenic reactivity decreased drastically upon blockage of the two tryptophans, as a result,

of definite unfolding of the molecule. ApoMb modified at tryptophan-7 and myoglobin prepared from it were immunochemically unchanged. It was therefore concluded that tryptophan-7 is not an essential part of an antigenic site in metmyoglobin (8).

Sperm whale apomyoglobin was digested with trypsin and the soluble peptides were fractionated by chromatography. Five peptides, which occupied mostly corners in the three-dimensional structure were purified and their immunochemical reactivities studied. These peptides occupied in the parent protein the corners A-B (i.e., the bend between helices A and B in the three-dimensional model), E-F, F-G and G-H and also most of helix B, all of helices F and C and the C-terminal hexapeptide. None of the peptides precipitated antibody and only inhibitory activities were detected. The greatest inhibitory activity resided in the segment A-B. This was followed, in degree of reactivity by segment E-F-G (i.e., corner between helices E and F, all of helix F and corner between helices F and G). The C-terminal hexapeptide was also reactive as was the segment comprising the bend G-H. The peptide corresponding to helix C showed no or very little inhibitory activity. It was therefore concluded that the foregoing corners on the surface of the molecule are present in antigenically active regions.

Apomyoglobin was also subjected to cleavage at tryptophan-7 and/or at the two methionine sites. The shortened apoprotein was immunochemically intact (relative to oxidized, uncleaved control). This, in addition to the finding that fragment 1-7 was inactive, suggested that the latter fragment is not part of an antigenic site in the intact molecule. There are indications, however, that sequence 1-7 might be important for the proper orientation of the reactive region(s) on fragment 1-55. Fragments 1-55 and 56-131 gave immune precipitates with antibodies to the whole protein. Fragment 132-153 showed only inhibitory activity with all the five sera tested. With a given antiserum, a greater portion of the reactivity appeared in the 'core' fragment (i.e. 56-131). However, the three fragments obtained by cleavage at the methionines accounted for almost all the immunochemical reactivity of the intact antigen. The significance of these findings is discussed (9).

PLANS FOR FUTURE

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

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BIOCHEMICAL PROPERTIES OF ANTIBODIES TO PURINE AND
PYRIMIDINE 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

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

We have been successful in purifying anti-thymidine antibody. This was accomplished by specific precipitation with homologous antigen followed by solubilization with hapten. The antibody was isolated in 70% yield from the supernatant by sodium sulfate precipitation of the antigen and subsequent dialysis. The technique is now being applied to the other antinucleoside antibodies.

Experiments are in progress to determine the effect of the antinucleoside antibodies on the DNA dependent-DNA polymerase system of a chick embryo preparation (P. Reichard, J. Biol. Chem. 234, 1244 (1959); H.K. Miller, et al, Biochem. 4, 1295 (1965). Anti-guanosine and anti-thymidine antisera have been found to inhibit the priming ability of DNA from calf thymus, *B. cereus* and *M. lysodeikticus*. Controls of normal gamma globulin and anti-bovine serum albumin antiserum were without effect. Additional DNA preparations are being isolated for use as primers. It is our purpose to determine whether inhibition can be related to the known specificities of our DNA reactive antisera. Antibody to the dinucleoside phosphates will also be tested. Purified anti-thymidine antibody is as effective as anti-thymidine antisera in inhibiting the priming ability of calf thymus DNA.

Specificity studies indicate that antibody to nucleotide can distinguish between nucleotide and nucleoside. Using double diffusion in agar, A-BSA and AMP-BSA exhibited identical reactions with anti A-BSA. Both reactions could be inhibited by A or AMP (23.5 μ moles/ml). On the other hand, as indicated by agar formation, anti AMP-BSA could distinguish AMP-BSA from A-BSA. The reaction with A-BSA was inhibited by

a concentration of A that did not abolish the reaction with AMP-BSA. Such differences were not encountered with anti C-BSA and Anti CMP-BSA. It is concluded that AMP-BSA possesses an antigenic determinant(s) in addition to those present in A-BSA while CMP-BSA (as compared to C-BSA) does not. Further studies with other nucleotide and nucleoside pairs are under way.

The specificity of antinucleoside antibodies was examined further by comparing the reaction of anti-cytidine (anti-C) and anti-thymidine (anti-T) antibodies with DNA's isolated from B. cereus, Ps. aeruginosa and the phage T2. The globulin fractions used in this study reacted only with the homologous nucleoside-protein antigen in gel diffusion and quantitative C' fixation studies. Anti-C reacted better with denatured Pseudomonas DNA (68% G-C) than with denatured B. Cereus DNA (38% G-C) as measured by C' fixation; the reverse was found with Anti-T. When T2 DNA, containing the modified base glucosyl hydroxymethylcytosine rather than cytosine was tested, the reaction with anti-C was eliminated while the reaction was still observed with anti-T. It may be concluded that immunochemical techniques can be utilized for the differentiation of DNA's of varying base composition.

PLANS FOR FUTURE

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

We also plan to continue purifying the various anti-nucleoside antibodies and hope to utilize them for the fractionation of DNA. Among the procedures contemplated for the fractionation of DNA is the use of chromatographic columns in which the medium is antibody immobilized on cellulose. Some of the techniques reviewed by Weliky and Weetall (Immunochem. 2, 293 (1965)) are being considered.

All of the above work was carried out in collaboration with Dr. S.M. Beiser of this Department.

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- (b) A.G. Garro, B.F. Erlanger, and S.M. Beiser (1967), "Anti-Pyrimidine Antibodies and Their Reaction with DNA's of Varying Base Composition." Fed. Proc. 26, 874.
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COMPARATIVE STUDIES ON THE IMMUNOGLOBULINS
OF MARINE FISHES

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ASSISTED BY

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) Establish the evolution of the structural gene classes coding for immunoglobulin heavy chains in the teleost fishes. (c) Study possible homology of the structure of invertebrate antibody-like proteins which are active in agglutination of mammalian red blood cells and certain bacteria.

ABSTRACT

None submitted.

COMPARATIVE IMMUNOCHEMISTRY

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WORK UNIT NO. NR 106 712

CONTRACT N00014-67-A-0380-0001

OBJECTIVES

To study immunochemical properties of homologous proteins.

ABSTRACT

Penguin ovomucoid (a mucoprotein which inhibits trypsin and α -chymotrypsin simultaneously and independently) has been found to be a mucoprotein containing high amounts of sialic acid (contains 5% sialic acid). This sialic acid has recently been specifically cleaved and removed from the ovomucoid enzymatically by the enzyme neuraminidase. We are presently testing the relative immunogenicity and antigenicity and the enzyme inhibitory capacities of the native and sialic acid-free proteins. Penguin ovomucoid has been found to have very low immunogenicity in the rabbit. Valid results on the immunogenicity of the sialic acid-free ovomucoid are not available at this time. Tests of trypsin inhibitory activities, however, show no differences between the native and sialic acid-free ovomucoids. The reactive sites with the enzyme thus must be far removed spatially from the sialic acid in the molecule.

The latter program on the penguin ovomucoid is the forerunner of a program on a study of a) a three-component system containing enzymes, inhibitor and antibody to enzyme or inhibitor or b) a four-component system containing the first three plus substrate.

The comparative immunochemistry of blood serum proteins of three penguins (Adelie, emperor and Humboldt) has been completed and is now in press. These blood serum proteins were also compared with the blood serum proteins of several other avian species. Gamma globulins have been prepared and are under study. The serum transferrins have been shown to be comparatively alkaline proteins with four or five multiple forms.

PLANS FOR FUTURE

To study:

- (a) The immunogenicity and antigenicity of penguin and chicken ovomucoids and their chemical derivatives.
- (b) The antigen-antibody interactions of rabbit anti-chicken lysozyme with chicken lysozyme and its derivatives.
- (c) The development of simple gel electrophoretic techniques for enzyme-inhibitor assays in blood and egg white and with purified materials using immunochemical interactions. The application of this technique for detecting very low concentrations of antibody in fluids is also planned.

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IMMUNOLOGICAL FUNCTION IN SEVERE THERMAL INJURY

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ASSISTED BY P. Casson, K. Kano, and A. C. Solowey

WORK UNIT NO. NR 106-733

CONTRACT Nonr 4503 (00)

OBJECTIVES

To evaluate specific changes in the immunological status 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-like responses.

ABSTRACT

Previous studies have indicated that tuberculin-sensitive guinea pigs subjected to 20 to 40% body surface area full-thickness skin burns develop marked losses of skin reactivity to intradermal challenge with tuberculin. In the present study, the cell migration technique of David was applied to an in vitro evaluation of mechanisms responsible for this non-reactivity to tuberculin after thermal injury. In the course of this study, capillary tubes were filled with post-burn peritoneal macrophages obtained from normal and from tuberculin-sensitive animals. These tubes were placed in Mackaness chambers filled with tissue culture media and tuberculin was added to the media. Control chambers were prepared in similar fashion without the addition of tuberculin. The chambers were incubated at 37° C for 24 hours, and the surface area of cell migration beyond the confines of the capillary tubes was calculated with a planimeter. The results indicate that, although severely burned guinea pigs consistently showed a marked inhibition in their peripheral or skin responses to tuberculin, peritoneal macrophages obtained from such animals retained the ability to respond to this antigen in vitro, with marked inhibition of cell migration resulting from the experimental techniques employed. The ability of peritoneal macrophages obtained from burned tuberculin sensitive guinea pigs to participate in this type of in vitro delayed hypersensitivity reaction suggests that the in vivo loss of skin reactivity to tuberculin after thermal injury may not be the result of a central interference with the burned host's ability to muster a cellular immunological response. Rather, it may represent a peripheral type of inhibition, either as a consequence of adrenocortical stimulation or as a result of the release of pharmacologically active agents at the site of injury.

The second portion of this study has been concerned with the immunological characterization of the hemagglutinating factor specific for rat erythrocytes which had been previously isolated in the thoracic duct lymph of inbred Fisher rats as a

result of thermal injury. Treatment with 2-Mercaptoethanol had no significant effect on the lymph hemagglutinating activity; heating at 56° C for 30 minutes also had no effect, but activity was inhibited after heating at 70° C. The agglutinating activity of post-burn thoracic duct lymph was completely absorbed from such lymph specimens by erythrocyte stromata of the Fisher strain. Human red blood cells had no effect upon the hemagglutinating activity of post-burn lymph. In an attempt to assess the antigenic characteristics of the hemagglutinating factor present in burn lymph, eluates from Fisher erythrocyte stromata incubated with positive lymph specimens were tested in Ouchterlony plates against rabbit antibody to rat serum. One distinct line of precipitation was formed by such eluates, which merged in an identity reaction with a line formed by Fraction II of pooled rat serum. Eluates obtained from rat erythrocyte stromata incubated with normal rat lymph gave consistently negative results. Immuno-electrophoretic studies of rabbit antibody produced by immunization with rat erythrocytes agglutinated by burn lymph, and tested against pooled rat serum and against the IgG fraction of rat serum yielded a major precipitation line consistent with the IgG nature of the factor present in post-burn lymph. These results indicate that thoracic duct lymph samples obtained from Fisher rats after thermal injury contain an agglutinating factor directed against rat erythrocytes, which has the serological properties of rat IgG. The consistent absence of hemagglutinating activity in the serum of burned rats suggests that the agglutinating factor may be absorbed by host erythrocytes after release into the systemic circulation. Positive direct Coombs tests obtained with erythrocytes of burned rats in the course of this study, as well as the results reported by McCarthy and his associates support this possibility. This observation may provide an experimental approach to studies of hematologic complications of the burn syndrome. It also provides evidence that severe burns may induce a state of autosensitivity to altered tissue constituents in the injured host.

PLANS FOR FUTURE

(a) Further study of the etiology of the peripheral inhibition of in vivo tuberculin hypersensitivity following thermal injury, with particular reference to the possibility that serum-bound agents may be involved in the mediation of this "peripheral" effect; (b) Further investigations of the development of mechanisms of autoimmune disease as a result of thermal injury, with particular reference to the possible influence upon the host's ability to respond to skin allografts and to bacterial infection.

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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

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

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

Eighteen different primate species have been typed for Gm factors present at the human γ_{2b} and γ_{2c} loci. Gm factors are absent in lower primates. The Gm factors associated with γ_{2c} molecules, namely, Gm(s), (c⁵), (b⁰) evolve first in the Cercopithecoidea species. Gm(z) occurs together with Gm(a) on human γ_{2b} molecules, but in baboon sera Gm(z) occurs in the absence of Gm(a). The simultaneous occurrence of Gm(a) and (z) in the same sera is established in Hominoidea. Gm(b³) is present only in gorillas, orangutans, and chimpanzees; while Gm(b⁴) and (c³) were detected in chimpanzees. Polymorphism for the factors studied exists only in orangutans and chimpanzees. The data show a close correlation between taxonomic classification and the evolution of Gm factors (f). Tryptic peptide maps demonstrated the presence of the "a" peptide in Gm(a+) non-human primate IgG (h).

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

γ_{2a} (γG_2), γ_{2b} (γG_1), γ_{2c} (γG_3), and γ_{2d} (γG_4) subgroup antigens of the heavy chains of γG immunoglobulins; and (3) the hereditary antigens, the various Gm and Inv factors which are associated with the heavy and light chains respectively. The cross-reactivity of γ , α and μ heavy chains and the kappa and lambda light chain antigens of humans and various non-human primates, has been related to evolutionary status of the various species (1).

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

PLANS FOR FUTURE

(a) 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 will 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 and to study the biochemical differences for the several types of heavy chains unique to γA -globulins. 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 and of similar γA -anti- γA allotypic 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.

CURRENT REPORTS AND PUBLICATIONS

- (a) D. C. Tormey, R. M. Kamin and H. H. Fudenberg (1967), "Quantitative studies of phytohemagglutinin-induced DNA and RNA synthesis in normal and agammaglobulinemic leucocytes." *J. Exp. Med.*, 125, 863-872.
- (b) D. C. Tormey, H. H. Fudenberg, and R. M. Kamin (1967), "Effect of prednisolone on synthesis of DNA and RNA by human lymphocytes in vitro." *Nature*, 123, 281-282.
- (c) H. H. Fudenberg, R. Kamin, S. Salmon, and D. C. Tormey (1967), "Quantitative abnormalities in lymphocyte metabolism in patients with immunoglobulin deficiency." In Nobel Symposium 3 GAMMA GLOBULINS. Edited by Johan Killander, Almquist & Wiksell, Stockholm, pp. 585-596.
- (d) H. H. Fudenberg (1967), "Genetically determined abnormalities in antigen-antibody interaction." In HUMAN GENETICS, J. Neel and J. Crow, Editors, Johns Hopkins University Press, Baltimore (In Press).
- (e) E. C. Franklin and H. H. Fudenberg (1967), "Rheumatoid factor and the Gm and Inv groups." In PROGRESS IN RHEUMATIC DISEASE, Grune & Stratton, Inc., New York (In Press).
- (f) E. van Loghem, J. Shuster, and H. H. Fudenberg. "Gm factors in non-human primates." *Vox Sang.* (In Press).
- (g) H. H. Fudenberg, E. R. Gold, G. N. Vyas, and M. R. MacKenzie. "Human antibodies to human IgA globulins." *Immunochemistry* (In Press).
- (h) E. van Loghem, J. Shuster, H. H. Fudenberg, and E. C. Franklin. "Phylogenetic studies of immunoglobulins: Evolution of Gm factors in primates." *Ann. N. Y. Acad. Sci.* (In Press).
- (i) J. Shuster, N. L. Warner, and H. H. Fudenberg. "Cross-reactivity of primate immunoglobulins." *Ann. N. Y. Acad. Sci.* (In Press).
- (j) G. N. Vyas, H. H. Fudenberg, H. M. Pretty and E. R. Gold. "A new rapid method for genetic typing of human immunoglobulins." *J. Immunol.* (Accepted).

Published subsequent to 1967 report:

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- K. D. Wuepper, and H. H. Fudenberg (1967), "Moniliasis, "autoimmune" polyendocrinopathy, and immunologic family study." *Clin. Exp. Immunol.*, 2, 71-82.

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

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ASSISTED BY A. Crowder, and T. Berndt

WORK UNIT NO. NR 106-753

CONTRACT N00014-67-A-0120-0003

OBJECTIVES

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

ABSTRACT

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

In an effort to test the first possibility the enzyme at one branch in the pathway, Dopa oxidase, has been purified and characterized. The enzyme oxidizes not only dopa but tyrosine and dopamine. It has three molecular variants which can be separated by chromatography on DEAE-cellulose. Its sedimentation constant (all three variants) is about 115. It may be present *in-vivo* in an inactive form which can be activated by ultraviolet radiation or trypsin. Activation does not change sedimentation constant. All metabolites in the pathway have been tested for their possible roles as modulators of enzyme activity and both accelerators and inhibitors have been found. These results have been used to produce a computer model and to test the possibility that switching is accomplished by modulation of enzymic efficiency by endproducts or intermediates. The computer predicts that this is not possible.

Another key enzyme in the pathway, dopa decarboxylase, has been partially purified in anticipation of experiments to characterize it with respect to its behavior in the presence of intermediates and endproducts of the tyrosine pathway.

The possibility of switching at the translational level is now being studied using cell free protein synthesizing systems whose polysomes derive from frog skin - a rich source of dopa oxidase. These polysomes support protein synthesis and preliminary experiments indicate that some

of this protein is dopa oxidase. Polysomes in this system can be partially precipitated by a very specific antibody for dopa oxidase. Other antibodies are not effective as precipitants. The presumption is that the precipitated polysomes contained sufficiently long nascent tyrosine oxidase protein fragments to impart antigenic specificity to them. If this is true it will be possible to examine the polysomes of various tissues with these reagents and to make quantitative estimates of Dopa oxidase m-RNA in various cell types. If there is a lack of correlation between rate of Dopa oxidase production and amount of polysomes capable of priming that synthesis, then the possibility may be entertained that switching occurs at the translational level.

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

PLANS FOR FUTURE

- (a) To confirm that polysomes specifically precipitated by anti-Dopa oxidase are capable of priming for the synthesis of Dopa oxidase.
- (b) to make quantitative estimates of Dopa oxidase m-RNA in different cell types.
- (c) To produce computer models which test the possibility that switching in the tyrosine pathway during development occurs at the translational level of protein synthesis.

THE ROLE OF THE THYMUS IN THE IMMUNE RESPONSE

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ASSISTED BY

WORK UNIT NO. NR 106-765

CONTRACT N00014-67-A-0128-0001

OBJECTIVES

1. To determine the relative contribution to primary function and/or restoration of function of thymic cells, thymic humoral factor and thymic subcellular particles. 2. To study the role of the thymus in determining the genetic constitution of antibody.

ABSTRACT

Many investigators have ascertained the importance of the thymus in the development of the immune response. Progress on detailing its precise role has been difficult because of lack of a reliable quantitative assay of thymic function. We have been interested in two related areas as listed under objectives.

The production of thymocytes within the thymus proceeds at a very rapid rate but the fate of these thymocytes is uncertain. Indeed, there is good evidence suggesting that most of the thymocytes produced never leave the thymus. We initially hypothesized that antibody variability could be a result of mutation within the thymus and that the thymus in some unknown way could screen out harmful mutations but utilize the useful mutations. If this is true with lesser amounts of thymus present at critical times there should be a decreased ability to make antibodies of differing specificities and with more thymus present more antigenic sites could be recognized with an increase in the number of specificities. Neither of these turned out to be true. Small amounts of thymus as we and others already knew was perfectly adequate to provide normal levels of antibody to a mouse. Twenty to thirty additional lobes of thymus in adult or neonatally thymectomized animals gave no additional information to the animal when tested in a cross reacting cow-horse-sheep red cell system. In fact some animals with multiple thymus grafts showed an evanescent wasting disease with decreased ability to form antibody against the immunogen. We did note however, that the immune response in neonatally thymectomized animals replaced with multiple grafts never recovered until the grafted thymus became architecturally "normal". In animals immunosuppressed with cyclophosphamid this was also noted. For this reason, we are repeating some of the millipore filter type experiments, using thymocyte free reticular epithelial tissue and normal thymus (both enclosed in cell tight chambers) to document levels of recovery and to

histologic
correlate this recovery with the appearance of all lymphoid tissues.

We have directed our attention to our second question by the following experiments. Observations which we have confirmed several times told us that random bred mice when compared with inbred mice (a) formed higher levels of antibody to a similar antigen dose (b) were more difficult to immunosuppress (c) recovered from x-ray more rapidly than inbred strains. These facts were difficult to reconcile on the thymus mutation hypothesis. Smithies has recently suggested on the basis of the critical analysis of sequence data from twenty-seven humans and three mouse Bence Jones proteins that somatic recombination between the elements of antibody gene pairs may explain antibody variability. This might explain our observation because outbred mice heterozygous for antibody genes might have more potential variations in their antibodies than inbred strains homozygous for antibody genes. We are consequently testing the antibody potentials of homozygous versus heterozygous mice and have found inbred mice strains that differ from each other and their F-1 hybrids in gamma globulin production. We are now making thymic and bone marrow chimerics in which we are testing for the relative contribution of thymus and/or bone marrow to the gamma globulin type. Data at this time is not sufficient to include in this report.

A second approach to this problem is also being utilized. Intra-uterine thymectomized rabbits and normal intrauterine rabbits are being implanted with thymus of known allotypes but yet differing from the host rabbit allotype. After birth, gamma globulin type is being examined. We anticipate that if the thymus is actively contributing to the genetic makeup of the gamma globulin the specificity of its allotype will be present.

CURRENT REPORTS AND PUBLICATIONS

- (a) W.A. Kiskien, M.D. and Nancy Swenson, "A technique of intra-uterine thymectomy in the rabbit." In press "Surgery".
- (b) Antigen specific immunosuppression in the adult mouse - manuscript in preparation.

THE ANTIGENICITY OF CONJUGATED PEPTIDES

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WORK UNIT NO. NR 106-782

CONTRACT N00014-67-C-0513

OBJECTIVES

(a) To study the feasibility of producing effective neutralizing antibodies to small molecular weight toxins (eledoisin and saxitoxin being prototypes) by conjugation with polymethylmethacrylate particles and poly-L-lysine, and (b) to test the neutralizing ability of these antibodies in vivo against acute toxicity and in vitro against the pharmacological actions of these toxins.

ABSTRACT

This research is just now getting started. Immunization of rabbits with the polymethylmethacrylate-peptide antigen is proceeding. After 42 days of immunization, sera from injected animals do not show precipitating antibodies. The presence of neutralizing antibodies has not yet been studied.

PLANS FOR FUTURE

To continue studies as indicated in the objectives. A readily available analogue of eledoisin with equivalent biological activity but having only six amino acids in the peptide chain will be used as an antigen. Cross reactions with this antigen and eledoisin will be studied.

CURRENT REPORTS AND PUBLICATIONS

ANTIGENICITY OF CONJUGATED PEPTIDES AND RELATED TOXIC MOLECULES. ELEDOSIN AND RELATED PEPTIDES AS HAPTENS; Midwest Research Institute, Watt, Semiannual Status Report, December 8, 1967.

IMMUNO-CHEMICAL STUDIES ON K ANTIGEN OF
VIBRIO PARAHAEMOLYTICUS

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ASSISTED BY K. Kuroda and S. Iida

WORK UNIT NO. NR 106-791

CONTRACT DAJB17-6' -C-0025

OBJECTIVES

(a) To define the optimal conditions for isolating the K antigen of Vibrio parahaemolyticus, (b) to establish the relationship between hexosamine content and serological activities of the K antigen.

ABSTRACT

Three methods, (a) fractional precipitation of culture supernatant with ammonium sulfate, (b) phenol-water extraction from cell bodies according to the method of Westphal, (c) condensation of supernatant of boiled cell suspension, were compared for the isolation efficiency of K antigen from the same quantity of the culture of Vibrio parahaemolyticus A 55 K⁺.

Variations were observed in yields of the K antigenic preparations by the various methods. A good yield was obtained by boiling the cell suspension: 30 mg of the purified K antigen per 4 g dry weight cells, after subsequent purification by zone electrophoresis.

A close association of hexosamine content and serological specificity was observed in all the purified K antigens obtained by the various isolation methods.

PLANS FOR FUTURE

To elucidate the chemical constitution responsible for immunologic specificity of the K antigen

CURRENT REPORTS AND PUBLICATIONS

G. Omori, M. Iwao, S. Iida and K. Kuroda (1966),
"Studies on K antigen of Vibrio parahaemolyticus."
Biken Journal, 9, 33-43.

STUDIES IN TOXICOLOGY

As usually employed, the term toxicology generally refers to a class of research of a somewhat different nature to that intended here. Basic research of naval importance in toxicology is conceived as covering the investigation of the biochemical basis for toxic action, particularly under conditions likely to obtain in future naval operations. Thus, the influence of pressure and similar factors (Schwartz, Soloway), or of chronic exposure to low levels of potentially toxic materials, the recognition of subclinical effects of materials which may not be usually thought of as hazardous and the effects of unusual portals of entry are matters of concern. It is our hope to build up the program in this area during the coming years since it is one which may have serious implications for the success of missions which are becoming technologically feasible, and is one not adequately covered by the interests of most existing supporting agencies.

ADVISORY CENTER ON TOXICOLOGY

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

ASSISTED BY

Director and Staff

WORK UNIT NO. NR 303-366

CONTRACT Nonr 2300(29)

OBJECTIVES

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

ABSTRACT

(a) Sixty-five requests for information and advice requiring extensive time and effort have been received to date in this calendar year. There have also been over 200 miscellaneous requests involving less time and effort. These represent a continuing increase in the workload. The subject matter has, as always, been quite broad in terms of the circumstances of exposure and the variety of chemicals. Many sponsors have submitted problems involving potential exposures of the public. In accordance with our standing policy, all problems involving criteria and standards for human exposures have been referred to the Committee on Toxicology. (b) The Center has continued to enlarge its coverage of the literature. A portion of its collection is now being placed on microfilm where it is just as readily available but with a valuable reduction in storage requirements. (c) Excellent cooperation has been received from industrial, academic, and governmental laboratories in providing up to the minute information on the results of current toxicity studies.

PLANS FOR FUTURE

The Advisory Center on Toxicology plans (a) to continue to fulfill its obligations to its sponsors in the most efficient manner possible, (b) to work with the Committee on Toxicology and other agencies of the National Academy of Sciences to provide leadership in the field of toxicology for the benefit of the sponsoring agencies.

ADVISORY CENTER ON TOXICOLOGY

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

ASSISTED BY

Director and Staff

WORK UNIT NO. NR 303-450

CONTRACT Nonr-2300 (15)

OBJECTIVES

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

ABSTRACT

Information on the toxicity of a variety of materials of construction has been obtained from the literature and from cooperating manufacturers which has been used as the basis for comments and advice by the Center and by the NRC Committee on Toxicology. Several atmospheric pollutants encountered as a result of fleet operations have been similarly evaluated.

PLANS FOR FUTURE

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

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

(a) It was previously shown in this study that murine hepatic lysosomes were not labilized in the presence of 1 atmosphere absolute of helium. Further studies with these particles were carried utilizing the following test atmospheres along with a pH 6.5 sucrose media: Helium at 6 atmospheres absolute; oxygen at 1 atmosphere absolute; oxygen at 6 atmospheres absolute. Determinations were made to determine the escape of acid phosphatase from the lysosomes and the increased permeability of the lysosomal membrane to the substrate, p-nitrophenylphosphate. As was seen with the helium at 1 atmosphere absolute, the additional 3 atmospheric conditions revealed no significant differences in comparison with air with regard to lysosomal stability over a 30 to 180 minute period. This was unexpected. In vivo studies by others has revealed that low oxygen tension within intact tissues results in labilization of lysosomes. In the in vitro studies reported here the absence of oxygen resulted in no increase in labilization as compared to air nor did the increased oxygen tensions result in a stabilization. It is known from studies by others and has been confirmed in these studies that lowering of the media to pH 5 results in an extensive labilization of the particles in vitro. The lowering of oxygen tension in vivo may result in lysosomal labilization due to the known secondary lowering of cellular pH rather by removal of a primary stabilizing effect oxygen might have on the membrane itself.

(b) A study was initiated to determine the effects of helium/oxygen atmospheres on capillary permeability during the development of experimentally induced edema. Edema was induced by the subcutaneous injection of a 5% Brewer's Yeast solution into the plantar surface of the rat's paw. Radio-iodinated serum albumin (RISA) was administered intravenously. At the end of the experimental period water and plasma protein content were determined for the extravascular space of the paw. When animals pressurized to 300 feet in a He/O₂ mixture, PO₂ 0.3 atm (abs), were

compared to sea-level controls no differences were observed in the development of edema over a 6-hour period.

PLANS FOR FUTURE

- (a) Effects of antiinflammatory drugs on the edema model developed in animals kept under satiated diving conditions;
- (b) Effects of hyperbaric helium/oxygen atmospheres on the drug-metabolizing microsomal enzyme systems.

Oxygen Metabolism: Metabolic Implications
in Hyperbaric Systems

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ASSISTED BY (Mrs.) A. S. Widman

WORK UNIT NO. NR 303-811

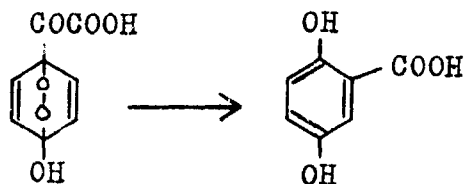
CONTRACT Nonr-8212

OBJECTIVES

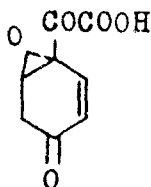
The objective is the determination of the possible biochemical formation of biologically-active oxygen intermediates, such as peroxides, epoxides and hydroperoxides, first under normal physiological conditions and then hyperbaric systems.

ABSTRACT

The work is just beginning, and as a start, we have undertaken to work out the conditions necessary to synthesis O^{18} - labeled p - hydroxyphenylpyruvic acid with the label in the phenolic function. The immediate goal is to assess the lability of this hydroxyl function in the biochemical transformation of p - hydroxyphenylpyruvic acid to homogentisic acid. Definitive evidence of this type would point to the possibility that the following transannular peroxide occurs as an intermediate.



Failure to show the occurrence of such a structure would prompt studies designed to show the feasibility that an epoxide with such a structure might be a labile intermediate in the above biochemical sequence.



PLANS FOR FUTURE

The ultimate goal is to determine not only the existence of such highly reactive structures but the effect of hyperbaric oxygen conditions upon their levels in mammalian systems.

EARLY DETECTION OF TOXIC EFFECTS OF ORGANIC CHEMICALS

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Kingston, Rhode Island

ASSISTED BY H. Shah

WORK UNIT NO. NR 303-812

CONTRACT N00014-68-A-0215

OBJECTIVES

To study the biochemical and pharmacological effects which are produced by chronic inhalation of organic chemicals, such as methylchloroform, prior to the appearance of gross pathological changes. Methylchloroform was selected for investigation because of its frequent use as solvent and degreaser in naval installations.

ABSTRACT

Significant and reproducible reduction of the duration of hypnosis induced by hexobarbital or pentobarbital occurred after methylchloroform inhalation by mice. This effect was reversible within 72 hr after the termination of inhalation. Continuous exposure for 24 hr or three repeated eight hour exposures at 16 hr interval were effective in reducing hypnosis. However, six daily four hour exposure periods produced no effect. The duration of hypnosis produced by barbitol or chloral hydrate was not affected by methylchloroform inhalation. Hexobarbital and pentobarbital are detoxified by oxidative enzyme systems localized in the hepatic reticular endothelium. Barbitol, not a substrate for these enzymes, is excreted unchanged. Chloral hydrate is metabolized by alcohol dehydrogenase localized in the soluble part of the liver cells. This pharmacological evidence suggests that methylchloroform induces reversible changes in the hepatic reticular endothelium and thereby increases the activity of hepatic microsomal enzymes responsible for the oxidative metabolism of hexobarbital and pentobarbital. Studies on the activity of liver microsomal enzyme systems further support this hypothesis. Methylchloroform inhalation increased the *in vitro* activity of the enzymes responsible for the side-chain oxidation of hexobarbital and demethylation of aminopyrine. The effect on demethylation was less pronounced. Since the microsomal nitro-reductase was not altered, the effect appears to be specific for the oxidative enzyme systems. Blockade of the methylchloroform effects by ethionin pretreatment suggests that the methylchloroform inhalation increases microsomal enzyme synthesis in the liver. The reduced hexobarbital hypnosis and increased rate of drug metabolism are effects specific to the inhalation of methyl-

chloroform. The intraperitoneal administration of methylchloroform, even in very small doses (25 ul/mouse), produced hepatotoxicity. The barbiturate hypnosis was prolonged and the liver microsomal enzymes were depressed after intraperitoneal injection of methylchloroform. The hepatotoxic effects of intraperitoneal methylchloroform were enhanced by dimethylsulfoxide and diminished by the dilution with olive oil.

Since methylchloroform inhalation produced specific and reversible changes in the liver microsomal enzymes, suitable tests may be devised to detect pre-pathological body changes produced by methylchloroform.

PLANS FOR FUTURE

The work summarized above was initiated at the University of Kansas by Harbans Lal, assisted by Hasmukh Shah, under Contract N00014-66-C0006 (NR 303-723) and is being continued at the University of Rhode Island. Future work will include an extension of the studies on liver microsomal enzymes and studies on the pharmacological sensitivity of other species as altered by the methylchloroform inhalation. Investigations will be conducted on the biochemical and physiological mechanisms by which methylchloroform inhalation alters liver microsomal enzymes.

Tests which can be used clinically to detect drug effects on liver microsomal enzymes will be explored.

CURRENT REPORTS AND PUBLICATIONS

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

STUDIES IN TOXINOLOGY

The sea is particularly rich in plants and animals producing toxins and venoms which may be a hazard or a nuisance. These substances may present direct threats to the efficiency or even the lives of naval personnel which must be met with knowledge of the nature of the toxic material (Tu, Shapiro, Abbott, Feigen) and with appropriate protective measures and treatment (Russell). The class of compounds represented is characterized by striking pharmacological activity, which offers promise of potential usefulness in medicine, pest control and other contexts (Chanley, Copley). There is, in addition, a large possibility that studies in this field will lead to new understanding based on the development of comparative biochemistry which will have an impact in the other fields of interest listed in this volume.

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

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& L. Hadji.

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

A. TOXINS

1. Tetanus: In previous studies it had been shown that the effects of crude preparations of tetanus toxin in stimulating an increased frequency of miniature end plate potentials (MEPPs) were due to the action of a molecular species which was physically and antigenically distinct from the classical lethal tetanospasmin. Ultracentrifugal analyses of the best fraction available last year showed the lethal fraction to have a Svedberg coefficient of 7 whereas the most potent MEPP-fraction was associated with 4S particles. This year, further preparative chromatographic separations were made on the MEPP-rich starting material (Fraction IV). Chromatography on Sephadex G-200 gave 3 major peaks. The yields of repeated runs were separately pooled on the basis of elution diagrams and each pool was concentrated by pressure-dialysis. No appreciable MEPP activity was found in the first 2 peaks; it appeared to be concentrated in Peak III, which had no lethal activity. This peak had only one antigenic determinant and gave a Svedberg coefficient of 2; but all of the lethal toxicity of the parent was quantitatively recovered in Peaks I and II which contained 5S and 4S molecules, respectively. The MEPP material accounted for only 7% of the total protein of the starting material.

2. Streptolysin: In appropriate concentrations the activated toxin causes a transient decrease in the rate and amplitude of contraction of the isolated atrium accompanied by an increase in the rate of repolarization of the atrial action potential. The severity of the effects depend upon the dose of toxin, and their magnitude decreases with succeeding challenges, suggesting that Streptolysin O may exert its action on the isolated atria through the release of an intermediate from tissue stores. This year, detailed studies of the mode of cardiotoxic action suggested that the chronotropic and inotropic changes exhibited by the isolated atria when they were challenged either in the presence of a blocking agent (atropine) or in the presence of anti-cholinesterases (eserine or DFP) were due to the release of acetylcholine. The electrical changes in single atrial cells - increased resting potential, accelerated repolarization, as well as the

generally negative results observed during ventricular impalements - were consistent with the acetylcholine hypothesis. In spite of the presence of powerful tissue cholinesterases, sufficient quantities of material were obtained by incubating large numbers of challenged atria with toxin to render pharmacological identification of Ach unequivocal.

3. Sea Urchin Toxin: The crude pedicellariar toxin of *Tripneustes gratilla* obtained from 1,000 specimens in 1966 and 2,000 specimens during 1967 was variously fractionated, and the fractions further purified by chromatography on Sephadex. The enzymological properties of these materials were studied with respect to toxin concentration, substrate concentration, and temperature. The kinetics of the reaction system with respect to whole plasma and to its pseudoglobulin fractions are of a complex order owing to (1) "natural" kinin formation of the substrate, (2) to the inactivation of the reaction product (and of synthetic bradykinin) by the toxin, and (3) to the existence of more than one enzyme in the "purified" material. Plots of first order velocity constants against the square of substrate concentration were linear suggesting the existence of substrate "modifiers". The dependence of reaction velocity on enzyme concentration was not of the classical type. The temperature optimum of the system was 26°C. A study of the reactions of various Cohn fractions with more purified enzymes suggested that the substrate furnishing the kinin activity on guinea pig tissues was α_2 -globulin and conclusive proof was obtained in studies with immunoelectrophoretically pure α_2 -macroglobulin. Parallel assays on guinea pig and rat tissues suggested that the substrate responsible for the oxytocic effect in the rat, on the other hand, is a β -globulin. Rabbit antibodies, produced in response to inoculations of formalin-toxoids in Freund's adjuvant, gave normal quantitative precipitin curves with "active" antigens (or toxoids) and they fixed complement. Immunoelectrophoresis showed 2 major antigens, the more immunogenic being concentrated in the 2/3 SAS fraction while the other was present among the 1/3 SAS materials. Biologically, the antisera showed quantitative antitoxic action in the usual protection and neutralization tests in whole animals as well as on isolated tissues. Toxin-induced inhibition of active Na transport in the toad bladder was also neutralized by the antitoxin.

B. ANAPHYLAXIS

1. Ragweed: Evidence was obtained that rabbit anti-ragweed conferred passive sensitivity on cardiac and intestinal tissues of the guinea pig. The degree of sensitization, estimated by histamine release, was shown to depend on the [AB] in the bulk phase. For a constant degree of sensitization the response to the whole antigen could be quantitatively inhibited by the presence of a dialyzable ragweed hapten during challenge with the non-dialyzable antigen.

2. Penicillin: Positive Schultz-Dale reactions were obtained to penicilloyl poly-lysine in isolated guinea pig tissues sensitized with rabbit antibodies to penicilloyl-rabbit serum albumin (anti-Pen RSA) and to penicilloyl Rabbit γ -globulin (anti-Pen RyG). The reaction could be quantitatively blocked by ϵ -aminocaproic acid and by the penicilloyl derivative of the condensation product between DAB (1, 4-diaminobutane) and fluorescein isothiocyanate. The eventual aim of these studies is to set conditions for the determination of the kinetic behavior, binding affinities, and heterogeneity constants of "allergic" antibodies to penicillin by measurements of fluorescence polarization.

3. Fc Fragments: Quantitative reversed passive sensitization with Fc fragments of rabbit γ -globulin was demonstrated in the *in vitro*-sensitized

heart challenged with ovine antiserum prepared against these fragments. The concentration of Fc fragments required to produce 50% sensitization was found to be 0.07 mg/ml and the liminal concentration was 3×10^{-4} mg/ml.

PLANS FOR FUTURE

A. TOXINS: (a) to continue purification of MEPP factor of tetanus toxin, and enzymes of sea urchin toxin; (b) to continue studies on mode of biochemical action of the various enzymes present in SUT on purified natural substrates and on artificial substrates to determine the specific point of attack; (c) to expand the immunochemical studies of these 3 component (antigen-antibody-enzyme) systems; to study the mechanism of Ach release by streptolysin under various experimental conditions.

B. ANAPHYLAXIS: To continue studies of the mechanism of tissue sensitization under "minimal" conditions, i.e. Fc fragments and Fab fragments having anti Fc specificity; to continue studies of the penicillin anti-penicillin system with blocking haptens.

CURRENT REPORTS AND PUBLICATIONS

R. L. Parsons, G. A. Feigen, W. W. Hofmann, and Bruce Reitz, "Presynaptic Effects of Crude Tetanus Toxin, Guanidine, and Temperature at the Mammalian Neuromuscular Junction", Fed. Proc. 25, 1966.

G. A. Feigen, D. J. Prager, C. B. Nielsen, and G. G. Vurek, "Role of Temperature in Anaphylactic Sensitization of the isolated atrium and ileum: A Study of the Variables affecting Estimates of the Activation Energy". 'International Symposium on the Biochemistry of the Acute Allergic Reactions' Punta Ala, Italy, June 1967, to be published by Blackwells, December 1967.

G.A. Feigen, E. Sanz, Roberta L. Meyers, and D. H. Campbell, "The Specific Inhibition of *in vitro* Anaphylaxis and Histamine Release by Dialyzable Material from Ragweed Pollen", Int. Arch. Allergy, 32:174, 1967.

G. G. Vurek, D. J. Prager, and G. A. Feigen, "Antibody Concentration and Temperature as Determinants of *in vitro* Sensitization and Histamine Release in Isolated Cardiac Tissues" (Accepted by J. Immunol.)

BIOPHYSICAL AND BIOCHEMICAL STUDIES
OF MARINE TOXINS

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ASSISTED BY

WORK UNIT NO. NR 305-743

CONTRACT Nonr 3985(02)

OBJECTIVES

- (a) To study the biological actions of naturally occurring toxins,
- (b) to purify and identify their chemical composition.

ABSTRACT

Studies have continued on the toxic factors in the algae Prymnesium parvum and Gymnodinium breve.

(a) In Prymnesium, emphasis has been placed on the processes of inactivation of the toxic factors. The toxin is purified by a series of processes which include extraction by spinning off the cells followed by their lyophilization, pigment and lipid removal with acetone and dissolution of the toxin in alcohol. The toxin is assayed in three ways, (1) hemolysis of red blood cells, (2) inactivation of the response of guinea pig gut muscle to acetylcholine and, (3) fish toxicity. Although solutions of the toxin have no noticeable absorption peak in the visible range, the toxicity decreases markedly when the solutions are exposed to bright lights.

Studies have been carried out by Dr. Dutta, Mr. Arthur Baeder, and myself on the kinetics of this inactivation and on the effects of the external environment. Inactivation studies have been made on all three assay systems under conditions of light and dark, under conditions aerobic and anaerobic, and at room temperature and 0°C. The experimentation is of necessity lengthy and is still in progress. It is certain, however, that while light does speed inactivation, the factors of temperature and oxidation are dominant. At 0°C and in nitrogen the toxin is very stable.

(b) Studies on Gymnodinium breve have been delayed by problems of mass culture. Although test tube colonies thrive, the cells are much less viable in large flasks, being very sensitive to temperature fluctuations. With the slow growth rate, the chance of contamination is high.

Toxicity studies have been extended to the central nervous system of cockroaches. Topical application sends the ventral nerve chain into a steady high frequency oscillation of over 200 discharges per second which persists for long periods. The precise mode of action is being investigated but is in agreement with studies on the motor nerve and muscle endings where the toxin induces depolarization.

(c) Other toxins. Two other studies of natural toxins are being carried out on the same program.

Mr. William Kem, a graduate student and Fellow, has been studying the neurotoxins present in a phylum of marine worms (Rhynchocoela), with the intention of utilizing them on the analysis of membrane excitation phenomena. One toxin, "amphiporine," has been purified and studied to the extent that a structure has been proposed. Structural information was obtained by a combination of spectral measurements (UV, IR, NMR, and mass spectrum) and chemical tests on a picrate salt (MP 175°). The proposed structure is a 3-substituted pyridine compound and its properties are presently being compared with the synthetic compound. This neurotoxin induces repetitive spike activity in arthropod axons and also causes contraction of the frog rectus abdominis (d-tubocurarine is antagonistic to this effect). The mechanisms for these actions will be investigated by intracellular microelectrode methods. Another neurotoxin, "nemertine" is also under investigation.

Mrs. Ann Snyder, also a graduate student and Fellow, has been engaged on the Gymnodinium breve toxin project. At the same time, she has been studying the action on excitable membranes of a toxin found in the hemolymph of a potato beetle. This toxin has been purified by Drs. Shaw and Fraenkel of our Entomology Department and is a polypeptide or protein of about 20,000 M. Wt. It has a toxic paralyzant action on mice which is slow in action, it is toxic to other arthropods. Blockage occurs at nerve muscle junctions but the most dramatic action is on the vertebrate heart which ceases contraction very quickly with no depolarization.

(d) When this contract was first originated, it included studies on the energetics of nerve tissue. This work is being continued although without financial support.

The problem of what is involved when an action potential occurs is understood in electrical and ionic terms but nothing still is known of how the membrane changes occur. All we know has been obtained from thermal measurements which indicate a heat liberation with depolarization and absorption proportional to repolarization. We are studying this problem still, using Garfish olfactory nerve which has a large number of small non-medullated fibers of uniform size. This work, we feel, is important and significant and hope eventually for its support.

PLANS FOR FUTURE

Algal toxins In early 1968, Dr. Zvi Paster is joining the group from the Hebrew University of Jerusalem as a postdoctoral fellow. He has specialized in study of a purification of the toxin from Prymnesium parvum. He has isolated and purified one component, and will undertake chemical identification of the compound as well as study its physiological action.

BIOCHEMISTRY OF PHYSIOLOGICALLY ACTIVE PRINCIPLES IN ECHINODERMS

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WORK UNIT NO. NR 305-757

CONTRACT Nonr 3128(00)

OBJECTIVES

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

ABSTRACT

An examination of the nmr spectra of holothurin A and desulfated holothurin A, each of which shows strong signals (ca. 1.70 ppm) for methyl groups on a double bonded carbon with their corresponding dihydro-derivatives, which lack this signal and have in its place a new doublet centered at 0.85 ppm ($J = 6$ cps), has established that the glycosides of holothurin A contain in the main open side-chains, terminating with an isopropylidene group. Enzymatic solvolysis of desulfated holothurin A furnished the neo-holothurinogenins (20 %) and neo-holothurin xylosides (80 %). The neo-holothurinogenins represent the steroid moiety as it exists in the holothurins. Chromatography of the acetylated neo-holothurinogenins (on Silica gel) gave ca. 6 compounds, one of which (main product) furnished on strong acid hydrolysis the known holothurinogenin I. This compound thus represents the steroid moiety as it exists in a particular holothurin of holothurin A mixture. Interestingly from the holothurin xylosides (see above), there was obtained primarily holothurinogenin U on strong acid hydrolysis. It would therefore appear that in the enzymatic hydrolysis of holothurin A, only those glycosides, which give holothurinogenin I, are completely split with release of the 4 sugars (xylose, glucose, 3-O-methylglucose, and quinovose), while those glycosides, which furnish holothurinogenin U are only affected to the extent of removal of the sugars mentioned above, excluding xylose. The mild hydrolysis of holothurin A with methanolic HCl furnished a variety of mono-methoxylated and dimethoxylated neo-holothurinogenins. Examination of the nmr spectra of this mixture revealed the presence of differently located methoxyl substitution. It was established that one of the

methoxyl groups was on the side-chain (addition to the isopropylidene group), while the location of the other has not yet been ascertained. We are investigating the methoxylated neo-holothurinogenins, since they may be prepared in high yield and are at present separating them by column chromatography, so that we may establish the interrelationship with the known holothurinogenins. Further studies on the role of the sulfate charge in the irreversible interaction of holothurin A with chemoreceptors have been carried out in conjunction with Dr. Friess of the Naval Medical Research Institute, Bethesda, Md. One of the principal differences between holothurin A and desulfated holothurin A is the reversibility of blocking action of desulfated holothurin and irreversibility of holothurin A.

We have recently examined the toxic principle, Asterosaponin A and B, from the starfish *Asterias amurensis* (sample kindly provided by Prof. Hashimoto, University of Tokyo, Japan) and have confirmed his conclusion that, while these compounds are steroid glycosides, which closely resemble the holothurins, they are substantially different in the nature of their steroid moiety. Interestingly, however, their activity re: nerve-diaphragm closely parallels that of holothurin.

The occurrence in the oceans of the world of these related neuro-poisons, their significance and effect on the ecology of the area are subjects of importance and deserve further investigation.

PLANS FOR FUTURE

To extend our biochemical studies to physiologically active principles of marine origin.

CURRENT REPORTS AND PUBLICATIONS

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STUDIES ON THE COAGULANT ENZYMES OF FRESH SNAKE VENOMS

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ASSISTED BY J. Bonner

WORK UNIT NO. NR 305-776

CONTRACT NOOO14-67-A-0449-0001

OBJECTIVES

This investigation will study venoms from various land and marine snakes for procoagulant activity. Venoms exhibiting procoagulant activity will be fractionated and studies done to characterize the active component chemically, physico-chemically and in regard to toxicity.

ABSTRACT

Several species of snakes are known to produce venoms which cause the blood to clot. Envenomation by these snakes often induces profound circulatory disturbances, e.g. shock, gangrene and necrosis.

At the time of this report, eighty-three venom extractions from *Bothrops atrox* have been obtained. We expect to obtain another hundred extractions of *Bothrops atrox* venom. Fifty *Bothrops jararaca* have been purchased and we expect to have sufficient venom to begin studies in March or April 1968. Small quantities of venoms from *Naja naja*, *Bungarus caeruleus*, *Vipera russellii*, *Echis carinatus* and *Cerastes cornuta* have also been obtained.

All venoms used have been frozen within thirty minutes of collection in liquid nitrogen (LN₂). Venoms are stored over LN₂ at -130°C. LN₂ freezing and storage has been shown to be superior to ordinary deep freezing and lyophilization.

We are trying to develop a standardized format for the characterization of procoagulant venoms. Such a study includes the following major phases: 1) characterization of the whole venom, 2) isolation of the coagulant enzymes, and 3) characterization of the isolated coagulant enzymes by biological and physico-chemical methods.

Whole venom from *Bothrops atrox* has been characterized as follows: 1) quantity of venom per extraction, 2) specific gravity, 3) total solids, 4) bound H₂O, 5) colloid solids, 6) spectra, 7) coagulant enzyme content, 8) discontinuous electrophoresis pattern under various conditions, 9) toxicity (intravenous and intraperitoneal, mouse), 10) microbiological assay, and 11) immunodiffusion (Ouchterlony). The fresh *Bothrops atrox* venom has been shown to hydrolyze only the fibrinopeptide A bond of fibrinogen and not the B fibrinopeptide bond as does thrombin.

Fractionation studies are underway employing ion exchange and gel-permeation chromatography. Physico-chemical and kinetic studies await the availability of a homogeneous, high specific activity fraction.

PLANS FOR FUTURE

During the coming year, our studies will center on the venoms of *Bothrops jararaca* and *Bothrops atrox*. Other venom may be collected, but they will be stored in LN₂ for later use. The kinetic study of the *Bothrops* venoms and chemical studies on other venoms are projected for the last half of the second and the whole third contract years.

AFLATOXINS IN MAMMALIAN CELL SYSTEMS

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WORK UNIT NO. NR 305-779

CONTRACT N00014-67-C-0252

OBJECTIVES

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 mammalian tissue culture cells; and
2. The synthesis of the different RNA components.

ABSTRACT

Aflatoxins have been produced by culturing Aspergillus flavus, ATCC No. 15548 in growth medium consisting of 20% sucrose and 2% yeast extract. The compounds have exhibited hepatotoxic and potential carcinogenic properties. The biological effects on cell growth, cell morphology, mitotic division, and DNA synthesis in cultured human embryonic lung cells have been reviewed recently.

We have undertaken a study of the kinetics of RNA synthesis in the sub-cellular fractions of HeLa S cell cultures exposed to different levels of aflatoxin B₁. At higher concentrations (1 and 4 ppm) of aflatoxin B₁ the specific activity of RNA found in the nuclear fraction rapidly decreased from four to twelve hours of incubation. At lower concentrations (0.1 and .5 ppm) specific activity decreased after eight hours of incubation. The specific activity of RNA in the mitochondrial fraction decreased rapidly from four to twelve hours of incubation at the 4 ppm aflatoxin level, whereas at lower aflatoxin levels the specific activity did not begin to decrease until after 8 hours of incubation. The specific activity of RNA in the microsomal fraction decreased from 4 to 8 hours of incubation and increased from 8 to 12 hours of incubation. The trend of the specific activity changes of RNA in the soluble fraction were opposite. The effect of different levels of aflatoxin on the specific activity of RNA in each sub-cellular fraction varied with the time of exposure of the cells to aflatoxins.

PLANS FOR FUTURE

1. To produce sufficient quantities of aflatoxins.
2. To continue work on the kinetics of DNA, RNA, and protein synthesis of mammalian cells in tissue culture exposed to aflatoxin.

CURRENT REPORTS AND PUBLICATIONS

Chung, R. A., R. W. Brown, L. H. Beacham, and I-Lo Huang. Amino Acid Metabolism of Different Mammalian Cell Lines. Canad. J. Biochem. 44:1145-1157 (1966).

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

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WORK UNIT NO. NR 305-780

CONTRACT N00014-67-0299-0005

OBJECTIVES

(a) To isolate the toxic principles of sea snake venom, (b) to determine chemical properties of toxic principles, (c) to investigate enzymatic properties of sea snakes of different geographical origin and of different species and genus.

ABSTRACT

Sea snakes were captured in Southeast Asia and the Far East and the venoms were extracted from the venomous glands. The yields of venom varied considerably depending upon the species of snakes. Yield and toxicity according to LD₅₀ are as follows: Enhydrina schistosa from the Gulf of Thailand (8.1 mg venom/snake), Pelamis platurus from Formosa (2.0 mg venom/snake, LD₅₀ 0.18), Lapemis hardwickii from the Gulf of Thailand (5.2 mg venom/snake, LD₅₀ 0.71), Aepyurus eydouxii from the Gulf of Thailand (0.6 mg venom/snake), Hydrophis cyanocinctus from Hong Kong (2.1 mg venom/snake). The extractions of other sea snake glands and toxicity test are still in progress.

Cytolytic activity of snake venoms from each family was tested on bovine spleen cells and mouse embryo cells in tissue culture. In contrast to venoms of Crotalidae and Viperidae, snakes (Hydrophiidae) and Elapidae venoms did not damage the cells.

In order to study the property of exopeptidase, venoms of 49 snakes (49 species and subspecies and 4 families) were investigated using L-leucyl- β -naphthylamide as substrate. Sea snake venoms as well as the venoms of other families hydrolyzed this substrate. The enzyme which hydrolyzes this substrate is quite common in snake venoms.

Antigenic properties of sea snake venoms were studied by immunodiffusion. Enhydrina schistosa (ES) venom from Malaya (the Strait of Malacca) formed one strong precipitin band against its own antibody. Pelamis platurus venom from northern Formosa had cross-reaction with anti-ES but the precipitin band did not fuse with that of ES. Venoms of Lapemis hardwickii from the Gulf of Thailand showed 2 precipitin lines of which none of them fused with that of ES venom while one of the lines fused with that of P. platurus. E. schistosa venom also fused together with H. cyanocinctus. It thus appears that L. hardwickii and P. platurus are phylogenetically closer to each other than to E. schistosa. However, E. schistosa appears closer to H. cyanocinctus than to the other snakes.

PLANS FOR FUTURE

(a) Snake venoms will be fractionated and the toxic principle will be isolated for further chemical study. (b) The presence or absence of various enzymes in sea snake venoms will be investigated. (c) Immunological properties of sea snake venoms of different geographical origins and their isolated components will be further studied using immunodiffusion and immunoelectrophoretic techniques.

CURRENT REPORTS AND PUBLICATIONS

(a) A. T. Tu and P. M. Toom (1967), "The presence of a L-leucyl- β -naphthylamide hydrolyzing enzyme in snake venoms." *Experientia*, 23, 439-440

(b) A. T. Tu, P. M. Toom, and S. Ganthavorn (1967), "Hemorrhagic and proteolytic activities of Thailand snake venoms." *Biochemical Pharmacology* 16, 2125-2130.

(c) A. T. Tu and D. S. Murdock (1967), "Protein nature and some enzymatic properties of the Lizard Heloderma suspectum suspectum (Gila Monster) venom." *Comparative Biochemistry and Physiology*, 22, 389-396

(d) A. T. Tu and P. M. Toom (1967), "Hydrolysis of peptides by snake venoms of Australia and New Guinea." *Australian J. of Exper. Biol. and Med. Sc.*, in press.

(e) A. T. Tu, P. M. Toom, and D. S. Murdock (1967), "Chemical differences in the venoms of genetically different snakes." *Animal Toxins*, Edited by F. E. Russell and P. R. Saunders, Pergamon Press, Oxford, England, page 351-362.

(f) A. T. Tu and P. M. Toom (1967), "Hydrolysis of peptides by *Crotalidae* and *Viperidae* venoms." *Toxicon*, in press.

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(i) A. T. Tu, J. A. Reinoso, and Y. Y. Hsiao (1968), "Peroxidative activity of hemepeptides from horse heart cytochrome c." *Experientia*, in press.

STINGRAY VENOM

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ASSISTED BY

WORK UNIT NO. NR 305-786

CONTRACT N00014-67-C-0391

OBJECTIVES

To Isolate and Characterize the Venom of the Stingray.

ABSTRACT

None Received.

SCULPIN TOXIN

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ASSISTED BY

WORK UNIT NO. NR 305-793

CONTRACT N00014-67-C-0390

OBJECTIVES

To study the sculpin, its activities, predator-prey relationships, habitats, etc. as well as the isolation and characterization of the toxin and development of suitable countermeasures.

ABSTRACT

None received.

THE CHEMISTRY OF COELENTERATE TOXINS AND THEIR
MODE OF ACTION

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ASSISTED BY

WORK UNIT NO. NR 305-807

CONTRACT NO0014-67-A-0298-0014

OBJECTIVES

Purification of coelenterate toxins. Characterization of the same toxins: molecular weight, isoelectric point, etc. Study of non-protein components of coelenterate venoms, particularly tetramine. Determination of the conditions of toxin attachment to the nerve membranes. Extension of previous physiological investigations to vertebrates. Determination of the site and mode of action of toxins.

ABSTRACT

The work is just beginning. The following first steps are to be employed:

a) Modification of a small-scale purification of Condylactis toxin to batch-wise techniques. Characterization of the toxin by physicochemical means. Purification of toxin from at least one other coelenterate, possibly Dactylometra. In these studies standard chromatographic techniques, gel-filtration, ion-exchange, adsorption, will be used.

b) Tetramine assays and extraction by thin-layer chromatography. Physiological investigation of the effect of tetramine on the action potential, particularly as compared with tetraethyl ammonium ion. This can be approached by standard electrophysiological methods, particularly intracellular recording using microelectrodes.

c) Comparison of the action of numerous other toxins with the action of Condylactis toxin on action potentials in crustacean nerves. This also will be by standard intracellular recording although some voltage-clamping will be undertaken.

d) Comparison of this plateau-forming action on crustacean nerves with neurophysiological action on vertebrate nerve and muscle. Techniques will be similar. Since some of these toxins attach irreversibly to the nerve membrane and appear to affect only one nerve parameter, the conditions for attachment, and the kinetics as well, will be investigated.

AUTHOR INDEX

Author	Page	Author	Page
Abbott, B. C.	86	Mcrita, R. Y.	20
Albanese, A. A.	32	Nakada, H. I.	16
Araki, G. S.	18	Nasatir, M.	22
Atassi, M. Z.	54	Nat'l Acad. of Sciences 74,75	
Callaghan, O. H.	34	Neurath, H.	4
Chanley, J. P.	88	Nowinski, W. W.	40
Chung, R. A.	92	Omori, G.	72
Converse, J. M.	62	Papernmaster, B. W.	59
Copley, A. L.	90	Rapaport, F. T.	62
Erlanger, B. F.	57	Rice, F. A. H.	38
Feeney, R. E.	60	Rittenberg, D.	25
Feigen, G. A.	83	Roberts, E.	10
Fudenberg, H. H.	64	Russell, F. E. 96,97	
Fuller, G.	80	Schwartz, S. L.	76
Gaffron, H.	31	Segre, D.	50
Graff, S.	27	Shapiro, B. I.	98
Hastings, J. W.	12	Singer, T. P.	6
Haurowitz, F.	44	Soloway, A. H.	78
Kabat, E. A.	46	Steiner, R. F.	41
King, T. E.	9	Stern, R. L.	78
Kisken, W. A.	69	Trapani, I. L.	52
Lal, H.	80	Triplett, E. L.	67
Iachini, B. W.	90	Tu, A. T.	94
Mahler, H. R.	29	Uyeki, E. M.	36
Mayer, M. M.	48	Watt, D. D.	71
Meeuse, B. J. D.	14	Williamson, M. B.	26
Moore, W. J.	29		

INDEX OF CONTRACTOR INSTITUTIONS

American University	38
Burke Relief Foundation	32
California, University of, Berkeley	59
California, University of, Davis	60
California, University of, San Francisco	6, 64
California, University of, Santa Barbara	16, 67
Chicago Medical School	34
City of Hope Medical Center	10
Colorado State University	94
Columbia University	25, 27, 46, 57
Florida State University	31
Harvard University	12, 98
Illinois, University of	50, 86
Indiana University	29, 44
Johns Hopkins University	48
Kansas University Medical Center	36
Loyola University	26
Los Angeles County Hospital	96, 97
Midwest Research Institute	71
Mt. Sinai Hospital	88
National Academy of Sciences	74, 75
National Jewish Hospital, Denver	52
Naval Medical Research Institute	41, 76
New York Medical College	90
New York University Medical Center	62
New York, State University of	54
Northeastern University	78
Oregon State University	9, 20
Osaka City Institute of Hygiene	72
Rhode Island, University of	80
San Francisco State College	18
Stanford University	83
Texas, University of	40
Toledo, University of	22
Tuskegee Institute	92
Washington, University of	4, 14
Wisconsin, University of	69