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PROGRESS REPORT
ABSTRACTS



MICROBIOLOGY BRANCH
Office of Naval Research



JANUARY 1968

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FOREWORD

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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 F. Acker

ROBERT F. ACKER, Ph.D.
Head, Microbiology Branch
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ENVIRONMENTAL MICROBIOLOGY

Research listed in this section is concerned with microbiology as it applies to the operating environment. It is important for the Navy, for example, to know the nature of microbial transformations in the oceans and how they affect man's performance and man's equipment in the depths of his "inner space." Studies under this program help establish which organisms are responsible, and under what specific conditions, for the initiation and/or stimulation of fouling, corrosion, or any other form of microbial deterioration in the marine ecology.

A STUDY OF THE MICROENVIRONMENT AND METABOLISM
OF FISH GNOTOBIOTES

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ASSISTED BY N. Ronald Walters

WORK UNIT NO. NR 103-652

CONTRACT Nonr-228(31)

OBJECTIVES

- (a) Surgical derivation and maintenance of a colony of germfree Xiphorhorous helleri, a species of ovoviviparous tropical fish;
- (b) A study of the anatomical, physiological and immunochemical aspects of the germfree fish vs. the conventionally reared fish;
- (c) A determination of the specific modes of microbial pathogenesis by selective contamination of the germfree host's environment.

ABSTRACT

The surgical excision of prenatal Xiphorhorous helleri by techniques developed in our laboratory has permitted the consistently aseptic, viable delivery of the test host. More than three hundred neonates have been successfully cesarian-derived to date. The continuing difficulties of rearing the germfree fish beyond ca. three months is believed due to factors similar to those experienced earlier with germfree rodent hosts: inadequate nutrition and postnatal maladaptation to the germfree environment.

We have repeatedly found that the surgically derived fish in control flasks which have become contaminated with ambient bacteria from the surgical area did not develop a 'tail spiking' syndrome which precedes the death of the germfree fish. Current studies utilizing these bacterial cultures may demonstrate that their presence provides a required nutritional cofactor or assimilates substrate toxic to the physiology of the germfree host.

Experiments have continued in an effort to provide a more adequate nutritional source for the germfree host using synthetic or defined media. While synthetic, diluted sea water presently used appears to be an adequate aqueous environment, greater control over buffering capacity and gas exchange continues to be sought with different salt mixtures.

The investigation of our host's serum proteins using cellulose acetate electrophoresis in various buffer systems has been expanded to include a comparative study of a number of marine fishes and mammals. We feel that this technique may be a useful method for higher marine life speciation.

PLANS FOR FUTURE

Continuation of the above ongoing studies with emphasis upon the resolution of developing an adult germfree fish colony.

CURRENT REPORTS AND PUBLICATIONS

N.R. Walters and M.D.Appleman (1967), "Propagation and Comparative Study with a New Germfree Host: A Tropical Fish, Xiphorhorous helleri", Bacteriol. Proc., p. 68.

VIROLOGICAL AND RELATED PROBLEMS IN MARINE ANIMALS

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UNIVERSITY OF MIAMI SCHOOL OF MEDICINE
CORAL GABLES, FLORIDA

ASSISTED BY

WORK UNIT NO. NR 302-476

CONTRACT NONR 4008(05)

OBJECTIVES

THE TWO MAJOR OBJECTIVES OF THIS PROJECT ARE (A) BIOCHEMICAL AND MORPHOLOGICAL STUDIES ON THE REPLICATION OF LYMPHOCYSTIS VIRUS IN TISSUE CULTURES; AND (B) IMMUNOLOGICAL STUDIES WITH GRUNT FIN AGENT, AN ORPHAN VIRUS OF MARINE FISH CELL ORIGIN.

ABSTRACT

STUDIES WITH LYMPHOCYSTIS VIRUS. WE HAVE PREVIOUSLY REPORTED THE ISOLATION OF LYMPHOCYSTIS VIRUS FROM TUMORS OF FISHES OF THE FLORIDA STRAITS, ALL ISOLATIONS BEING MADE IN CELL LINES DERIVED FROM FIN TISSUES OF THE BLUE STRIPED GRUNT, A MARINE FISH. OF 9 SUCH CELL LINES OF VARYING IN VITRO AGES AND PASSAGE LEVELS, 7 (AGED 7 WEEKS TO 21 MONTHS, 4 TO 74 SUBCULTURES) WERE EQUALLY SUSCEPTIBLE TO THE CYTOPATHIC EFFECTS OF THE VIRUS; ONE (1 WEEKS OLD, 5 SUBCULTURES) WAS MUCH LESS SUSCEPTIBLE; AND IN ONE (7 YEARS OLD, 260 PASSAGES) THERE WAS NO CYTOPATHOLOGY.

BIOCHEMICAL STUDIES OF SOME OF THE EARLY EVENTS IN THE IN VITRO GROWTH OF THE VIRUS HAVE BEEN MADE. THESE INCLUDE DETERMINATION OF THE SYNTHESIS OF NUCLEIC ACIDS AND PROTEINS, AS MEASURED BY RATES OF INCORPORATION OF ^{14}C ADENINE AND ^{14}C LEUCINE AND BY BIOCHEMICAL ASSAYS. PEAKS OF RNA AND PROTEIN SYNTHESIS RESPECTIVELY OCCURRED 4 AND 5 DAYS AFTER INFECTION, AND THERE WERE AT LEAST 2 PEAKS OF DNA. DESPITE THEIR INCREASED RATES OF SYNTHESIS (AS COMPARED WITH THOSE IN EQUAL POPULATIONS OF NORMAL CELLS) THERE WAS NO ACCUMULATION OF THESE MACROMOLECULES, SUGGESTING THAT SYNTHESIS DURING EARLY INFECTION IS ACCOMPANIED BY A HIGH TURNOVER RATE. IN CONTRAST, ESTABLISHED IN VIVO TUMORS CONTAINED SIGNIFICANTLY LARGER AMOUNTS OF NUCLEIC ACIDS AND PROTEIN PER UNIT WEIGHT THAN DID NORMAL FISH TISSUES. THESE IN VITRO AND IN VIVO BIOCHEMICAL FINDINGS ARE COMPATIBLE WITH MICROSCOPIC OBSERVATIONS OF INFECTED TISSUE CULTURES: (A) IN THE EARLY STAGES OF INFECTION, THE CYTOPATHOLOGY CONSISTS ONLY OF ROUNDING AND INCREASED REFRACTIVENESS OF THE CELLS. ONLY A FEW OF THESE CELLS ARE ENLARGED, AS OBSERVED IN SITU AND BY SIZE DISTRIBUTION IN A COULTER COUNTER. (B) LATER STAGES OF INFECTION ARE CHARACTERIZED BY ENORMOUS CELLULAR HYPERTROPHY (DIRECT OBSERVATIONS) AND BY MARKEDLY INCREASED AMOUNTS OF DNA AND RNA (SHOWN BY ACRIDINE ORANGE STAINING).

THE SIGNIFICANCE OF THESE FINDINGS LIES IN THE FACT THAT CELLULAR GIANTISM IN THE MORPHOLOGIC AND BIOCHEMICAL SENSES IS APPARENTLY THE RESULT OF SOME LATE EVENTS WHICH, AT LEAST IN TISSUE CULTURES, CAN BE SEPARATED FROM THE EARLY EVENTS ASSOCIATED WITH VIRUS INFECTION. THIS

OPENS A NEW APPROACH TO INVESTIGATIONS ON LYMPHOCYSTIS VIRUS WHICH, IN ADDITION TO BEING ONE OF THE FEW VIRUSES CAUSING DISEASE IN FISHES, POSSESSES THE INTRIGUING FEATURES OF (A) BEING A DNA-CONTAINING VIRUS WHICH REPLICATES IN THE CYTOPLASM, AND (B) INDUCING PROFOUND CHANGES IN THE CELL (ENORMOUS CELL HYPERTROPHY; ACCUMULATION OF RNA; SYNTHESIS OF A NEW STRUCTURE, A CAPSULE) THAT ARE NOT ENCOUNTERED IN OTHER VIRUS-CELL INTERACTIONS.

STUDIES WITH GRUNT FIN AGENT (GFA). THE ISOLATION AND SOME OF THE PROPERTIES OF THIS AGENT HAVE BEEN PREVIOUSLY DESCRIBED, AS HAS OUR DIFFICULTY IN PRODUCING A GOOD ANTISERUM IN ANY OF SEVERAL ANIMAL SPECIES. MORE RECENT ATTEMPTS AT ELICITING AN IMMUNE RESPONSE HAVE BEEN MORE FRUITFUL. INOCULATION OF A TOTAL OF APPROXIMATELY 10^8 TCID₅₀ OF CRUDE VIRUS, ADMINISTERED IN 15 DOSES OVER A PERIOD OF 5 1/2 MONTHS, RESULTED IN SIGNIFICANT ANTIBODY PRODUCTION IN 2 OF 4 RABBITS AND 2 OF 4 GUINEA PIGS, THE POST-IMMUNIZATION SERUM NEUTRALIZATION TITERS BEING 1:25-1:125. SERA OF OTHER ANIMALS INOCULATED ON THE SAME SCHEDULE WITH NORMAL CELL LYSATES WERE WITHOUT SIGNIFICANT NEUTRALIZING ACTIVITY. MARINE FISHES, HOWEVER, SHOWED NO DEMONSTRABLE ANTIBODY RESPONSE TO GFA WHEN INOCULATED AT 7-10 DAY INTERVALS OVER A PERIOD OF TWO MONTHS.

CHROMOSOMAL ANALYSES OF MARINE FISH TISSUE CULTURE CELLS. ONE OF THE MOST INTERESTING ASPECTS OF THE STUDIES OF MARINE FISH CELL LINES IS THE OBSERVATION THAT THE CELLS REMAIN NEAR DIPLOID UPON PROLONGED IN VITRO CULTIVATION. KARYOTYPE ANALYSES IN OUR LABORATORY AND IN COLLABORATION WITH DR. J.D. REGAN OF THE OAK RIDGE NATIONAL LABORATORY SHOW PRIMARY CULTURES TO HAVE 48 CHROMOSOMES, ALL TELOCENTRIC. WITH TIME THERE IS A REDUCTION IN CHROMOSOMES TO A MODAL NUMBER OF 46 WITH 44 TELOCENTRICS AND 2 METACENTRICS. CELLS OF ONE LINE, REEXAMINED AFTER MORE THAN 250 PASSAGES OVER A PERIOD OF 7 YEARS, WERE STILL FOUND TO BE CHARACTERIZED BY THIS PSEUDODIPLOID STATUS OF 44 TELOCENTRIC AND 2 METACENTRIC CHROMOSOMES, INDICATING THEM TO BE KARYOTYPICALLY HIGHLY STABLE. MOREOVER, GRUNT FIN CARRIER CULTURES CHRONICALLY INFECTED WITH GFA AND INFECTIOUS PANCREATIC NECROSIS (IPN) VIRUS HAVE SHOWN NO DEVIATIONS FROM THIS NORM.

PLANS FOR FUTURE

AS A COMPLEMENT TO THE RESULTS SUMMARIZED ABOVE, BIOCHEMICAL AND MORPHOLOGICAL STUDIES WILL BE MADE OF THE LATER EVENTS IN LYMPHOCYSTIS REPLICATION IN TISSUE CULTURES. IT IS ALSO PROPOSED TO CHARACTERIZE THE NUCLEIC ACIDS FOUND IN THE LYMPHOCYSTIS GIANT CELLS. FLUORESCENT ANTIBODY TESTS WILL BE EMPLOYED TO LOCATE THE VIRUSES IN CHRONICALLY INFECTED CULTURES WHICH ARE DOUBLE-CARRIERS OF BOTH IPN VIRUS AND GFA. IN ADDITION, FURTHER EFFORTS WILL BE MADE AT PURIFICATION OF GFA AND DETERMINATION OF ITS PROTEIN CONTENT.

CURRENT REPORTS AND PUBLICATIONS

- (A) SIGEL, M.M., A.R. BEASLEY AND M.C. LAUNER. CYTOLOGICAL AND CYTOCHEMICAL STUDIES ON LYMPHOCYSTIS IN TISSUE CULTURE. IN VITRO, 2:153, 1966.
- (B) BEASLEY, A.R. AND M.M. SIGEL. INTERFERON PRODUCTION IN COLD-BLOODED VERTEBRATES. IN VITRO, (IN PRESS).
- (C) LOPEZ, D.M., A.R. BEASLEY, L.S. DIETRICH AND M.M. SIGEL. BIOCHEMICAL AND MORPHOLOGICAL ASPECTS OF THE EARLY PHASE OF LYMPHOCYSTIS INFECTION IN TISSUE CULTURE. FOR PRESENTATION AT THE 1968 ASM MEETING.

ISOLATION AND IDENTIFICATION OF PHOTOSYNTHETIC SULFUR BACTERIA

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ASSISTED BY Suharjo Haditirto

WORK UNIT NO. NR 103-480

CONTRACT Nonr-3969(00)

OBJECTIVES

(a) To isolate and maintain as many photosynthetic sulfur bacteria as possible, and other photosynthetic bacteria isolated incidental to this effort, (b) to devise methods and procedures for the characterization and identification of these organisms.

ABSTRACT

During the past year a new, 24-lane thermal-gradient block has been constructed using conventional refrigeration, and work has been initiated on thermoelectrically cooled and heated thermal-gradient blocks. Determinations of the temperature optima and limits, the pH limits, and the salt-concentration limits for the growth of Rhodospseudomonas gelatinosa, R. spheroides, R. capsulata, R. palustris, and R. viridis on complex media and on a defined medium containing different carbon sources has been completed. Similar studies have been initiated with Chromatium D and C. warmingii. Studies on an anaerobic biotype of Chlorella vulgaris which appeared in an enrichment culture for photosynthetic bacteria have been completed. This organism appears to inhabit the same ecological niche as that inhabited by the non-sulfur photosynthetic bacteria.

PLANS FOR FUTURE

To continue the above work, concentrating on the photosynthetic sulfur bacteria.

CURRENT REPORTS AND PUBLICATIONS

Edwin H. Battley and Suharjo Haditirto (1967), "Studies on the anaerobic growth of a biotype of Chlorella vulgaris." Antonie van Leeuwenhoek, 33 (in press).

BIOCHEMICAL INVESTIGATIONS OF A MARINE BACTERIUM AND ITS VIRUSES

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ASSISTED BY Lee Ann French (terminated 9/1/67) Edwin Walker (9/15/67 to present) and Mary Anne Hutchison

WORK UNIT NO. NR NR 103-611

CONTRACT NOnr 4413(00)

OBJECTIVES

We have previously reported the isolation and partial characterization of five viruses which infect Serratia marnorubra. These viruses are all large (ca 3400 A in overall length) and give small plaques and burst sizes. We have been trying to further characterize these viruses, to determine whether they are five distinct viruses, or five isolates of the same virus. Some additional work has been done on the metabolism of the host cells in respect of tryptophan synthesis as well.

ABSTRACT

Virus work. In view of previous work showing very long latent periods for each of these viruses, and the small size of plaques which made counting difficult, a study of the effect of host cell density in the lawns on plates was undertaken. The results of varying bacterial density in the lawns are shown in Table 1.

Table 1. Effect of host cell density on plaque size

Host cell density/ 3 ml lawn	7.4×10^8	7.4×10^7	7.4×10^6	7.4×10^5
Plaque size	1 mm and smaller	1-2 mm	2 mm	plaque barely visible

It is interesting to note that at a lawn density of 7.5×10^6 bacteria, the plaques are larger, more easily counted and the estimates of virus numbers are thus more exact and reproducible. It seems likely that disparities in our earlier work may have resulted from bacterial overgrowth which obscured some plaques and made enumeration difficult. This would be expected at high lawn density, and with viruses with latent periods longer than the generation time of the host cells. Secondly, the small burst size would further contribute, since few cells surrounding a lysed cell would become infected.

Previous attempts failed convincingly to distinguish these viruses from one another, although several parameters suggested that we were dealing with more than one virus. The electron microscope morphologies are somewhat different for several of them, two of the viruses consistently give mixed bursts containing a smaller flexible tailed virus, and early immunological work all suggested that there were at least three viruses.

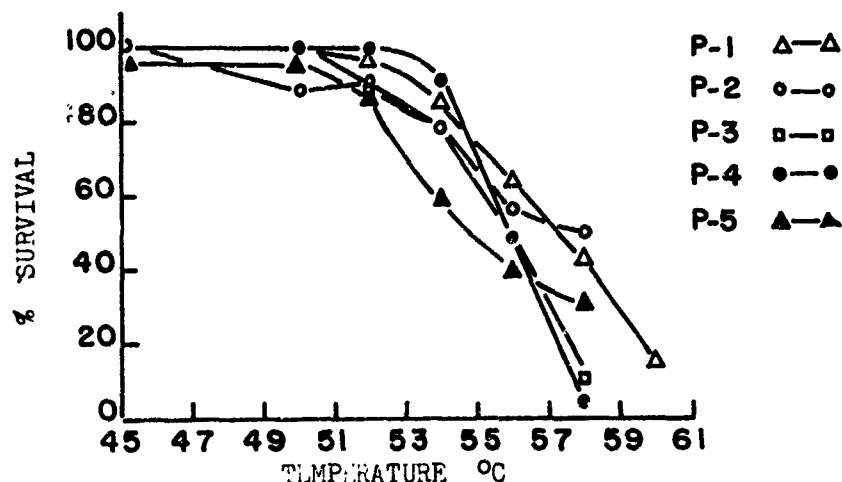
Two major lines have been attempted to add information on this question. The first is an extension of the immunological studies. Antisera have been prepared against each of the five viruses, and K values determined for each virus with both its homologous antiserum and the other four heterologous antisera. Results of these studies are in Table 2.

Table 2. K values of antiserum inactivation

	Phage tested				
	P1 ϕ	P2 ϕ	P3 ϕ	P4 ϕ	P5 ϕ
Anti P1	321.5	276.2	160.1	137.3	101.7
Anti P2	163.9	492.7	306.6	894.0	890.0
Anti P3	169.4	964.1	1461.0	1190.0	1628.4
Anti P4	-	554.9	377.4	920.0	460.1
Anti P5	261.8	1759.1	1209.8	653.7	1277.9

These data contribute little to the discrimination among the two alternatives. If these are separate viruses, they are closely related immunologically. There are some suggestions of differences among them, but certainly nothing sufficiently clear-cut to allow sweeping conclusions.

The second line of investigation was an assessment of the temperature lability of each of the viruses. These experiments were done in two ways. 1) Each virus was exposed to a variety of temperatures, for a fixed period of time and then quick chilled and assayed. A graph of pooled results of several experiments is presented in Figure 1.



There was some variation in the shape of the survival curves, and some indication of subtle difference among the five viruses. In order to amplify these subtle differences, a time of exposure study was set up using a fixed temperature. 56° C was chosen since most of the viruses showed between 40 and 60% survival at five minutes treatment at that temperature. 2) Each virus was carefully assayed to determine starting titer. Aliquots of 1 ml were delivered into a series of tubes, and the whole series placed in a water bath at 56° C. Tubes were removed at periodic intervals,

chilled and assayed. The time of treatment required to yield 50% survival are shown in Table 3.

Table 3. 50% inactivation times of P viruses held at 56° C

P-1	3 min.
P-2	16 min.
P-3	13 min.
P-4	6 min.
P-5	4 min.

The results of these studies confirm and extend the implications of the previous types of studies, that we are dealing with at least three viruses. None of these parameters by itself seems adequate to distinguish any given virus from another, but taken together, they support the notion that more than one virus with five separate isolations is involved here.

Tryptophan biosynthesis: The five enzymes of tryptophan biosynthesis have been demonstrated in Serratia. The chemical characteristics of the assays are so similar to those used for Escherichia coli as to be unworthy of note. The aggregation pattern of the enzymes as evidenced by migration in sucrose gradient centrifugation is markedly different, however. Anthranilate synthetase does not aggregate with PRtransferase as in E. coli, but migrates separately from all other enzymes. Moreover, PRtransferase, PRA-isomerase and InGPsynthetase all are found in the same fractions in the gradient. It is tempting to speculate that they do in fact form an aggregate, which is different from that found in E. coli. In E. coli, InGPsynthetase and PRAisomerase activities are both found in a single polypeptide chain.

Recent investigations have centered around the mode of derepression, in an attempt to establish whether or not we are dealing with an operon. Four of the five enzymes have given data consistent with coordinated derepression, suggestive that they might reside in an operon. The fifth enzyme, PRAisomerase in preliminary studies has not behaved in a coordinated fashion. There are two hypotheses which may be advanced to account for these results. 1) The gene responsible for directing synthesis of this enzyme does not reside in the same regulatory unit, or 2) the gene product is dependent upon aggregation with one of the other enzymes to become active.

Data derived from a study of the enzymatic activities of various mutants in our collection would favor the latter hypothesis. When the enzymatic activities of each of seven mutants in the tryptophan pathway were assayed, the results in Table 4 were obtained.

Table 4. Enzyme activities of tryptophan mutants

	Anthranilate Synthetase	PR Transferase	PRA Isomerase	IGP Synthetase	T'ase B	Suspected Lesion
53	+	+	+	+	+	T'ase A
82	+	-	-	-	-	?
143	+	-	-	-	-	?
150	+	+	+	-	+	IGP'ase
152	+	-	+	+	+	PRT'ase
160	+	+	+	+	-	T'ase B
199	+	+	+	+	-	T'ase B

It is clear from the table, that mutations affecting other of the activities in the tryptophan pathway simultaneously affect the PRA isomerase activity. Mutants 82 and 143 behave as though they were deletion or nonsense mutants in one of the genes, which lead to loss of all activities distal to the site of the mutation. Obviously final confirmation will come only when we are able to map the mutant genes in question, but the above results strongly support the second hypothesis to explain the apparent non-coordination.

PLANS FOR FUTURE

The virus work will proceed along lines to further distinguish among the viruses. High titer preparations have been made for DNA analysis. We propose in the near future to establish the base composition by two independent methods. Density gradient centrifugation will be carried out using a reference band of E. coli DNA, and melting temperatures will be determined. From these data, base compositions may be determined, and if they vary sufficiently, may provide the necessary final parameter in the description of the viruses.

The tryptophan work will also proceed along lines of isolating more mutants for analysis of enzyme composition, attempts at in vitro complementation studies among various pairs of mutants, and in the long range planning we will attempt some enzyme purification.

A STUDY OF INORGANIC SALTS REQUIREMENTS AND EFFECT ON
THE MORPHOLOGY OF MARINE BACTERIA

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WORK UNIT NO. NR 103-667

CONTRACT Nonr-4810 (00)

OBJECTIVES

(a) To investigate the role of inorganic ions in maintaining integrity of cellular structure of marine bacteria; (b) to determine fine structural changes imposed on obligately psychrophilic marine bacteria by heat treatments; (c) to probe the mechanisms determining the morphology of marine bacteria; and (d) to use the techniques of numerical taxonomy, electrophoretic analysis and DNA homology for identification and classification of marine bacteria.

ABSTRACT

Studies of the obligate marine psychrophile, Vibrio marinus MP-1, and the moderate psychrophile, Vibrio marinus PS-207, have revealed pronounced effects of heat treatment on the cellular fine structure. Whether heat treatment was drastic, i.e., subjection to temperatures of 40C or 50C for several minutes, or minimal, 24.5C for one minute, there was initially a rapid lysis of the more sensitive cells of the population, followed by gross changes in morphology of the remaining intact cells of the heat-treated population. Figure 1a shows the typical gigantic, rounded-up cell of V. marinus MP-1 observed after one minute at 24.5C. Membrane damage also occurred as seen in Figure 1b. V. marinus MP-1 grown at 4C was much more thermosensitive than when grown at 18C. It has been observed also that the cytoplasm of cells of MP-1, harvested after 48 hours at 4C, appeared more densely granular than cells after 48 hours growth at 18C. See Figure 2a. (Temperature range of MP-1 is 0C - ca. 24C.) After gentle heat treatment (10 minutes at 25.5C), membrane limited inclusions were observed in heat-treated 4C grown cells (Figure 2b). The inclusions were more numerous and of greater diameter with increasing time of exposure (up to 90 minutes) to elevated temperature (25.5C).

Structural effects resulting from growth in media of reduced ionic strength, i.e., concentration of NaCl, KCl, and/or MgCl₂ of 10⁻¹ or 10⁻² X that of standard seawater, were varied, depending greatly on the given strain studied. In general, without Mg⁺⁺, cells demonstrated walls and membranes of aberrant, "wrinkled" appearance.

Thin sections prepared from a variety of bacteria, viz., Pseudomonas, Achromobacter, Vibrio, Halobacterium, Sarcina, Agrobacterium, Rhizobium, Hydrogenomonas, Cytophaga, and Caulobacter spp., have revealed interesting comparisons of wall, membrane, and cytoplasmic structures. The halophilic cocci which have been examined possess extremely thick walls (~330 Å). Figure 2c is a section of Sarcina morrhuae OH 5.23. Cytophaga strain CB243 revealed the presence of numerous mesosomes, Figure 2d. CB243 was isolated from estuarine waters of Chesapeake Bay.

Electron micrographs of a Gram-negative bacterium isolated from the Chesapeake and Ohio Canal, strain COC-21, revealed a double-layered cell wall structure typical of the Gram-negative bacteria. The bacterial mucopolysaccharide included a hexosamine component, D-quinovosamine. The organism was identified and classified as Achromobacter georgiopolitanum N. SP. The overall DNA base composition, determined by buoyant density measurements in cesium chloride, was 41 moles %.

Properties of bacteria isolated from sediment samples collected at depths from 9,400 to 10,400 meters in the Philippine and Marianas trenches of the Pacific Ocean were determined. Thirty-eight isolates, for which 116 characteristics were determined for each isolate, were studied. Adansonian analysis revealed five distinct phenetic clusters, four of which were identified as species of Pseudomonas. The 38 strain data were compared with data for 132 marine strains previously subjected to computer taxonomic analysis.

PLANS FOR FUTURE

(a) To continue the Adansonian and molecular taxonomic studies of marine bacteria; (b) to continue studies of ionic conditions resulting in aberrant cell morphology; and (c) to determine the effects of growth temperature, nutrition, and suspending medium on the fine structure of obligate marine psychrophiles during heat treatment.

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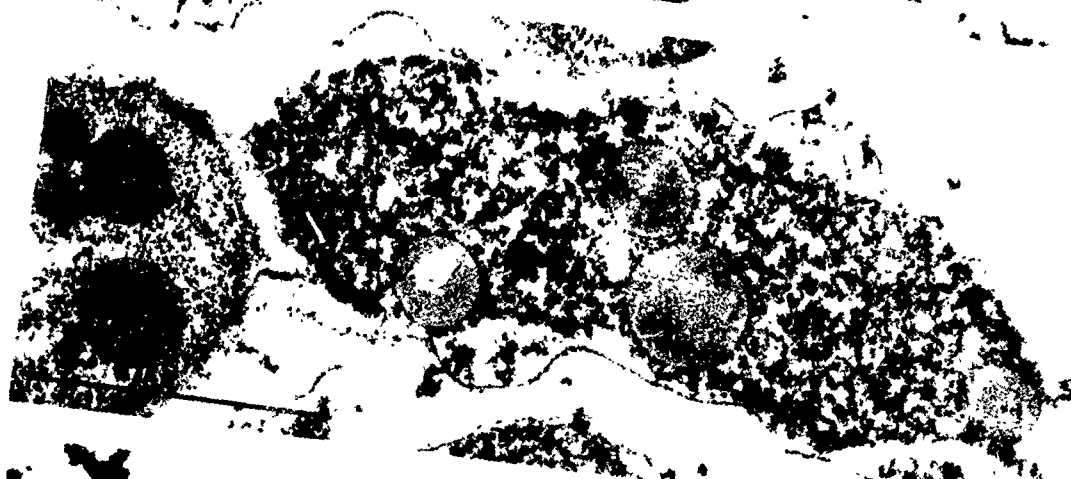
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2a



2b



2c



2d

12 a

HALOPHILIC BACTERIA

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WORK UNIT NO. NR

CONTRACT F61052 67 C 0085

OBJECTIVES

(a) To determine control mechanisms in metabolic pathways of halophiles, (b) to compare these mechanisms with those of ordinary microorganisms, (c) to study flagellar components, (d) to isolate and investigate DNA components.

ABSTRACT

Halobacteria are red, rodformed, polarly flagellated organisms, with an absolute requirement for at least 12 % salt for growth, able to live in saturated brine. Intercellular salt concentrations are of the same order of magnitude as Intracellular, with KCl as the major intercellular salt. (Christian & Waltho, Biochem. Biophys. Acta 65 506 (1962)). All halobacteria enzymes studied to date show zero activity in the absence of salt, with optimal activities sometimes at saturated salt concentration. One has established the presence of some enzymes of the Arginine pathway (arginine desimidase, ornithine transcarbamylase, argininosuccinase. (Dundas & Halvorson, J. Bact. 91 113 (1966)). (a) One has settled on Ornithine transcarbamylase (OTC) as a suitable enzyme for studying control mechanisms in the Arginine pathway. OTC has been studied in cell free extracts with specific activities of about 10^{-3} μ M citrulline formed per minute per γ protein. Activity is lost on dialysis against salt-free media, and is not regained on redialysis against 25 % NaCl or KCl. The enzyme shows maximum activity in about 20 % KCl, activity is always higher in the presence of KCl than with the same concentrations of NaCl. OTC activity is slowly lost in crude extracts at 37°C in 20 % KCl, but the presence of 0.1M ornithine stabilizes the enzyme under the same conditions. Carbamyl phosphate or citrulline do not seem to influence enzyme stability. The enzyme has been purified to a specific activity of about 10^{-1} μ M citrulline formed per minute per γ protein, by fractionation precipitation with acetone. The purified enzyme also lost activity when dialysed against low salt concentrations, but preliminary results indicate that this loss of activity is due to loss of protein during dialysis.

Enrichment cultures to obtain Halobacteria prototrophic for arginine and with simple nutritional requirements are

hampered by extensive crossfeeding. Strains prototrophic for Arginine have been obtained, but they all exhibit rather exacting nutritional requirements, several aminoacids being necessary for satisfactory growth. (b) Halobacterium salinarium strain 1 is a polarly flagellated rod. Flagella can be sheared from the organisms by treatment of the culture in a waring blender. Fractional centrifugation has been used to harvest the flagella, but such preparations are heavily contaminated with cell debris regardless of how carefully the fractional centrifugation is carried out. Incubation of cultures at room temperature without aeration allows the accumulation of considerable amounts of flagella, unattached to bacteria. These flagella can be harvested by centrifugation, and the preparations appear nearly pure as controlled by electron microscopy. The flagella show strong tendency to parallel aggregation in media with high salt concentrations. Lowering the salt concentration to about 8 % NaCl causes dissociation of the flagellar aggregates, without any apparent disorganization of the structure of individual flagella.

PLANS FOR FUTURE

(a) To proceed with the purification of OTC, studying what mechanisms are effective in the control of enzymatic activity in vitro. To study in vivo control of OTC activity using Arginine auxotrophic mutants, preferably with simple nutritional requirements. (b) To develop methods for preparation of larger samples of pure Halobacterium flagella. To establish the amino-acid composition of the flagella and to study the effect of pH, temperature and salinity of the environment on flagellar structure. (c) To study the cross-hybridization of DNA and RNA from various Halobacterium species, to isolate different DNA species from Halobacterium salinarium strain 1 and investigate which RNA species can hybridize with the different DNA isolated.

CATABOLISM OF ALGINIC ACID AND MANNURONIC ACID
BY MARINE AND TERRESTRIAL MICROBIAL ISOLATES

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WORK UNIT NO. NR 103-624

CONTRACT Nonr-3677(03)

OBJECTIVES

(a) To isolate marine and terrestrial microorganisms that can catabolize alginic acid and its major subunit, mannuronic acid, (b) to identify and classify these isolates, (c) to elucidate the catabolic pathways utilized for the microbial dissimilation of these compounds, and (d) to compare these findings to those reported by other investigators for other living systems.

ABSTRACT

Gram-positive alginolytic bacterial species were isolated from soil. Two of these, identified as Arthrobacter and Streptomyces species, were studied. Resting Arthrobacter cells oxidized alginate only when they had been pre-induced. Alginolytic activity was demonstrated in cell-free extracts from non-induced cells, but this activity was much increased by growth in alginate. Glucose appeared to inhibit alginate utilization by Arthrobacter resting cells.

Catabolism of alginate by Arthrobacter and Streptomyces is similar, if not identical, to that of the Pseudomonas of Preiss and Ashwell, which produced a series of unsaturated oligo-uronides and the unsaturated monomer 4-deoxy-L-erythro-5-hexoseulose uronic acid. Further degradation involved an NADPH-linked dehydrogenase to produce 2-keto-3-deoxy gluconate.

Evidence was presented for the pathway of glucuronate dissimilation by Arthrobacter, showing isomerization to fructuronate as the first step. Then reduction occurred via an NADPH-linked dehydrogenase to produce mannonate, followed by probable dehydration to form 2-keto-3-deoxy gluconate. The cofactor involved differs from that previously reported in glucuronate degradation by Escherichia coli. Alginate was found to be an inducer for glucuronate isomerase in Arthrobacter but not in Streptomyces.

The Arthrobacter and Streptomyces isolates and an alginolytic Vibrio, although unable to grow in mannuronate, all produced enzymes capable of degrading mannuronate. Cell-free extracts from these three species produced immediate logarithmic oxidation of NADPH in the presence of mannuronate, suggesting direct reduction of mannuronate.

Evidence against direct reduction of mannuronate was also obtained,

with some indications that fructuronate is an intermediate. It was proposed that fructuronate might remain enzyme-bound and thus not appear in chromatographic analysis.

The product of dehydrogenase activity in the presence of mannuronate and Arthrobacter cell-free extracts appeared to be mannonate. The cofactor involved here was NADPH; no activity was noted with NADH. The next mannuronate breakdown product is 2-keto-3-deoxy gluconate, according to the evidence obtained. Optimal pH for dehydrogenase activity of a partially-purified Arthrobacter crude enzyme was 6.8.

The catabolism of mannuronic acid by a soil isolate tentatively identified as a species of Aeromonas was also studied. Evidence was obtained that mannuronate was directly reduced to mannonate without prior isomerization to fructuronate. While either NADH or NADPH could serve as cofactor, the rate of the reaction was several fold faster when NADP served as cofactor.

PLANS FOR FUTURE

Studies will be continued to further elucidate the catabolic pathway of mannuronate dissimilation by Aeromonas. Attempts will be made to partially purify the key enzymes involved and to isolate and identify the key intermediary metabolic products.

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THE MICROBIOLOGY OF MANGANESE NODULES

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WORK UNIT NO. NR NR 103-655 CONTRACT Nonr-591(22)

OBJECTIVES

(a) To investigate the role of living organisms in the formation and degradation of manganese nodules, (b) to determine the activity of microbial enzymes in manganese oxidation, reduction, precipitation, and solubilization in the ocean, (c) to explore the role of chemical phenomena of nonbiological origin in manganese transformations in the marine environment.

ABSTRACT

We examined the enzymatics of manganese accretion to nodules and some effects of pressure and temperature upon the process. We also examined the effect of MnO_2 reduction on pyruvate and lactate production on two nodule cultures, and tested the capacity of one of them to be induced with respect to a missing electron-transport component.

(a) Manganese Accretion

Cell-free extract from our mesophilic strain of Arthrobacter 37 (temperature optimum: 18 - 25 C) was shown to catalyze Mn^{II} oxidation. Controlling the composition of the electrolyte environment at the time of harvesting and testing of enzyme activity was important. The enzyme activity was oxygen dependent and required NaHCO_3 in the reaction mixture. The enzyme had a temperature optimum around 17.5 C. Increased amounts of extract caused increased amounts of Mn^{II} to be oxidized in 3 hr. The enzyme was inhibited by HgCl_2 and p-chloromercuribenzoate but not by atebaine. The enzyme activity in crude extract was slowly destroyed by heat. This destruction was faster in 3% NaCl solution than in natural seawater. The extract was rich in nucleic acid which could be separated to a large extent from the enzyme-containing protein fraction by gel-filtration in 3% NaCl through Sephadex G-150. Separation of the nucleic acid from extract prepared in natural seawater by gel-filtration was much poorer. Similar manganese-oxidizing enzyme activity was obtained in cell-free extract from our psychrophilic strain of Arthrobacter 37 (temperature optimum: 4 - 10 C). The enzyme had a temperature optimum between 4 and 10 C. Gel-filtration through Sephadex G-150 gave a separation of enzyme protein from nucleic acid similar to that with extract from our mesophilic strain.

Mn^{II} oxidizing activity by whole cells of mesophilic Arthrobacter 37 at room temperature was inhibited between 5,000 and 10,000 psi in natural seawater and between 10,000 and 15,000 psi in 3% NaCl medium. The same culture exhibited no activity at atmospheric pressure, 1,000 or 5,000 psi at 10 C. Whole cells of the psychrophilic strain of Arthrobacter 37 at

10 C exhibited Mn^{II} oxidizing activity at atmospheric pressure, 1,000, 2,000, and 5,000 psi. The results with the psychrophilic strain showed that manganese oxidation at temperatures and hydrostatic pressures found in the depths of the open ocean is possible.

(b) Manganese Oxide Reduction

The study on chemical and physical changes in the culture medium resulting from bacterial MnO₂ reduction was extended to coccus 32. In the presence of MnO₂ the coccus caused a significant pH drop to 5.6 in 11 days and Eh changes varying between 394 and 514 mv at an initial glucose concentration of 0.6%. Pyruvate and lactate production in the presence and absence of MnO₂ was followed for both, Bacillus 29 and coccus 32. With Bacillus 29, total lactate and pyruvate production was greater with MnO₂ than without. Lactate production by coccus 32 was negligible, but total pyruvate production was greater with MnO₂ than without.

Previous work with Bacillus 29 had shown the electron transport system for MnO₂ reduction to be incomplete when the Bacillus had not been reducing MnO₂ just prior to testing. Recent findings suggest that the missing component can be induced by incubating the Bacillus in a medium containing 4.35×10^{-3} M MnSO₄·H₂O, 0.87% glucose and 0.04% peptone for 5 hr. Of the ingredients, Mn²⁺ and either glucose or peptone were essential. Chloramphenicol in preliminary experiments seemed to inhibit the induction.

PLANS FOR FUTURE

(a) To attempt further purification and characterization of the manganese oxidizing system, (b) to study further the induction of the missing component in the electron transport chain in MnO₂ reducers not adapted to MnO₂, (c) to extract, purify and characterize the MnO₂ reducing enzyme system, (d) to continue the study on hydrostatic pressure effects on Mn^{II} oxidation and MnO₂ reduction by appropriate organisms and their enzymes.

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STUDY OF ALGAL CHARACTERISTICS THROUGH THE FRICTION-REDUCING EFFECT

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WORK UNIT NO. NR 103-699

CONTRACT P. O. 8-0030

OBJECTIVES

(a) To study Chaetoceros didymus and other marine diatoms, dinoflagellates and bacteria which exude high-polymer organic materials into the sea through measurements of the friction-reducing effect, and (b) study the chemistry of algal polysaccharides.

ABSTRACT

A number of marine diatoms, dinoflagellates and bacteria have been shown to reduce the friction in turbulent pipe flow. This effect has been traced to high molecular weight polysaccharides exuded into the culture medium by the organisms. The biological reason for the high-polymer excretion is not known, but the practical effects of this action include drag changes in towing tanks due to algae and bacteria, and possible errors in ship powering measurements on the measured mile.

In addition to the study of friction-reducing metabolites produced by laboratory cultures of algae and bacteria, work has continued on the relationship of slicks on the ocean to concentrations of polysaccharide-producing organisms. Samples of water have also been taken aseptically from several hydrodynamic testing facilities, cultured in the laboratory to concentrate the different species of algae and bacteria, and checked for friction-reducing capabilities. The principal experimental technique used in the study is the measurement of fluid friction using a miniature pipe flow apparatus.

The following are the detailed accomplishments under this project during 1968:

1. About 20 different friction-reducing microscopic algae are now available in culture in our laboratory. About 20 different friction-reducing bacterial strains are also on hand.
2. Three types of algal release of friction-reducing materials have been identified. (1) Liberation of polysaccharide during active growth, and rapid decay following cell disintegration (as in Chaetoceros), (2) Liberation of materials from cell breakdown (as in Prorocentrum micans), and (3) Liberation of materials during growth, which do not subsequently break down (Porphyridium sp.).
3. High-molecular weight components were detected in ocean slicks by the friction-reduction technique. Water 10 cm below the slick did not show the effect. In addition, cultures started using material from the natural slicks as the inoculum have shown friction reductions as high as 17%.

4. Samples of towing tank flora have been obtained aseptically from two British tanks: the National Physical Laboratory tank at Feltham, and the Admiralty Research Laboratory tank at Teddington. The predominate alga in the Feltham tank was a green species which in culture in our laboratory produced a friction reduction of about 58%. Recent measurements in this tank have indicated a fluctuating water resistance and it is apparent that this could be due to the algal population. Bacterial samples from the two tanks when subjected to further culturing in broth yielded species giving friction reductions of 20-30%. Hence, it seems likely that the towing tank flora can strongly influence measured ship model resistance.

5. Samples of water have also been obtained aseptically from:

David Taylor Model Basin, Carderock
Garfield Thomas Water Tunnel, Pennsylvania State
Underwater Sound Laboratory, New London
Underwater Sound Reference Division, NRL, Orlando

Evidence of friction-reducing ability has been obtained from bacteria from each of the above sources.

6. In conjunction with Dr. A. T. Ellis of Caltech, studies were made of the effect of algae cultures and polymer solutions on cavitation inception in a water tunnel. (Cavitation is local "boiling" of the fluid in regions of high velocity and reduced pressure. The intense noise and erosion damage associated with collapse of the vapor bubbles is of great interest in military and commercial applications.) It was found that a very dilute solution of algae gave a significant lowering of the cavitation inception point. It is possible that the effect of algae or other contaminants in the water accounts for the large differences in cavitation inception reported for the same test shape in water tunnels in different locations around the world.

7. Chemical studies were undertaken on Chaetoceros didymus polysaccharide and methods worked out for its structural determination. The final accomplishment of the polysaccharide structural determination was deferred, however, due to accidental contamination of the stock culture.

PLANS FOR FUTURE

(a) Continue the study of Chaetoceros and other marine algae and bacteria exhibiting the friction-reducing effect. Determine the chemical structure of a typical polysaccharide and estimate its molecular weight.
(b) Measure friction-reducing properties of slick-producing blooms in the ocean, and attempt to correlate the flora of slicks with friction-reduction.
(c) Continue sampling of towing-tank flora and those of other major hydrodynamic facilities to identify micro-organisms which affect friction and cavitation inception. Possible controls for such micro-organisms will be investigated.

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BACTERIAL FLORA OF MARINE MAMMALS

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WORK UNIT NO. NR 302-686

CONTRACT N00014-66-C0274

OBJECTIVES

a) To continue isolation and characterization of bacteria from various sites of marine mammals, b) to determine presence of PPL organisms in marine mammals, c) to determine presence of antibodies to specific bacteria encountered in wild marine mammals, and d) to make comparative studies of marine mammalian bacteria and human bacteria.

ABSTRACT

The techniques for culture of bacteria have been established for different temperatures (6°, 20° and 37° C.) and under different gas pressures to simulate both the changes in altitude and changes in depth of the environment. Special chambers have been devised to create the environmental conditions desired. Similar bacteria of human and marine mammalian origin are carried through the different experiments simultaneously.

PLANS FOR FUTURE

a) Continual effort to refine the studies of bacteria and the specific immunological features will proceed with attempts to isolate PPL organisms, b) infections of underwater workers will continue to be investigated in reference to bacteria of marine mammals and the ocean environment, c) samples of ocean water in areas of capture site will also be investigated to provide an appraisal of the ocean environment, d) studies will be undertaken to determine the feasibility of metabolic by-product studies by gas chromatography.

THE MEMBRANES OF MARINE AND
TERRESTRIAL THIOBACILLI

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WORK UNIT NO. NR 103-547

CONTRACT Nonr-00014-67-A-
0158-0002

OBJECTIVES

To determine the (a) chemical nature of the cytoplasmic membranes of marine and terrestrial Thiobacilli, with special emphasis on the phospholipids, (b) the chemistry of the extracellular organic compounds in the medium after growth and (c) the intracellular pH of the cells.

ABSTRACT

Cell envelopes of Thiobacillus thiooxidans were prepared by osmotic lysis of spheroplasts. Spheroplasts were prepared by treatment of stationary phase cell suspensions of T. thiooxidans in 0.1 M Tris buffer (pH 7.0) with 0.2 mg/ml muramidase, followed by raising the pH to 9.0. Envelope fragments were washed until free of isocitric dehydrogenase activity and lyophilized.

The gross composition of the envelopes, calculated as % dry weight, was as follows: protein, 65-70%; total lipid, 13-15%; free lipid, 6%; carbohydrate 20-22%; amino sugar 1.4%; total phosphate 0.65%; total nitrogen 7%.

Glucose, galactose and glucosamine were the only carbohydrates detectable with paper chromatography.

The phospholipids present and their relative concentrations, based on % of total phospholipid phosphate present, were: phosphatidyl glycerol, 45%; diphosphatidyl glycerol, 10%; phosphatidyl serine, 21%; phosphatidyl ethanolamine, 15%; phosphatidyl-N-monomethyl ethanolamine, 9%.

Two-dimensional paper chromatography of acid hydrolysates of the envelopes, followed by spot elution and colorimetric ninhydrin determinations, indicated the presence of large amounts of aspartic and glutamic acids, relative to the other amino acid components. The number of amino acids present was typical of a protein hydrolysate. With the exception of the amino acid composition, the chemistry of T. thiooxidans envelopes was found to be similar to that of Gram-negative heterotrophs.

The phospholipid composition of the extracellular organic compounds in the spent medium qualitatively reflects the composition of the envelopes.

PLANS FOR FUTURE

(a) The chemical analysis of the envelopes of T. thiooxidans will be continued to present as complete and reliable an analysis as possible (b) several other Thiobacilli will be analyzed for comparison (c) the respiratory characteristics of T. thiooxidans will be studied to relate them to sulfur uptake and (d) the internal pH of the cell will be estimated in several ways.

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DECOMPOSITION OF ORGANIC MATERIALS BY
MARINE BENTHIC BACTERIA

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WORK UNIT NO. NR 103-633 CONTRACT

Nonr-477(41)

OBJECTIVES

(a) To study the role of microorganisms in the benthos food chain, (b) to study the distribution, taxonomy, and activity of bacteria from the benthos in relation to assimilatory and degradative processes, (c) to study the chitinase system of selected chitinoclastic bacteria, and (d) to identify lytic agents active against sediment bacteria and to study associated genetic exchange phenomena.

ABSTRACT

Surface sediments and waters from Puget Sound were examined for total heterotrophic bacteria, bacterial types and the bacterial-biochemical activity during three extended and four one-day cruises. Total bacteria counts in sediments varied among the Puget Sound stations, but generally ranged from 10^3 to 10^5 per gram sediment. On rare occasions, counts up to 10^6 bacteria per gram were encountered. Chitinoclastic bacteria made up 1-20% of the total bacteria, which agrees with previous observations. Intertidal sediments from various areas had consistently higher total and chitinoclastic counts than Puget Sound sediments. The intertidal sediments, taken mainly from oyster-bed areas, had total counts of 10^6 to 10^8 bacteria per gram with chitinoclastic bacteria accounting for 13% to 50% of the total numbers. Bacterial numbers in water samples were generally lower by 10 to 100 times than for sediments from the same stations. Agar digesters usually made up less than 0.1% of the total bacteria in all samples. Total biochemical activity measurement, with multiple tube enrichments, showed different levels of activity for a number of organic substrates. The utilization of glucose and galactose, and the reduction of nitrate occurred at high levels, whereas, cellulose digestion and gelatin liquification occurred at much lower frequencies in the same samples.

During the past year, 87 chitinoclastic bacteria isolated from Puget Sound sediments, waters and fauna have been studied. All were gram negative, motile, asporogenous rods, with a predominance of *Vibrio* types. All isolates grew and showed chitinoclastic activity between 8°C and 22°C. However, at 35°C, only 13 of 87 grew and none grew at 42°C. The examination of additional chitinoclastic isolates from intertidal sediments, waters and shellfish was also initiated. Preliminary results suggests again the predominance of *Vibrios*. Chitinoclastic *Cytophagas*, which were rare in Puget Sound samples, and a chitinoclastic *Spirillum* have been isolated from intertidal areas. Also, a *Vibrio* showing simultaneous chitin and agar digestion was isolated.

Chitinoclastic activity appears to be dependent on aerobiosis. Under anaerobic growth conditions, chitinoclastic activity was either poor or absent. Only a single culture showed comparable activity under aerobic and anaerobic growth. The effect of various organic compounds on chitinoclastic activity was examined. Glucosamine and N-Acetylglucosamine, at 0.25% levels, stimulated activity in a majority of cultures. In contrast, glucose at the same concentration showed marked inhibition of chitinoclastic activity. Ribose, starch, cellobiose, acetate and citrate had varied effects. Attempts at routinely assaying chitinase activity in cell-free growth medium have not been successful. Chitinase preparations obtained by ammonium sulfate precipitation are now being tested.

The model sea bed system was used to investigate the assimilatory capabilities of a composite of Puget Sound sediments. Analysis of the sediments, after contact with a large volume (ca 17,000 mls) of flowing sea water, showed no significant increase in organic carbon, as compared to control sediments. This observation is in contrast with previous findings.

We have isolated Vibrio parahaemolyticus, a bacterium responsible for major outbreaks of food-borne illness in Japan, from Puget Sound intertidal waters. Numerous strains of this bacterium have been isolated from sea water, marine sediments and oysters (Crassostrea gigas) using a newly formulated starch agar medium. The cultures have been shown to be identical to Japanese strains of V. parahaemolyticus and clearly distinguishable from the related V. alginolyticus and V. anguillarum. Two bacteriophages specific for some strains of V. parahaemolyticus have been isolated by enrichment of oyster homogenate with Japanese strains of this bacterium. One Puget Sound isolate was susceptible to lysis by both bacteriophage.

PLANS FOR FUTURE

Further experiments, using the model sea bed system, will examine the assimilation of dissolved organic materials from sea water by marine sediments. Chitin decomposition by surface sediments from various areas will also be investigated using the same system. The in situ decomposition of natural chitin in intertidal sediments is also planned. Examination of the chitinoclastic activity of isolates from Puget Sound and the Washington Coast will continue. Purification of chitinase and the assay of its activity will be carried out with selected isolates. Investigation of V. parahaemolyticus and its bacteriophage will be continued with the hope of acquiring a sufficient number for typing purposes. The investigation of cross inhibition and the lytic phenomenon between marine bacteria and on genetic exchange processes will be continued.

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PROTEOLYTIC ENZYME PRODUCTION BY MARINE BACTERIA

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WORK UNIT NO. NR 103-602

CONTRACT Nonr-610(05)

OBJECTIVES

1. To determine the distribution of proteolytic bacteria in various marine localities; 2. To study the characteristics of proteolytic marine bacteria and the factors that influence their protease production; 3. To isolate, purify and characterize the extracellular proteases of marine bacteria.

ABSTRACT

Biochemical and physical properties of proteolytic and nonproteolytic marine bacteria were examined in a continuing program to separate, identify and select active cultures, and to expose patterns that are characteristic of proteolytic marine bacteria. It was noted in these and previous studies that when standard bacteriological techniques were used to detect carbohydrate fermentation, no gas production was observed with any marine cultures. The underlying reasons for the failure to observe gas production were investigated. Failure of marine bacteria to produce detectable amounts of CO₂ during fermentation was shown to be a property of the marine bacteria, rather than a function of the medium. Attempts to induce hydrogenlyase activity in several cultures were unsuccessful. CO₂ production under aerobic conditions was observed in Warburg respirometry studies of 24 cultures. However, under anaerobic conditions only one of the above cultures produced a sizable amount of CO₂. This culture was Aeromonas proteolytica, a proteolytic bacterium originally isolated from the gut of a wood-boring isopod. CO₂ production occurred only after adaptation of the culture to anaerobiosis and glucose utilization. Most of the other cultures fermented glucose vigorously, as evidenced by acid production, but produced at most trace amounts of CO₂. CO₂ production by A. proteolytica in Warburg vessels has been confirmed with a CO₂-adsorption train.

The cultural and biochemical characteristics of several proteolytic marine bacteria were studied in detail. The factors influencing the production of their proteases were investigated, and the isolation and characterization of their enzymes was carried to various stages of completion. A. proteolytica, and four Bermuda cultures, B-30, B-118, B-207 and B-279, received the most attention. A medium was developed for A. proteolytica that favored production of endopeptidases almost to the exclusion of the aminopeptidase normally found in culture filtrates. The proteolytic enzymes of B-118, a deep-sea bacterium, were resolved by zone electrophoresis, and an aminopeptidase and an endopeptidase were separated

on DEAE-cellulose columns. Some characterizations of the enzymes have been carried out. The endopeptidase preferentially attacks on the amino side of leucine residues. The structures of B-30, an in-shore *Aeromonas*, and B-207, a new marine *Pseudomonas*, were investigated by electron microscopy of whole and thin-sectioned organisms. Enzyme production by both organisms was studied. Maximum enzyme yields were obtained by varying the composition of the medium and cultural conditions. The nitrogen source, pH, rate of aeration, incubation temperature, and age of the culture had pronounced effects on the types and amounts of enzymes synthesized. Four endopeptidases were produced by B-30. The major endopeptidase was isolated and characterized. It is a serine protease that requires calcium for activation. Calcium was shown to activate and to stabilize the enzyme against heat denaturation. Enzyme activation effects were most notable above 40°C. Spectrophotometric, electrophoresis, and gel filtration evidence for either conformational or quaternary structure changes in the presence of calcium was obtained. Enzyme specificity studies indicated a preference for the amino groups of tyrosine, phenylalanine, leucine, and alanine. The proteolytic enzymes of B-207 have been separated by gel filtration and ion-exchange chromatography on DEAE-cellulose into two endopeptidase and one aminopeptidase. The aminopeptidase is relatively heat stable and can best be characterized as a zinc-requiring leucine aminopeptidase. Evidence was obtained to indicate that the aminopeptidase is an intracellular enzyme that is released when cells autolyze, while the endopeptidases are extracellular and are released into the medium during the early phase of growth.

A search for collagenases in marine bacterial filtrates was instituted, and new sources of chromoproteins were investigated. The production and stability of the chromoproteins of several Cyanophyta were compared with *Porphyra leucosticta*. Several Cyanophyta will serve as good sources of chromoproteins if laboratory production can be increased. A difference in the stability toward proteolytic attack of Cyanophyta and Rhodophyta phycoerythrins was observed.

PLANS FOR FUTURE

We plan to continue the characterization of marine proteolytic bacteria and the proteases that they produce. Our principal immediate efforts will be directed toward: (1) complete identification of the most active cultures with which we have been working, (2) complete characterization of the proteolytic enzymes that have now been separated, (3) studies on the mode of action of purified proteases, (4) characterizations of other active cultures that are in our collection or that will be added to the collection by field studies, (5) continuation of studies on the influence of chemical and physical factors on enzyme production, (6) understanding respiration and fermentation of marine proteolytic bacteria.

CURRENT REPORTS AND PUBLICATIONS

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(b) T. Sipos and J. R. Merkel (1967), "Proteolytic enzymes of a marine bacterium," *Bacteriol. Proc.* p. 45.

INTERRELATIONSHIPS IN ENRICHMENT CULTURES
OF AQUATIC AND MARINE MICROORGANISMS

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WORK UNIT NO. NR 103-487

CONTRACT Nonr 477 (43)

OBJECTIVES

(a) To carry out studies on steady-state enrichment cultures of aquatic and marine microorganisms and (b) to carry out definitive studies on the properties of little known groups of aquatic and marine bacteria, including stalked and budding bacteria, marine vibrios, and aquatic myxobacteria.

ABSTRACT

Investigations of bacteria living in sea water have concentrated on the nitrifying bacteria, particularly those oxidizing ammonia to nitrite. Steady-state enrichments and batch cultures have been employed in this study, and bacteria different in some respects from those reported by other investigators appear to be involved. Increased ammonia input to some continuous cultures has resulted in a higher proportion of cells existing free in the culture fluid rather than attached to the vessel walls. Several cultures of ammonia oxidizing bacteria have been obtained from these enrichments which are free of heterotrophs as judged by the lack of growth on complex organic nutrients. Two morphological types have been observed microscopically, ellipsoids and what appear to be vibrios. Efforts to separate these two forms, one of which may be heterotrophic, are in process and the possibility that one or both is obtaining nutrients from the other is being considered. Growth of ammonia oxidizing bacteria on solid medium has not been obtained despite the use of highly purified agar and incubation under conditions of low oxygen tension. When liquid medium for ammonia oxidizers was added to a gelled layer of Ionagar #2 and inoculated from active batch cultures nitrite was not produced although it was formed in controls which lacked the agar.

Oxidation of ammonia and nitrite appears to be limited by the area available on the vessel walls. As more substrate is added the surface becomes effectively saturated and more cells appear in the fluid, but the accumulation of substrate in the culture also tends to be inhibitory, with the result that the concentration of unattached ammonia oxidizing bacteria has not been found to be above 1×10^7 per ml.

Pure cultures of Sphaerotilus natans isolated from steady-state enrichments require other factors than or in addition to vitamin B₁₂. Exploration of the possibility that the nutrient factors required are provided by other bacteria in the enrichment culture is underway.

Studies on the degradation of lignin sulfonic acid in steady-state cultures have continued. Sea water cultures are now more active than

formerly in decreasing the concentration of added purified lignin sulfonic acid. Enrichment levels have been increased to facilitate characterization of bacteria responsible for this activity.

Studies have continued on marine vibrios from a number of sources using the method of DNA homology as well as conventional cultural methods. The method of DNA homology is now being employed for investigation of a collection of marine myxobacteria. A paper has been submitted to the Journal of Bacteriology dealing with the employment of membrane filters in investigation of DNA homology.

PLANS FOR FUTURE

In the study of bacteria oxidizing ammonia in sea water an effort will be made to separate the two kinds of bacteria coexisting in batch cultures, to determine whether either is nutritionally dependent on the other, or heterotrophic, and otherwise characterize them. Additional kinds of solid media will be tested for suitability in obtaining colonial growth. The isolation and description of nitrite oxidizing bacteria in steady-state cultures will receive increased attention. Investigations of the degradation of lignin sulfonic acid in sea water will continue. Additional work will be done on the nutrition of some strains of Sphaerotilus. Studies on marine vibrios and marine myxobacteria using the method of DNA homology are being continued.

BIOCHEMISTRY AND PHYSIOLOGY OF DENITRIFICATION
BY MARINE BACTERIA

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WORK UNIT NO. NR 103-538

CONTRACT Nonr-3677(01)

OBJECTIVES

(a) To study the enzymes involved in the terminal reductive steps in denitrification and (b) to contrast the cytochromes synthesized in response to the presence of nitrate as opposed to nitrous oxide as the electron acceptor.

ABSTRACT

Gas chromatographic methods were previously developed and used to separate mixtures of gases of known composition, as well as CO_2 and N_2 from denitrifying cultures of Pseudomonas perfectomarinus. The bacteria were found to release N_2 from either nitrate or N_2O , when grown anaerobically. The gases were swept out with helium.

Using an elevated quantity of nitrate in the culture medium (1% as opposed to 0.1%) we were able to discern small quantities of N_2O in the gases from P. perfectomarinus after prolonged incubation only.

Cell-free extracts of cells grown on sea water-tryptone-yeast extract broth with nitrate as final oxidant were obtained by passage of the bacteria through a French pressure cell. The extracts were clarified by two centrifugings at $34,000 \times g$ for an hr. Other extracts were prepared by alumina grinding in the cold. Reaction mixtures containing the first extracts were supplied with malic acid, NADP, flavins and nitrate. A sample of the pellet from the first centrifuging was added. This mixture was incubated in a modified, double-lobed Thunberg tube that had a rubber-capped port inset through which samples were drawn. These samples of the gases over the mixture were obtained with an air-tight syringe and injected into the gas chromatograph. After prolonged incubation N_2O was observed as a product of this reaction mixture.

Incubation of the extracts from alumina-ground cells was carried out in tubes attached to the sampler used for assaying gases from cultures. The composition of the reaction mixture was the same but the mixture was swept with helium from the time the extract was added. The gases swept into the sampler and injected into the analyzer were found to contain NO very soon after incubation began. This oxide

persisted for 20 min, but was not discernible after 28.

In addition to the revelation of these gases as products of cell-free extracts in these reaction mixtures, the most significant finding of this part of the study has been the inadequacy of sampling devices for the investigation of gas releasing enzymes. Exclusion of any contaminating air is desirable because one of the products sought is N_2 . Moreover, a minimum of 8 min after injection of samples is required to insure sensing of N_2O . Thus, the frequency of submitting samples to analysis is set. We are currently working on design of a device that will permit rapid taking of small samples that can be held air-tight until time for analyses can be gained.

The cytochrome contents of cells of P. perfectomarinus grown with a variety of terminal oxidants are being measured with the hope that a paradigm of the absorption spectra will shed light on the participation of the various pigments in NO_3^- , NO_2^- , NO and N_2O reduction.

PLANS FOR FUTURE

(a) To determine the differences in cytochrome content and identity in cells grown with nitrate, NO or N_2O as terminal electron acceptor; (b) relate the cytochromes observed to the various steps in denitrification (nitrate respiration); (c) design devices for rapid, air-tight samples of effluent gas to be used for obtaining measurements of products of short-term enzymatic experiments.

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(b) James M. Barbaree and W. J. Payne (1967). Products of denitrification by a marine bacterium as revealed by gas chromatography. Marine Biology 1:136-140.

ECOLOGICAL REQUIREMENTS OF ISOTRICHA SPP.

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WORK UNIT NO. NR 103-634

CONTRACT Nonr 4709(00)

OBJECTIVES

(a) To determine the ecological requirements of Isotricha spp., (b) to determine the biochemical behavior of isotrichs under various environmental conditions in the Ecoanalyzer.

ABSTRACT

A systemic approach has been followed in determining the physical and chemical requirements of I. intestinalis and I. prostoma for growth in vitro in axenic culture. These requirements are: (a) pH between 6.8 - 7.0, (b) oxidation-reduction potential between -200 and -350 mv, (c) osmotic pressure corresponding to 111 mmol, (d) temperature between 35-39 C, (e) surface tension near 60 dynes, (f) a culture medium containing 40 nutrilites in proper concentrations. Factorial-design growth assays were employed to determine the nutrilitite requirements, with isotrich populations enumerated by electronic cell counter. Essential amino acids were determined by comparison of amino acid analyses of defined medium 4 before and after growth of isotrichs in it (with environmental conditions held constant by the Ecoanalyzer culture apparatus), plus analysis of hydrolyzed isotrichs; amino acid analyses were conducted on a continuous gradient amino acid analyzer.

Fermentative ability of isotrichs under various environmental conditions has been checked by Ecoanalyzer analysis. In general, any conditions other than the above listed optimal parameters for growth cause a marked decrease in metabolic activity of the isotrichs.

PLANS FOR FUTURE

No further plans for ecological studies on Isotricha spp. are being made.

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(b) D. W. Zimmerli (1967), 'An electronic counter for population and size distributions of microscopic particle suspensions.' M.S. Thesis, Iowa State University, Ames, Iowa

STUDIES ON THE ROLE OF POLYPHOSPHATES
IN DIVIDING CELLS

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WORK UNIT NO. NR NR 103-68

CONTRACT Nonr-233 (71)

OBJECTIVES

(a) To determine the effects of temperature shifts on lipid synthesis in Tetrahymena Pyriformis, (b) to study lipid stability in Tetrahymena, (c) to identify the three phosphatides which accumulate in synchronized Tetrahymena, (d) to examine the fatty acid composition of marine and fresh water organisms, (e) to study polyphosphate metabolism in Polytoma and Tetrahymena and (f) to investigate the effects of RNA hydrolysis on cell size.

ABSTRACT

(a) Lipid metabolism in synchronized Tetrahymena. Using a rapid radioassay technique for lipid biosynthesis (Byfield, Henze and Scherbaum, 1967) we studied the effects of synchronizing temperature shifts on Tetrahymena. The uptake of labeled glycerol and ethanolamine was stimulated in marked contrast to either protein or RNA synthesis. This suggested that the induction of temperature synchrony was probably not associated with alterations in lipid metabolism. This was further confirmed by investigations on the mechanism of action of Triparanol. Studies using the latter drug (an inhibitor of cholesterol synthesis in mammalian cells) showed that massive replacement of lipid exogenously did not affect the induction of synchrony while the addition of Triparanol (at concentrations inhibiting synchronous division) resulted in significant inhibition of protein synthesis in Tetrahymena. (b) However, an extension of our previous studies aimed at identifying the three phosphorous containing compounds which accumulate in synchronized Tetrahymena resulted in a partial characterization of these compounds. They appear to represent either partially synthesized or breakdown products of phospholipids suggesting that slight aberrations in lipid metabolism may occur during synchronizing shifts. (c) Preliminary attempts at studying polyphosphate content in synchronized Tetrahymena have also been started. Cultivation studies indicate that rigorous control over growth conditions are important if significant amounts of polyphosphates are to be isolated. These suggest that polyphosphates are probably not important phosphogens in Tetrahymena. (d) Finally, we have studied the effects of stimulating mRNA hydrolysis in Tetrahymena on cell size and viability. We have found that periods of rapid template hydrolysis are associated with marked cellular edema and death. It has been proposed that this may be the primary source of cell death in anoxia and other non-physiologic states (Byfield and Scherbaum in preparation).

PLANS FOR FUTURE

These studies will be continued in an effort to relate the effects of temperature shifts to the mechanism of induction of synchrony and the biochemical requirements for division.

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4. Ito, J., Lee, Y.C., and Scherbaum, O.H., 1967, Macronuclear microtubules in synchronized Tetrahymena, submitted to Exptl. Cell Res.
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MEDICAL MICROBIOLOGY

Understanding of the mechanisms whereby microorganisms produce disease in the host is the objective of the research reported in this section. Such an approach, although basic in nature, is producing results which will have important application in the field of military medicine. New methods for preventing, controlling, and treating diseases of importance to the Navy and the Marine Corps may well emerge. Some of the work described is supported jointly by the Office of Naval Research and the Research Division of the Bureau of Medicine and Surgery.

STUDIES OF INFECTIOUS VIRAL NUCLEIC ACIDS

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To develop methods of production, stabilization and assay of infectious nucleic acids from several viruses in quantities sufficient to carry out further objectives. (b) To determine infectivity and immunogenicity of viral nucleic acids. (c) To correlate aerosol behavior with alterations in physical and chemical properties of viral nucleic acids.

ABSTRACT

It was previously reported that when mice were exposed to aerosols of different strains of the encephalomyocarditis (EMC) group of viruses, it became evident that certain strains were lethal while others were immunogenic and this phenomenon was correlated with plaque size. Furthermore, responses in mice exposed to aerosols of viral RNA proved to be similar to that observed when using different strains of intact virus; i.e., viral RNA behaved like the intact virus from which it was derived.

Recent studies have shown that differences in pathogenicity for the EMC group viruses were also correlated with plaque size, state of the virus (intact or as RNA), and the presence or absence of circulating antibodies. With aerosols of Mengo 37A virus, a small plaque-forming immunogenic strain, virus was recovered from the lungs, intestines, spleen, liver and blood. Pathologic changes occurred in the lungs and heart. Mice exposed to lethal aerosols of Mengo or Columbia-SK (Col-SK) (large plaque-forming strains) yielded virus from every organ. However, with lethal Mengo RNA aerosols there was a delayed appearance of virus in the intestinal tract. Mice exposed to lethal Mengo RNA, Col-SK and Mengo virus aerosols exhibited similar pathologic changes which occurred only in brain and liver tissues. Challenge with lethal Col-SK or Mengo aerosols resulted in no deaths in Mengo 37A-immunized mice. However, challenge virus was recovered from the lungs, intestinal tract, spleen, liver and blood. Pathologic changes were also observed in lung and liver tissues. Mengo RNA aerosol exposure of mice immunized with Mengo 37A resulted in virus being isolated from lung tissue only.

PLANS FOR FUTURE

(a) To study the relationship of thermostability to aerostability; i.e., the effect of relative humidity and temperature on thermostable and thermolabile EMC virus strains and their viral RNA. (b) To study the effect of relative humidity shifts on the airborne stability of EMC viruses or their infectious RNAs. (c) To determine the possible relationship of

nucleic acid structure; i.e. RNA and DNA, double or single stranded, as related to airborne stability of intact viruses. (d) To further explore means of production and assay of infectious nucleic acids and the extension of EMC findings to other virus groups. (e) To assess statolon-induced resistance of mice to lethal virus aerosols.

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A STUDY OF FACTORS INFLUENCING VIRAL PARASITISM

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WORK UNIT NO. NR 302-352 CONTRACT N00014-67A-0077-0004

OBJECTIVES

To study factors that influence viral parasitism: (a) Intrinsic host factors as revealed by study of cellular constituents, (b) non-specific resistance to viruses induced by immunologically different materials, and (c) immunity produced by combination of live viruses.

ABSTRACT

The initial proposal to search for induction of non-specific resistance to viruses induced by immunologically different materials led to the concept that heterotypic pairs of viruses protected against each other. Heterotypic viral protection assumed greater importance because the basis of protection appeared to be a sensitization of cells that produce immunoglobulins into a state of secondary response by the inducer virus to all others heterotypically related. The broad reactivity of a heterotype apparently was based on possession of a common antigen because infective virus was not necessary. In further study, antigenic analysis of heterotypes proceeded with the idea of determining whether a fraction might be prepared which would induce this commonality of protection. For this study, the bovine virus diarrhea (BVD) virus - hog cholera (HC) virus heterotypic pair was chosen.

A first requirement for antigenic analysis was directed toward growing virus in the highest possible concentration. Tissue cultures of BVD virus were prepared and semicarbazide was added to medium in an attempt to increase growth. A doubtful increase was obtained but there was no decrease. Secondly, various attempts were made to concentrate and purify virus. A number of procedures were tested, and the procedure finally adopted was to concentrate virus by centrifugation. Thirdly, chemical treatment with the Tween 80-ether-urea procedure was used for fractionation. Finally, a test system more plentiful and cheaper than pigs was sought in the use of guinea pigs and rabbits. The guinea pig has not yet produced satisfactory results, but since both BVD virus and HC virus infect rabbits, heterotypic response produced in them has made possible a greater number of tests. A fraction of BVD virus was prepared that appeared to be non-infectious when tested in tissue culture and in calves. It induced effective protection in pigs because the pigs did not die when given lethal HC virus. Furthermore, antibody produced by sequential inoculation of BVD virus followed by HC virus was found to be 7S type rather than the 19S which followed primary stimulation.

A study of factors that influenced the production and retention of viral immunoglobulins was initiated in a Beagle colony with the view of establishing a genetical basis. Distemper vaccine virus was the antigen that was given puppies after maternal distemper antibody had disappeared. Then, after establishing that the vaccine had immunized, serum samples were procured 3 months after vaccination and tested for antibody content. Thus far matings have been made that produced puppies with high antibody (approximately $\log 10^3$) and with low antibody (less than $\log 10^2$). In fact, a few litters contained puppies that failed to show any antibody. These results suggest interesting prospects of establishing selected lines of antibody producers.

The combination vaccine in cattle that contained 5 antigens (3 live viruses, 1 live organism and 1 bacteria) was given to beef calves when they were 7 months of age. At that time and every 7 months afterwards, these animals have been weighed and serum samples taken. The combined vaccine produced antibody to all constituents. A determination of actual value of vaccination has not been established because after 21 months natural challenge has not occurred.

PLANS FOR FUTURE

Now that an effective fraction has been procured, a commonality of antigens will be studied:

- 1) Particulate antigens in the fraction resulting from the degradation of the BVD virus by Tween 80-ether-urea treatment will be separated by differential centrifugation in gradients of various densities by available methods beginning with caesium chloride and potassium tartrate solutions.
- 2) Particles present at selected densities will be tested for antigenic similarity to HC virus, and will be studied using electron microscopy methods.
- 3) Particles with demonstrated antigenic similarity will be further degraded enzymatically.
- 4) Attempts to characterize these particles will be made using electrophoresis and paper chromatography.

Genetic studies on production and retention of viral immunoglobulins will continue.

Evaluation of combined vaccine will continue.

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STUDIES ON THE FRACTIONATION OF PNEUMOCOCCAL TRANSFORMING FACTORS

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WORK UNIT NO. NR 103-327

CONTRACT Nonr-266(40)

OBJECTIVES

(a) To investigate methods for separation of transforming activities in a DNA preparation (b) to study the mechanism of transformation (c) to investigate biological activity of antibodies which react with DNA.

ABSTRACT

Dialysable fractions with transforming activity have been demonstrated in the B. subtilis, H. influenzae and D. pneumoniae systems. These materials appear to be less efficient than non-dialyzed DNA in mediating transformation, but compete strongly with non-dialyzable DNA as evidenced by inhibition studies. Sub- and super-critical heat inactivation determinations of target size in the H. influenzae and D. pneumoniae systems indicate a critical target about one-fifth that of non-dialyzed DNA.

The effect of anti-thymidine antibody on the in vitro priming activity of DNA was studied using a DNA polymerase from homogenized chick embryos. Globulin fractions prepared from antisera and specifically-purified antibody inhibited uptake of tritiated thymidine to the same extent relative to antibody content.

Attempts to inhibit transformation in the H. influenzae and B. subtilis systems using globulin fractions prepared from anti-purine and anti-pyrimidine antisera have been unsuccessful.

PLANS FOR FUTURE

- (a) Continue investigation of the dialyzable fractions including attempts to fractionate transforming activities.
- (b) Further studies on the biological activities of antibodies specific for purines and pyrimidines.

CURRENT REPORTS AND PUBLICATIONS

None

ANTIBACTERIAL EFFECTS OF HYPERBARIC OXYGEN

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ASSISTED BY

WORK UNIT NO. NR 103-664

CONTRACT N00014-66-CO189

OBJECTIVES

(a) To study the antibacterial effect of hyperbaric oxygen upon the growth of a variety of bacteria; (b) to investigate the effect of hyperbaric oxygen upon experimental bacterial infections and upon normal intestinal flora.

ABSTRACT

Characterization of bacterial growth under hyperbaric oxygenation is a basic need for rational utilization of hyperbaric oxygen therapy in medicine. Hyperbaric oxygen has been reported to exert a bacteriostatic effect. Accordingly, combined therapy with antibiotics and hyperbaric oxygen may be useful against some infections. This project has demonstrated that exposure of serial dilution assays of antibiotics to hyperbaric oxygen results in a combined effect which is best described as an enhancement of antibiotic activity. This enhancement has been shown employing Staphylococcus aureus ATCC #6538P with penicillin, streptomycin, tetracycline, oxytetracycline, kanamycin, and cephalothin. As identical enhancement has been shown when polymyxin B and hyperbaric oxygen were employed against six strains of Pseudomonas aeruginosa isolated from patients. Similar enhancement with hyperbaric oxygen and an iodophor solution suggests the occurrence of a general phenomenon with antibacterial agents. These findings suggest the possibility that the therapeutic effectiveness of an antibiotic administered at its maximal dosage may be increased if exposure of a patient to hyperbaric oxygen can also bathe the infecting microorganisms with oxygen.

PLANS FOR FUTURE

(a) To extend these *in vitro* observations to experimental infections in laboratory animals. (b) to characterize the effect of hyperbaric oxygen on the growth of pure cultures of a variety of bacteria and on mixed cultures such as occur in wound exudates and fecal suspensions.

CURRENT REPORTS AND PUBLICATIONS

(a) G.H. Bornside (1967), "Enhancement of antibiotic activity against Staphylococcus aureus by exposure to hyperbaric oxygen." Appl. Microbiol. 15, 1020-1024.

(b) G.H. Bornside (1967), "Exposure of Pseudomonas aeruginosa to hyperbaric oxygen: Inhibited growth and enhanced activity of polymyxin B." Proc. Soc. Exp. Biol. & Med. 125, 1152-1156.

HOST-PARASITE INTERACTIONS
AND GENETICS OF ACTINOPHAGES

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WORK UNIT NO. NR 103-490

CONTRACT Nonr-710(36)

OBJECTIVES

(a) To elucidate the mechanisms of action of photosensitizing heteroanthracenes, (b) to isolate and analyze subunits of Streptomyces venezuelae phage, (c) to characterize further a nocardiphage specific for one mating type of Nocardia and (d) to determine the genetic and physiologic factors controlling the relationships between temperate or virulent actinophages and their hosts.

ABSTRACT

The heteroanthracenes acriflavine, proflavine and methylene blue, and the triphenylmethane dye crystal violet inactivate Streptomyces venezuelae-phage MSP2 when exposed to bright light. Photoinactivation of phages by these dyes proceeds as a function of the product of light intensity and exposure time. The photodynamic killing of phages by these dyes is prevented by addition of deoxyribonucleate (DNA), ribonucleate, or one of several purine ribonucleotides. Photosensitization by proflavine is greater at pH 8 than at pH 5.5 whereas crystal violet gives the opposite response and methylene blue is independent of pH in this range. These dyes bind to DNA; the extent of binding over the pH range 5.5 to 8 corresponds to that for photosensitization. The extent of binding of the dyes to DNA is greater in 0.01 M phosphate than in 0.15 M NaCl. More crystal violet is bound by DNA containing 50% guanine and cytosine (GC) than by DNA containing 70% GC. Conversely, methylene blue and proflavine are bound more extensively to DNA with 70% GC than to DNA with 50% GC. Methylene blue, but not crystal violet or proflavine, cause photodestruction of guanosine and guanylate. Moreover guanylate protects phage MSP2 from photoiractivation by methylene blue. These data indicate that guanine residues are the photosensitive sites of methylene blue's action.

The gross binding of acridines to DNA is a function of the DNA composition; however, binding of acridines to DNA occurs in two ways. The initial binding involves intercalation and binding between two polynucleotide chains. The second type of binding is on the exterior of the DNA helix. Binding via intercalation will bring the two amine groups of the proflavine molecule contiguous to the phosphate radical in each of the DNA strands. External binding probably involves an electrostatic attraction between a single amine group in the proflavine molecule and a single phosphate radical in one of the DNA strands. Because the extent of gross binding is directly related to the GC content, the extent of intercalation is proportional to the adenine and thymine content. The acridine dyes

are not responsible for photodegradation of guanine derivatives, nor for changes in the molecular weight of treated DNA samples; neither can deamination of the primary amine group of the DNA be detected by the present assay methods. These findings indicate that neither photolysis of the DNA chain nor deamination are the photosensitive sites for acridine dyes' action.

PLANS FOR FUTURE

(a) To elucidate the photochemical mechanisms of action of proflavine and crystal violet, (b) to characterize further a nocardiphage specific for one mating type of Nocardia, (c) to isolate and analyze subunits of Streptomyces venezuelae phage, and (d) to determine the genetic and physiological factors controlling the relationships between temperate or virulent actinophages and their hosts.

CURRENT REPORTS AND PUBLICATIONS

(a) R. A. Kolstad and S. G. Bradley (1967), "Factors affecting replication of an actinophage for Streptomyces venezuelae", Develop. Indust. Microbiol., 8, 198-205.

(b) G. H. Brownell, J. N. Adams and S. G. Bradley (1967), "Growth and characterization of nocardiphages for Nocardia canicruria and Nocardia erythropolis mating types", J. Gen. Microbiol., 47, 247-256.

HOST-VIRUS RELATIONSHIPS IN TEMPERATE

SALMONELLA BACTERIOPHAGES

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WORK UNIT NO. NR 302-654

CONTRACT NOOO14-66-COO18

OBJECTIVES

To study the biological and physico-chemical properties of selected temperate Salmonella bacteriophages and to investigate by genetic, biological, physical and chemical means, relationships between the viruses and their lysogenic and sensitive hosts.

ABSTRACT

Hybridization studies between the DNA of phage P4/2 and that of its lysogenic host SP4 have indicated that, relative to the degree of complementarity between homologous DNAs, there is 55.6% complementarity between the DNAs of phage P4/2 and SP4 compared to 4.85% homology between phage P4/2 DNA and that of its sensitive host SP2. There is therefore approximately eleven and one half times more complementarity between phage P4/2 DNA and that of its naturally occurring lysogenic host than between the phage DNA and that of its sensitive host. We attribute this difference to the presence of the P4 prophage in the SP4 genome and its absence in the SP2 genome.

We have overcome many of the difficulties in producing large volumes of high titer lysates of the B class Salmonella phage P3/2. However, the fragility of this phage continued to necessitate modifications of almost every method used for further investigative analysis. Since density gradient centrifugation in cesium chloride resulted in only 1 - 2% survival, we resorted to the use of potassium tartrate in which the survival frequency was much greater. With potassium tartrate prepared phage we have obtained electron micrographs of phage P3/2 which show an hexagonal head about 48 m μ in diameter and a tail about 100 m μ long with 3 - 4 fibers 8 m μ long protruding in a brush-like fashion from the end of the tail. The diameter of the core is about 5 m μ .

In conjugation experiments we have been unable to transfer any chromosomally located genes from Hfr and F+ strains of E. coli to S. potsdam although we have demonstrated the transfer of F-lac from a suitable strain of E. coli. We have accomplished the transfer of

chromosomal genes from Hfr strains of S. typhimurium to S. potsdam but only at low frequency. Present results indicate that only short chromosomal segments have either been transferred or undergone recombination. Donor ability has not yet been transferred to S. potsdam.

PLANS FOR FUTURE

Continuation of (a) the physical and chemical analyses of phage P3/2 including complementarity studies between this phage and its lysogenic and sensitive hosts and (b) genetic experiments to develop a fertility system in S. potsdam similar to that in E. coli and other Salmonella. We have also commenced studies to investigate possible converting properties of the different classes of Salmonella phages. Studies with additional phages may be commenced.

CURRENT REPORTS AND PUBLICATIONS

- (a) L.R. Bullas (1966) Effect of age and temperature on the growth of a Salmonella bacteriophage. Bact. Proc. V98, p.150.
- (b) L.R. Bullas, A.J. Zuccarelli and R.L. Nutter. Ageing effect on phage particles leading to an increase in burst size. Nature. In press.

STUDIES ON VIRAL INTERFERENCE AND EXALTATION

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WORK UNIT NO. NR 302-745

CONTRACT N00014-67-A-0201-0001

OBJECTIVES

- (A) TO STUDY THE ESCAPE FROM CONTACT INHIBITION UNDER THE INFLUENCE OF HYDROCORTISONE.
- (B) STUDIES WITH VIRUS-INDUCED INTERFERON.
- (C) INTERACTIONS OF RAT VIRUS (RV) WITH ONCOGENIC VIRUSES.

ABSTRACT

PHASE A. (J.D. CONNOR AND P. TOCCI) ESCAPE FROM CONTACT INHIBITION UNDER THE INFLUENCE OF HYDROCORTISONE.

IN FOLLOWING UP THE RESULTS OF THE EFFECT OF HYDROCORTISONE ON PROTEIN SYNTHESIS USING ^{14}C GLYCINE (REPORTED LAST YEAR) WE HAVE FOUND THAT THE INCREASED INCORPORATION OF THE AMINO ACID WAS DEPENDENT ON THE PRESENCE OF HORSE SERUM IN THE MAINTENANCE MEDIUM. WITHOUT SERUM THERE WAS AN INITIAL INCREASE DURING A TWO-HOUR PERIOD. THEREAFTER, PROTEIN SYNTHESIS WAS DECREASED IN THE HORMONE TREATED CULTURES COMPARED WITH THE CONTROLS. WHEN ^{14}C OROTIC ACID WAS ADDED TO THE MAINTENANCE MEDIUM OF THE HORMONE TREATED CULTURES, THERE WAS A PROGRESSIVE INCREASE IN THE RADIOACTIVITY OF THE CYTOPLASMIC RNA OVER THE INTERVAL OF TWO TO 24 HOURS, INDICATING A STIMULATION OF THIS BIOSYNTHETIC PROCESS. SINCE HYDROCORTISONE TREATED CULTURES CONTAINED CELLS IN MITOSIS, WHEREAS THE CONTROL CULTURES DID NOT, IT IS POSSIBLE THAT THE INCREASED SYNTHESIS OF RNA AND PROTEIN MAY HAVE BEEN RESPONSIBLE FOR THE RESUMPTION OF CELL DIVISION. THE FINAL CONCLUSION REQUIRES THE MEASUREMENT OF DNA SYNTHESIS IN THE HORMONE STIMULATED CELLS. THIS IS PRESENTLY UNDERWAY.

PHASE B. (M.M. SIGEL, J.L. TRUDEN, L.S. DIETRICH AND B.S. MICHAELS) STUDIES WITH VIRUS-INDUCED INTERFERON.

OUR FINDINGS INDICATE THAT THE SPLEEN IS THE MOST IMPORTANT INTERFERON PRODUCING ORGAN IN THE MOUSE WHEN INTERFERON PRODUCTION IS STIMULATED BY NEWCASTLE DISEASE VIRUS (NDV). DE SOMER ET.AL. HAVE REPORTED THAT INTERFERON INDUCTION INHIBITS ANTIBODY PRODUCTION IN RATS. NO INFORMATION IS AVAILABLE ON THE NUMBER OF ANTIBODY PRODUCING CELLS UNDER THE INFLUENCE OF STIMULATION BY INTERFERON. LOWER ANTIBODY YIELDS COULD RESULT FROM LOWER RATES OF SYNTHESIS BY EACH CELL, BY FEWER CELLS PRODUCING, OR BY BOTH OF THE ABOVE. WE HAVE USED THE JERNE AND NORDIN TECHNIQUE FOR DETECTION OF INDIVIDUAL ANTIBODY PRODUCING CELLS AND HAVE TESTED THE EFFECT OF NDV STIMULATED INTERFERON ON THIS SYSTEM. PRELIMINARY EXPERIMENTS INDICATED THAT THE NUMBER OF ANTIBODY PRODUCING CELLS REMAINED THE SAME. (SHEEP RED BLOOD CELLS WERE USED AS ANTIGEN AND INTERFERON INDUCTION WAS 24 HR PRIOR TO

PLATING FOR ANTIBODY PRODUCING CELLS.)

PHASE C. (V.V. BERGS AND H. BAEZ) INTERACTIONS OF RAT VIRUS (RV) WITH ONCOGENIC VIRUSES.

INTERACTIONS, EXPRESSED BY INTERFERENCE OR ENHANCEMENT, BETWEEN RV AND THE ONCOGENIC ADENOVIRUS TYPE 12 (AD12) WERE STUDIED IN VITRO. IN ADDITION, EXPERIMENTS HAVE BEEN INITIATED TO INVESTIGATE THE POSSIBILITY OF RECIPROCAL INTERFERENCE BETWEEN RV AND THE MOLONEY LEUKEMIA VIRUS (MLV) IN VITRO AND IN VIVO. IN EXPERIMENTS WITH RV AND AD12 PRIMARY RAT EMBRYO CELL CULTURES WERE EMPLOYED AS A HOST AND THE TITERS OF PROGENY RV IN THE SUPERNATANT OF CULTURES WERE DETERMINED BY AGGLUTINATION OF GUINEA PIG ERYTHROCYTES. TIMING AND DOSAGE OF INFECTION AND SUPERINFECTION WITH THE TWO VIRUSES WERE VARIED AND THE RESULTS INDICATED THAT SIMULTANEOUS INOCULATION OF CULTURES WITH RV AND AD12 RESULTED IN 8- TO 16-FOLD HIGHER TITERS OF RV THAN IN CULTURES INFECTED WITH RV ALONE. THIS ENHANCING EFFECT WAS DIRECTLY RELATED TO THE AMOUNT OF AD12 IN THE INFECTING MIXTURE AND WAS ENTIRELY ABSENT IF THE DOSAGE OF AD12 WAS SMALL. THE ENHANCING EFFECT BY LARGE DOSES OF AD12 WAS ALSO PRESENT IF RV WAS INOCULATED AS LATE AS ONE TO FOUR DAYS POST-INFECTION WITH AD12. SOME BUT A LESSER ENHANCING EFFECT WAS OBSERVED IF RV INFECTED CULTURES WERE SUPERINFECTED WITH AD12 ONE TO FOUR DAYS LATER. FURTHERMORE, INTERFERENCE BY AD12 WITH THE REPLICATION OF RV COULD BE DEMONSTRATED, PROVIDED THE SUPERINFECTING DOSE OF RV WAS SMALL. THE ABOVE WORK CONFIRMS AND EXTENDS THE RESULTS REPORTED BY CHANY AND BRAILOVSKY AND SUGGESTS THAT UNDER CERTAIN EXPERIMENTAL CONDITIONS AD12 MAY INHIBIT RATHER THAN ENHANCE REPLICATION OF RV. PRELIMINARY EXPERIMENTS, DESIGNED TO EXPLORE POSSIBLE INTERFERENCE BETWEEN RV AND MLV UPON SIMULTANEOUS INOCULATION INTO IN VITRO AND IN VIVO HOSTS, INDICATED THE FOLLOWING: 1) MLV CAUSED AN EARLY, 8-TO 16-FOLD, REDUCTION OF HEMAGGLUTINATING TITERS OF RV IN RAT EMBRYO CELL CULTURES, AS COMPARED WITH CULTURES INOCULATED WITH RV ALONE. THE REDUCTION WAS APPARENT ONLY IF MODERATE OR SMALL DOSES OF RV WERE USED AND DIMINISHED BETWEEN THE 4TH AND 10TH DAY FOLLOWING INFECTION. NO REDUCTION OF TITERS OCCURRED WHEN A LARGE DOSE (E.G. 10^5 TCID₅₀) OF RV WAS USED. 2) IN A LIMITED ANIMAL EXPERIMENT, INOCULATION OF A MIXTURE OF RV AND MLV INTO NEWBORN SPRAGUE-DAWLEY RATS RESULTED IN AN APPROXIMATELY 60 PERCENT REDUCTION IN THE INCIDENCE OF LEUKEMIA. ONE HUNDRED PERCENT OF THE RATS INOCULATED WITH MLV ALONE DEVELOPED LEUKEMIA 85 TO 105 DAYS POST-INFECTION, WHEREAS ONLY ABOUT 40 PERCENT OF THE ANIMALS INOCULATED WITH THE MIXTURE OF RV AND MLV DEVELOPED THE DISEASE WITHIN THE ABOVE PERIOD OF TIME. THE REMAINING 60 PERCENT OF RATS ARE STILL ALIVE AND APPARENTLY HEALTHY AT THE PRESENT TIME, WHICH REPRESENTS 5 MONTHS POST-INFECTION. EXPERIMENTS ON A LARGER SCALE ARE IN PROGRESS TO VERIFY THIS PHENOMENON. IF CONFIRMED, IT MAY EXPLAIN THE LOW INCIDENCE OF LEUKEMIA PREVIOUSLY OBSERVED IN OUR STUDIES WITH A LEUKEMOGENIC AGENT OF RATS AND THUS PROVIDE EVIDENCE FOR THE POSTULATED INTERFERENCE BY A SECOND VIRUS (9H), ANTIGENICALLY RELATED TO RV, WHICH WAS ISOLATED FROM THE LEUKEMIC TISSUES. (V.V. BERGS, J. NAT. CANCER INST. 38:481, 1967)

CURRENT REPORTS AND PUBLICATIONS

CONNOR, J.D. AND MARTI A., REVERSAL OF CONTACT INHIBITION IN PRIMARY AMNION CULTURES BY HYDROCORTISONE, PROC. SOC. EXP. BIOL. AND MED. 123,730, 1966.

TRUDEN, J.L., SIGEL, M.M. AND DIETRICH, L.S., AN INTERFERON ANTAGONIST: ITS EFFECT ON INTERFERON ACTION IN MENO-INFECTED EHRLICH ASCITES TUMOR CELLS, VIROLOGY 33, 95, 1967.

THE LOCALIZATION OF THE PHOTOSYNTHETIC PIGMENT SYSTEM IN
RHODOSPIRILLUM RUBRUM & RELATED PROCARYOTIC SPECIES

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ASSISTED BY

WORK UNIT NO. NR 103-627

CONTRACT Nonr 3357 (05)

OBJECTIVES

To localize the photosynthetic membrane system and to ascertain the biophysical configuration of this system. Also to cytochemically localize the oxidative enzyme system in Bacillus spores.

ABSTRACT

The purpose of this investigation is to cytochemically localize the photosynthetic pigment system within the membrane of Rhodospirillum rubrum and related photosynthetic species. When R. rubrum is grown photosynthetically one is able to determine two bacterial chlorophyll-containing membrane components which are separable by density gradient centrifugation: a light band with a density of 1.13g/cc, and a heavy band with a density of 1.18g/cc. The three dimensional photosynthetic cytomembrane system of R. rubrum has been investigated to determine if the density difference between the light and heavy bands was due to a change in composition of the membrane, or if it was the result of photosynthetic material being incorporated into the interior of a three dimensionally complete membrane. The light band has been isolated and purified from photosynthetically grown cells of R. rubrum. This light band has been broken by sonication and osmotic shock, and after repeated density gradient centrifugation a band having a density of 1.18g/cc was isolated from the light band. It is possible to define the chromatophore membrane to contain an interior which is separated from a distinct exterior. Upon breakage of the light band, it is hypothesized that a soluble protein is released at the same rate as the disappearance of light bands. The heavy band formed by osmotic shock or sonication has the same bacterial chlorophyll to protein ratio indicating that the soluble protein is indeed released from the interior of the membrane system. Chromatophore or membrane vesicles isolated from Chloropseudomonas ethylicum also show a similar density change upon sonication and osmotic shock with the concomitant release of soluble protein.

In order to investigate the localization of the respira-

tory system in Bacillus species, a whole range of bacilli have been investigated both by chemical fixation and the new technique of freeze-etch. Recent investigation reveals a highly membranous exterior system in almost all Bacillus spore species studied. This membranous system has not been observed prior to these investigations and it is hoped to biochemically determine the chemical nature of this highly complex system.

PLANS FOR FUTURE

The plans for the future aim at continuing the work on the localization and characterization of the photosynthetic pigment system within a whole host of photosynthetic species. It is hoped that the actual biophysical make-up of the photosynthetic membrane system will be evaluated and characterized. Further, the experiments involving the genus Bacillus will characterize and localize the respiratory system of this genus.

ARBOVIRUS SEROLOGICAL CROSS REACTIVITY

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To systematically study the behavior of Langat virus under a variety of in vivo and in vitro conditions; (b) to utilize various passages of this virus to immunize various species of laboratory animals, and (c) to determine the serological cross-reactivity of the resulting antisera toward other members of the group B arboviruses.

ABSTRACT

Four strains of Langat virus obtained from other laboratories were utilized in this study. Extensive attempts to develop a plaque assay procedure for these viruses using mono-layers or suspensions of infected chick embryo cells were not successful. Success was achieved using the cell lines PK_{h13} or L. The viruses produced cytopathogenic effects in BHK-21, A1, ERK, VERO-MK cells, and in chick embryo monolayers which had been infected in suspension. Assay of the infectivity in mouse brain pools of each virus by various techniques indicated that suckling mice and PK_{h13} cells were the most sensitive indicators.

All passages of Langat virus in suckling mice both before and after Genitron 113 extraction were capable of hemagglutinating (HA) goose red blood cells (RBC) when procedures which had been worked out for arboviruses were utilized. Considerable variation in HA activity among the virus strains was encountered when other species of RBC were used. In general, tissue culture passaged virus did not exhibit HA activity under our conditions.

The sensitivity of various ages of mice to the viruses was also determined. Sucklings were equally sensitive whether inoculated via the intraperitoneal (ip) or intracerebral routes. However, as the animals aged a marked drop in sensitivity occurred especially when inoculations were made ip. It was also found that the transmission of each virus between litter-mates under natural conditions occurred quite easily.

The possibility that an interfering component is synthesized by certain cell lines infected with Langat virus was also demonstrated. In addition, certain of the above results gave good evidence that strains of Langat virus may vary significantly in their behavior or response to comparable experimental conditions.

PLANS FOR FUTURE

(a) Rabbit, guinea pig, and hamster Langat antisera have been prepared using a variety of immunizing procedures. Now that the behavior of

Langat virus has been characterized it will be possible to utilize various serological procedures to ascertain the degree of cross-reactivity of the above antisera to other group B arboviruses-- especially those of the tick-borne encephalitis complex. (b) In addition, attempts will be made to better understand the nature of the variability in behavior which has been encountered among the various strains of Langat virus.

CURRENT REPORTS AND PUBLICATIONS

(a) E. F. Deig, I. P. Rie, R. E. Cranford and H.M. S. Watkins (1967), "Production and assay of Langat arbovirus." Forty-first Tech. Prog. Rep., Nav. Biol. Lab., Rep. EE-6.

(b) E. F. Deig, I. P. Rie, R. E. Cranford and H. M. S. Watkins (1967), "Cultural and serological characteristics of Langat virus." Forty-second Tech. Prog. Rep., Nav. Biol. Lab., Rep. EE-7.

SURVIVAL OF SPORES IN DISINFECTANTS AT LOW TEMPERATURES

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To determine behavior of microbial spores at subzero temperatures, especially while in contact with disinfectants; (b) to develop new methods for assaying viability of treated (injured) cells.

ABSTRACT

Survivor curves of Bacillus globigii, kindly furnished by the Army Chemical Corps Biological Laboratory, Fort Detrick, Frederick, Md., were determined at temperatures ranging from 20 to -20 C and with concentrations of free available chlorine (FAC) from 20 to 2000 ppm. At initial concentrations of 10^8 spores per ml, survivor curves were not logarithmic; usually about 10^2 cells per ml, at temperatures above 0 C, survived for extended periods at initial FAC concentrations up to 200 ppm, despite the measured presence of at least 50 ppm of remaining FAC--a concentration that could initiate disinfection of 10^8 spores per ml. Below 0 C, initial kill was slow, then sometimes became more rapid, but total numbers seldom dropped below about 10^4 cells per ml. Slow addition of FAC was more effective than rapid addition. Dry spores in contact with frozen FAC were not killed by concentrations as high as 1000 ppm. Fifteen ppm of FAC destroyed the infectivity of Columbia SK virus for mice in less than ten minutes at 20 C; at 0 C, infectivity was reduced 3 to 6 logs.

A 200 tube, automatic turbidimeter, intended to plot the times at which the contents of each tube passes through a given turbidity value, has been built and is being tested. This measures a different parameter than standard colony assay methods (numbers that can form colonies).

PLANS FOR FUTURE

Tests of the turbidimeter will be continued. Disinfectant potential of FAC for virus at temperatures below 0 C will be examined. Methods for removing spores from surfaces for purposes of assay will be tested, specifically a membrane filter-vacuum system.

CURRENT REPORTS AND PUBLICATIONS

None.

GENETICS OF THE RICKETTSIAL AGENT: COXIELLA BURNETII

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WORK UNIT NO. NR 103-590

CONTRACT Nonr-4240(02)

OBJECTIVES

(a) To study the genetics of the rickettsial agent, Coxiella burnetii, (b) to obtain genetically marked strains of C. burnetii, (c) to carry out genetic recombinations employing these marked strains, (d) to carry out genetic transformations utilizing DNA from genetically marked strains and (e) to determine the degree of genetic homology between C. burnetii and other rickettsiae.

ABSTRACT

Rickettsiae, Coxiella burnetii Phase II in a 20% yolk sac suspension, with an LD₅₀ of $10^{-4.6}$ were inoculated into embryonated eggs. Mutants have been obtained which have the following resistance: to chloromycetin, 2,000 µg per egg; acromycin, 20 µg per egg; to aureomycin, 50 µg per egg; terramycin, 20 µg per egg. It must be stated that adequate stocks of these organisms have not yet been obtained and that these results are currently under re-investigation to confidently obtain resistant strains. Our work is now being zeroed in to one or two of the antibiotics. Simultaneously, L-cells have been titrated for sensitivity to chloromycetin. It was established that an upper limit of 10 gammas per ml could be used. A strain of C. burnetii Phase II (E) has been obtained by ultraviolet irradiation for 20 seconds and then passage twice in L-cells in the presence of 10 µg per ml of chloromycetin.

PLANS FOR FUTURE

Our plans remain to obtain a stable mutant of C. burnetii and propagate it in sufficient quantities so that we may be able to extract DNA from the rickettsiae for transformation experiments. More emphasis will be placed on the use of tissue cells as a propagation medium for the rickettsiae. Under immediate study is the use of Ehrlich ascites cells as agents for genetic studies with rickettsiae. M. Wiebe is in the process of developing techniques to hybridize rickettsial DNA to attempt elucidation of relationships between C. burnetii and other rickettsiae.

CURRENT REPORTS AND PUBLICATIONS

(a) Paretsky, D. (1967), "Biochemistry of Rickettsiae and Their Infected Hosts With Special Reference to Coxiella burnetii." Zentralblatt (in press)

(b) Tsung, Pi-Kwang and D. Paretsky (1967), "Biochemical and Ultrastructural Changes in Liver Endoplasmic Reticular Fractions During Q Fever." Acta Virologica (in press)

MODE OF FISSION OF THE BENZENE NUCLEUS IN MICROORGANISMS AND
REGULATION OF GROWTH AND DIVISION BY BENZENE DERIVATIVES

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WORK UNIT NO. NR 103-504

CONTRACT Nonr-3443(00)

OBJECTIVES

Investigate the influence of nitrobenzoic acid on the growth, division, and inducible enzyme synthesis of microbial cells.

ABSTRACT

o-Nitrobenzoic acid (ONB) delays synthesis of an inducible degradative enzyme, protocatechuate oxygenase, in *Pseudomonas fluorescens* and synthesis of β -galactosidase in *Escherichia coli*. ONB did not affect activity of induced cells or a constitutive succinoxidase system.

ONB did not decrease the viable cell count but did decrease the incorporation of selected labeled amino acids into the hot TCA soluble fraction of the cell. The uptake of amino acids or the inducer were not affected. Inhibition of the synthesis of protocatechuate oxygenase could not be reversed by a mixture of amino acids previously reported to relieve the ONB inhibition of growth of a species of *Flavobacterium* using *p*-nitrobenzoic acid as the carbon and energy source.

CURRENT REPORTS AND PUBLICATIONS

(a) N. N. Durham and J. R. Martin (1966). Influence of analogs on the microbial accumulation and oxidation of D- and L-tryptophan. *Biochim. Biophys. Acta*, 115, 260-266.

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(c) J. R. Martin and N. N. Durham (1966). Metabolism of D-tryptophan by a species of *Flavobacterium*. *Can. J. Microbiol.*, 12 1269-1282.

STUDIES ON PASTEURELLA PESTIS

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WORK UNIT NO. NR 302-100

CONTRACT Nonr 222(73)

OBJECTIVES

1. (a) Immunogenicity tests of Armed Forces Plague Vaccine (AFPV) in mice and guinea pigs. (b) Study of vaccine dose and challenge dose relationships in guinea pigs. (c) Comparison of immunogenicity of AFPV- and lauric acid-killed Pasteurella pestis in mice with pneumonic plague. (d) Use of Fraction I of P. pestis as a reference standard in evaluations of plague vaccines. (e) The value of various stabilizing agents in lyophilized plague vaccines. 2. The effects of normal and variously treated sera on enzymes of tricarboxylic acid and hexose phosphate oxidation.

ABSTRACT

1. (a) Potency tests continue as one criterion for the release of AFPV by NIH. (b) Preliminary work shows that vaccines may be assayed in guinea pigs without concomitant adjuvant and with a lower challenge dose of P. pestis than used heretofore. (c) Preliminary tests show that equivalent doses of lauric acid-killed P. pestis strain 1951P endowed mice with greater resistance to pneumonic plague than the AFPV which was killed with formalin. Lauric acid killed P. pestis by inactivating 6-phosphogluconic dehydrogenase. (d) Fraction I of P. pestis strain EV76 was prepared and tested for immunogenicity in mice over eight months. The ED₅₀ of the lyophilized Fraction I was 2.05 gamma with a range of from 1.2 to 2.4 gamma/mouse. The ED₅₀ in guinea pigs was 10 gamma/pig when adjuvant was used. (e) This project was recently contracted with NIH and work will start in January, 1968. 2. Normal rabbit and mouse sera inhibited 6-phosphogluconic and glucose-6-phosphate dehydrogenases and preliminary observations show a possible relationship with complement.

PLANS FOR FUTURE

1. Routine potency tests of the Armed Forces Plague Vaccine and efforts at test improvement will continue. Attempts to stabilize the potency of plague vaccines by the inclusion of carbohydrates, proteins, antioxidants and chelating agents in lyophilized preparations. 2. Further studies of the antienzyme effects of normal serum will be made to determine if activity is related to complement.

CURRENT REPORTS AND PUBLICATIONS

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(b) D. M. Eisler and R. J. Heckly (1967), "Anti-Pasteurella pestis factor in the organs of normal mice and guinea pigs IV. Possible mechanisms of action." J. Bacteriol., submitted for publication.

(c) D. M. Eisler, E. K. von Metz and R. J. Heckly (1967), "A mechanism of anti-Pasteurella pestis activity of some lipids. Bacteriol. Review.

THE NATURE OF RESISTANCE AND SUSCEPTIBILITY

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WORK UNIT NO. NR 302-267

CONTRACT Nonr-2121 (14)

OBJECTIVES

(a) To distinguish strains and species of phytopathogenic bacteria by biochemical criteria, principally enzymes, (b) to investigate the role of nutrition and inhibition (in the broad sense) in the host-parasite relation, and (c) to determine the nature of host-specificity with respect to tissue-macerating enzymes excreted by the parasite in vitro and in vivo.

ABSTRACT

The extracellular endopolygalacturonases (endoPG), pectin lyases (PL), and cellulases (Cx) of many phytopathogenic bacteria have been assumed to be responsible for the dissolution, maceration, or disintegration of host-tissue and, consequently, significant factors in their virulence. A similar situation is found for those animal pathogens known to excrete collagenases and elastases. Protocols developed for the phytopathogens have been successfully applied to these pathogens by Dr. J. W. Rippon, Department of Medicine (Dermatology), University of Chicago, and an active collaboration has been initiated.

Starch-gel zone electrophoresis had indicated that culture filtrates from phytopathogenic bacteria contained not more than 1-2 enzymes with endoPG, PL, or Cx activity. Multi-enzymic systems have now been detected for both bacterial and fungal culture filtrates by means of an inexpensive, efficient device fabricated in the laboratory for preparative polyacrylamide gel electrophoresis. Although fungal filtrates may be directly applied to the device, bacterial filtrates had to be processed by Sephadex column chromatography.

A survey of the available literature revealed that zymograms or protein-profiles obtained by starch-gel or disc electrophoresis using cellular extracts or culture filtrates might have taxonomic value. Cellular extracts from phytopathogenic bacteria gave esterase zymograms which appeared to characterize strain within a species as well as species from the same or different genera.

A protocol involving chemical mutagens has been developed to obtain prototrophic avirulent mutants of phytopathogenic bacteria. Such mutants will be used to determine whether avirulence may be correlated with the inability to produce or to induce specific tissue-macerating enzymes.

PLANS FOR FUTURE

(a) To complete survey of esterase zymograms for bacterial phytopathogens to determine their worth for taxonomic purposes, (b) to relate avirulence with specific tissue-macerating enzymes by means of avirulent mutants, and (c) to compare tissue-macerating enzymes from virulent and avirulent strains or mutants with respect to their induction, electrophoretic mobility, molecular weight, and serological specificity.

CURRENT REPORTS AND PUBLICATIONS

- (a) D. Knösel and E. D. Garber (1967), "Pektolytische und cellulolytische Enzyme bei Xanthomonas campestris (Pammel) Dowson." *Phytopath. Z.* 59, 194-202.
- (b) E. D. Garber and J. W. Rippon (1968), "Enzymes and proteins as taxonomic tools." *Adv. Appl. Microbiol.*, in press.

STUDIES ON EPIDEMIOLOGY AND ECOLOGY OF THE
VIRUS OF WESTERN EQUINE ENCEPHALITIS

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WORK UNIT NO. NR 103-674

CONTRACT Nonr 1288 (07)

OBJECTIVES

(a) To study the presence of naturally occurring virus and antibody of Western equine encephalitis in snakes and other cold blooded animals. (b) To establish the conditions in nature for virus transfer from mosquitoes to snakes, (c) to determine the reasons for cyclic viremia in infected snakes.

ABSTRACT

Declining temperature in late summer and early fall, along with close association of mosquitoes with snakes, appear to play a dominant role in the biting habits of Culex tarsalis mosquitoes in accepting snakes as blood meal hosts. Over 460 snakes were captured in three marshy areas, tagged and bled, then released in their native areas. Many of these snakes have been recaptured, bled, and released. Preliminary tests suggest that virus and antibody appear as cooler weather sets in in late summer and early fall, suggesting confirmation of laboratory studies relative to temperature.

A non-infectious, tissue cultured, purified WEE virus has been developed that produces excellent hemagglutination tests and is superior for hemagglutination inhibition tests for antibody detection.

A method has been developed to freeze preserve snake embryo cells and on testing six months after holding in liquid nitrogen, grow well in tissue culture and produce excellent virus yields.

PLANS FOR FUTURE

(a) To follow the presence of virus and hemagglutination inhibiting antibody in over 460 snakes, tagged, bled, and recaptured during the summer and fall of 1967; recapture as many as possible in the spring, summer, and fall of 1968; and test the blood for virus and hemagglutinating antibody. (b) To study the cyclic nature of virus in snakes, both hibernated and non-hibernated, using antibody detection and interferon detection, the latter being possible using the frozen snake embryo cells in tissue culture systems. (c) To continue studies on improving the specificity of the hemagglutination inhibition test for snake WEE antibody.

CURRENT REPORTS AND PUBLICATIONS

(a) L. P. Gebhardt, G. J. Stanton, and S. de St. Jeor (1966), "Transmission of snakes by infected Culex tarsalis mosquitoes." Proc. Soc. Exp. Biol. and Med., 123, 233-235.

(b) R. W. Sidwell, L. P. Gebhardt, B. D. Thorpe (1967), "Epidemiological aspects of Venezuelan equine encephalitis virus infections." Bact. Rev., 31, 65-81.

(c) G. J. Stanton (1967), Ph. D. Thesis, "The role of poikilothermic animals in overwintering Western equine encephalitis virus."

SPORE ULTRASTRUCTURE

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WORK UNIT NO. NR 302-531

CONTRACT Nonr 2587(08)

OBJECTIVES

The general objective has been to characterize the ultrastructure of bacterial spores, from cell anatomy to macromolecular fabric and with physical, chemical, and physiological methods. Where possible, the results have been extended to considerations of practical problems of infectious disease, sterilization, and preservation. Specific objectives are evident in the papers and individual projects undertaken, the more recent of which are given below.

ABSTRACT

Some confirmatory work on the chemical composition of the exosporium of spores of Bacillus cereus was undertaken. Several hundred milligrams of exosporium were separated and purified, then subjected to amino acid analysis. Somewhat different results were obtained than with previous preparations, both qualitatively and quantitatively, indicating either variability in chemical composition or in relative purity of the preparations. Promising results were obtained with use of sodium dodecyl sulfate and gradient centrifugation for separation of paracrystals from an unpurified preparation of disrupted spores.

A previous effort had been made (Gerhardt and Black, 1961) to characterize molecular sieving by intact spores. This was reexamined by a newly developed method in which a profile is made of the molecular weight distribution in a heterodisperse polyethylene glycol bath, before and after uptake. The results revealed a threshold porosity (i.e., the largest opening in the wall structure) ten-fold smaller than had been estimated previously. Polyglycols of molecular weight 10,000 and above appear to be excluded by the surface of intact B. cereus spores.

A review was prepared on the cytology of the anthrax bacillus. The sporulation cycle and cell structures were compared with those reported in the historical observations by Koch. Newly reported observations included the presence of an ordered macromolecular lattice in the ultrastructure of vegetative cell walls, a situation just now realized to occur in gram-positive as well as gram-negative bacteria.

PLANS FOR FUTURE

In previous published work (Gerhardt and Ribi, 1964) an interpretation of the surface ultrastructure of exosporium had been made from electron micrographs of phosphotungstate-stained fragments and from wide-angle X-ray diffraction patterns. We want to verify these deductions by use of a small-angle X-ray camera, which should detect periodicities in crystalline structure greater than 50 Å.

A further effort will be made to characterize the permeability of intact spores and perhaps isolated fractions, using the refined methodology now developed. We also seek to make a critical analysis, using deuterium, of the content and distribution of water in spores, as a key toward understanding the mechanism of thermoresistance as possibly explained by a dehydration mechanism.

CURRENT REPORTS AND PUBLICATIONS

(a) Gerhardt P. (1967), "Cytology of Bacillus anthracis". Fed. Proc. 26:1504-1517.

(b) Matz, L. L. (1967), "Chemical ultrastructure of the spore of Bacillus cereus". Ph.D. Thesis, University of Michigan, Ann Arbor, (Multilith copies available in request).

STUDIES OF INFECTIOUS DISEASES OF TAIWAN

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I. Emmanuel, M.D.

WORK UNIT NO. NR 103-724

CONTRACT Nonr-(G)-00044-66

OBJECTIVES

To study infectious diseases of the Far East in association with NAMRU-2 on Taiwan.

ABSTRACT

Utilization of the monkey eye model for trachoma infection has allowed demonstration that highly purified trachoma vaccine can be produced that exhibits a protective effect. Monkey eye infection completely similar to human infection has been demonstrated. Field studies with trachoma vaccines show short protective periods of 1 - 2 years. For other laboratory studies, investigations of congenital malformations and heart disease in Chinese, see list of publications.

PLANS FOR FUTURE

Present studies emphasize use of trachoma in the monkey eye model for evaluation of new vaccine preparations and utilization of the unique epidemiology of rubella on Taiwan to evaluate attenuated rubella vaccine.

CURRENT REPORTS AND PUBLICATIONS

(a) E. R. Alexander, S. P. Wang and J. T. Grayston (1967), "Further classification of TRIC agents from ocular trachoma and other sources by the mouse toxicity prevention test." Amer. J. Ophth., 63, 443-452.

(b) E. R. Alexander and J. W. Fresh (1967), "Prevention of anaphylactic death in the mouse toxicity prevention test for TRIC agents." Amer. J. Ophth., 63, 436-443.

(c) E. R. Alexander and W. T. Chiang (1967), "Infection of pregnant monkeys and their offspring with TRIC agents." Amer. J. Ophth., 63, 119-127.

(d) C. C. Huang, I. Emanuel, S. W. Huang and T. Y. Chen (1967), "Two cases of the De Lange syndrome in Chinese infants." J. Peds., 71, 251-254.

(e) H. M. Jenkin and S. C. Hung (1967), "Effect of vancomycin on the growth of psittacosis-trachoma agents cultivated in eggs and cell culture." Appl. Micro., 15, 10-12.

- (f) H. M. Jenkin, H. M. Yu and E. C. Suitor Jr. (1967), "Plaque neutralization of various arboviruses in monkey kidney cells (LLC-MK2)." *Bact. Proc.*
- (g) H. M. Jenkin and Y. K. Lu (1967), "Induction of interferon by the Bour strain of trachoma in HeLa 229 cells." *Amer. J. Ophth.*, 63, 84-89.
- (h) H. M. Jenkin (1967), "Comparative lipid composition of trachoma and psittacosis agents." *Amer. J. Ophth.*, 63, 61-72.
- (i) K. S. W. Kim and E. S. Boatman (1967), "Electron microscopy of monkey kidney cell cultures infected with rubella virus." *J. Virol.*, 1, 205-214.
- (j) G. C. Y. Lee, J. T. Grayston and S. P. Wang (1967), "Protective studies in mice and monkeys with an inactivated Japanese encephalitis virus vaccine grown in hamster diploid cell culture." *Proc. Soc. Exper. Biol. & Med.*, 125, 803-808.
- (k) C. Y. Lee and J. T. Grayston (1967), "A serological survey of neutralizing antibodies to selected enteroviruses on Taiwan." *J. Formosan Med. Assoc.*, 66, 448-452.
- (l) Y. B. Li, N. Ting, B. N. Chiang, E. R. Alexander, R. A. Bruce and J. T. Grayston (1967), "Electrocardiographic response to maximal exercise. Treadmill and double master exercise tests in middle aged Chinese men." *Amer. J. Card.*, 20, 541-548.
- (m) S. P. Wang, J. T. Grayston and E. R. Alexander (1967), "Trachoma vaccine studies in monkeys." *Amer. J. Ophth.*, 63, 119-127.
- (n) R. L. Woolridge, J. T. Grayston, I. H. Chang, K. H. Cheng, C. Y. Yang and C. Neave (1967), "Field trial of monovalent and of a bivalent mineral oil adjuvant trachoma vaccine in Taiwan school children." *Amer. J. Ophth.*, 63, 619-624.
- (o) R. L. Woolridge, J. T. Grayston, I. H. Change, C. Y. Yang and K. H. Cheng (1967), "Long-term follow-up of initial trachoma vaccine field trials on Taiwan." *Amer. J. Ophth.*, 63, 624-627.
- (p) R. L. Woolridge, K. H. Cheng, I. H. Chang, C. Y. Yang, T. C. Hsu and J. T. Grayston (1967), "Failure of treatment of trachoma with antibiotic eye ointment and oral sulfa drug alone or in combination with trachoma vaccine." *Amer. J. Ophth.*, 63, 551-557.
- (q) R. L. Woolridge, J. T. Grayston, E. B. Perrin, C. Y. Yang, K. H. Cheng and I. H. Chang (1967), *Amer. J. Ophth.*, 63, 287-294.
- (r) R. L. Woolridge and J. T. Grayston (1966), "Trachoma infection on Taiwan: Epidemiology, prevention and chemotherapy studies." *Australian J. of Med.*, II, 348-351.
- (s) I. Emanuel and S. W. Huang (1967. In press.), "Physical features of Chinese children with Down's syndrome." *Amer. J. Dis. Child.*

RESEARCH ON INFECTIOUS DISEASES OF TAIWAN
AND THE FAR EAST

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ASSISTED BY John Cross and W. D. Kundin

WORK UNIT NO. NR 103-730

CONTRACT Nonr-14-67-A-0103-0001

OBJECTIVES

(a) John Cross is studying parasitic diseases to determine the distribution, prevalences and ecology of parasitic diseases in Taiwan and the Far East. (b) W. D. Kundin is studying viral, rickettsial and bacterial diseases to determine the distribution, prevalences and ecology of microbial diseases in Taiwan and the Far East, with emphasis on ectoparasite-rickettsial and arboviral relationships. (c) John Cross and W. D. Kundin together are studying Zoonoses of Viet Nam.

ABSTRACT

Rodents in rural areas have been found more frequently infected with Angiostrongylus cantonensis than urban rats. Various species of terrestrial snails as well as aquatic snails have been found as vectors of the parasite. Heavy infections with the Taiwan strain of A. cantonensis have been found to cause death in Taiwan monkeys.

Prevalence surveys, animal autopsies and life cycle studies are being undertaken on a new species of Capillaria infecting man in the Philippines.

A new species of malaria in the Taiwan flying squirrel has been found and is under study.

Acute and convalescent materials obtained from hospitalized servicemen in Viet Nam are being tested for the presence of infectious agents and the appearance of antibodies against a variety of viral, rickettsial and bacterial agents at a rate of 25-35 patients per week. Successful diagnoses are being made on about half the cases fulfilling the criteria of an FUO.

Of the several score Viet Nam zoonosis materials tested, leptospirosis has been isolated from one rat, an unidentified agent from another rat, and scrub typhus from two rats and two chigger pools. Identification of the various rodents and ectoparasites are under way.

PLANS FOR FUTURE

- (a) Laboratory studies on the host-parasite relationships between various strains Angiostrongylus cantonensis and the Taiwan monkey.
- (b) To establish methods of sero-diagnosis for angiostrongyliasis in man.
- (c) Determination of the means of transmission and life cycle of Philippine Capillariasis.
- (d) Studies on the development of malarial parasites in arthropod tissue culture.

The FUO study is expected to continue pretty much in its present form for the duration of the Marine Corps involvement in Viet Nam. However, as time and opportunities present themselves, serological studies of rural Vietnamese and Montagnards will be initiated in order to determine the normal patterns of infections in the Marine Corps sector of Viet Nam.

The future of zoonotic studies in Viet Nam are, of necessity, flexible. As opportunities present themselves collections in other areas will be initiated. However, as long as the conflict persists, it will not be safe to do anything beyond piecemeal collections. With the advent of peace more systematic surveys and ecologic studies will be instituted. The plans for the future of zoonotic studies in Taiwan are more specific. The study will cover a five-year period. The first year will be mainly a screening of thousands of animal and human sera already on hand. The next two years will be mainly a trapping and processing of animals and ectoparasites for isolation studies, testing of sera from human populations at high risk, and pinpointing foci of infection. The next two years will be mainly ecological studies of the foci of infection and hospital studies of infections in man to determine how man gets involved in the cycle in order to determine how to interrupt or mitigate the cycle.

CURRENT REPORTS AND PUBLICATIONS

(a) John H. Cross (1967), "Review of Angiostrongyliasis in Taiwan." Seminar in Helminthiasis and Eosinophilic meningitis. Noumea, New Caledonia; 5-16 June 1967.

(b) P. K. Chang, J. H. Cross and S. S. Chen. "Aquatic Snails as Intermediate Hosts for Angiostrongylus cantonensis in Taiwan." (Submitted for Publication)

SURVIVAL MECHANISM OF DRY CELLS

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

To investigate biological and physical mechanisms that affect viability (survival) of dried cells and tissues, with the ultimate goal of being able to maximize or minimize survival.

ABSTRACT

Dried bacteria, in contact with oxygen, produce free radicals (FR) and this FR production, as measured by electron paramagnetic resonance, correlates with loss of viability. An aqueous extract of sonicated Serratia marcescens also produced FR when dried and exposed to air. Two components of this extract were needed for FR production by oxygen: a high molecular weight (HMW) compound (about 60,000), and a low molecular weight material (LMW) of less than 25,000. The principal component of the HMW material is a partially depolymerized deoxyribonucleic acid, though the activity could be due to an impurity in the preparation. The LMW component may have a structure similar to adrenaline, since a combination of adrenaline or propyl gallate and the HMW fraction produced FR when the mixture was dried and exposed to air. FR production was probably not caused by antioxidation because no FR were detected when p-hydroxybenzoic acid was used in place of propyl gallate. The direct involvement of these two components in survival mechanisms of dry organisms has not been demonstrated.

Various substances, such as sucrose, inositol or propyl gallate, generally increase the percentage of microorganisms surviving lyophilization and storage. Some apparent inconsistencies in protective effect were found to be related to a temporal response of the organisms to the changed environment produced by the additive. When an additive was mixed with suspensions of Serratia marcescens or other organisms and samples were removed and frozen at the shortest practicable intervals after mixing, the number of cells that survived both lyophilization and exposure to air varied rhythmically as a function of time between mixing and freezing. The number of surviving cells in the sample, removed and frozen 30 sec after mixing with 0.4% propyl gallate solution, was occasionally 100 times that in samples frozen 10 to 20 sec earlier or later. Other "peaks" in survival were observed at approximately 125 and 450 sec, but the times at which the peaks were observed were not constant from one experiment to the next. Rhythmic changes in resistance to lyophilization were also observed after the

addition of sucrose or after centrifugation and resuspension of cells. It appears that the bacteria responded physiologically to changes in their environment, and some of these responses had survival value.

PLANS FOR FUTURE

Studies are being continued in an effort to learn more about the free radical, or its precursors, and why the free radical produced by lyophilized bacteria is correlated with loss of viability, and to determine the mode of action of the current protective substances.

CURRENT REPORTS AND PUBLICATIONS

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(b) R. J. Heckly and R. L. Dimmick (1967), "Electron paramagnetic resonance in frozen and dried biological materials." *Nature*. In press.

BIOSYNTHESIS OF BACTERIAL CELL WALLS

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WORK UNIT NO. NR 302-432 CONTRACT Nonr-2702(00)

OBJECTIVES

To study the metabolic and physiological factors involved in the formation and function of bacterial cell walls and membranes.

ABSTRACT

The adverse effects of several B vitamin deficiencies on cell wall composition and the operation of amino acid transport systems in Lactobacillus plantarum and Streptococcus faecalis have led us to study the membrane lipids of these organisms in an effort to describe the metabolic basis of these structural and functional changes. The readily extracted lipids of L. plantarum have been separated into classes on TEAE ion exchange cellulose columns and each of these fractions has been sub-fractionated on thin layer plates. The components identified so far include phosphatidylglycerol, cardiolipin, a sulfatide, a series of glycosylglycerides, free fatty acids and a neutral lipid fraction containing at least six components. The so-called bound lipids, which could be liberated by extended application of our usual extraction procedure, were shown to contain the same components as the easily-extractable fraction. Biotin and pantothenic acid deficiencies markedly reduced the total amount of lipid in L. plantarum. There are several distinctive changes in the fatty acid components of the individual lipids which are now being studied in detail. Following completion of the descriptive phase of this study, which will include quantitation of all the lipid components, an attempt will be made to correlate the membrane lipid changes with alterations in permeability and transport properties of the respective cell types.

In addition to the lipid changes discussed above, several B vitamin deficiencies caused distinctive cell wall changes. A pantothenic acid deficiency, for example, markedly increased the sensitivity of the wall to lysozyme digestion and also increased its capacity to bind positively-charged proteins. The possibility that the net negative cell surface charge was increased by this deficiency was studied using a prototype model of a continuous particle electrophoresis system recently developed by Beckman Instruments, Inc. Normal cultures of L. plantarum were shown to be heterogeneous, containing two main populations of cells both bearing a negative charge. In confirmation of the lysozyme binding

studies, a pantothenic acid deficiency increased the negative charge of both populations. An advanced prototype model of this apparatus will shortly be made available to us which should greatly facilitate further work on this project.

Studies on the transport of the aminophosphonic acids analogous to the amino acids have been extended using tritiated substrates. Previous studies indicated that substances such as 2-amino-3-phosphonopropionic acid (APP) (analogous to aspartic acid) and 1-aminoethylphosphonic acid (1-AEP) (analogous to alanine) were transported by the constitutive amino acid carriers. S. faecalis, which contains two catalytic systems for the transport of dicarboxylic acids, could utilize only one of these, the so-called "high affinity" system for the transport of APP. As a consequence, a mutant lacking this system failed to transport APP at a measurable rate. Studies with tritiated APP established the relative resistance of this substance to metabolic alteration or utilization, indicating that it may serve as a useful analog for the study of this transport system.

PLANS FOR FUTURE

The following will receive major attention in the immediate future: (1) Conclusion of studies on the lipid constituents of nutritionally-modified L. plantarum and S. faecalis; (2) correlative studies on membrane lipid changes and alterations in cell function such as permeability, transport and cell wall formation; (3) metabolism and turnover of membrane lipids as a function of nutritional status; (4) continuous particle electrophoresis studies on cell wall structure changes induced by nutritional genetic and environmental factors.

CURRENT REPORTS AND PUBLICATIONS

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(b) J. T. Holden and N. M. Utech, "Actinomycin D inhibition of amino acid transport in Streptococcus faecalis." Biochim. et Biophys. Acta 135, 351 (1967).

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THE BIOLOGY OF MYCOPLASMA

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WORK UNIT NO. NR. 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

- (a) To study the nutritional requirements for growth of mycoplasma of human origin, both those strains which ferment glucose such as Mycoplasma pneumoniae and Mycoplasma fermentans, and the nonfermenting strains such as Mycoplasma hominis, type I and Mycoplasma salivarium.
(b) To determine the role of lipids in the growth and survival of these organisms.

ABSTRACT

Although the lipids of egg yolk were found earlier to support maximal growth of Mycoplasma neurolyticum in the presence of 0.25% bovine serum albumin, these lipids were not found essential for growth of all of the human species. It became apparent in those studies that the growth requirements of each species are different. An additional difficulty developed when it was learned that the lipids prepared from egg yolk are toxic for certain mycoplasma species unless they are added with serum albumin.

In a search for substances which will replace animal serum as a nutrient for these microorganisms, skim milk and gelatin were examined. Since both materials can be sterilized by autoclaving, their addition to media would provide an improvement over the use of serum. With M. hominis growth comparable to that obtained with horse serum was found when 2% skim milk was added to broth. The use of 2% skim milk in agar media resulted in comparable counts with M. hominis, although the size of the colonies was somewhat smaller. Another disadvantage of this addition to agar was the resulting opacity of the medium. However, with some lots of horse serum the inclusion of both 2% skim milk and 10% horse serum resulted in larger colonies and higher counts than with horse serum alone.

PLANS FOR FUTURE

We plan to examine other species of mycoplasma of human origin, in an effort to determine the composition of culture media which will permit more sensitive detection and growth of these microorganisms.

CURRENT REPORTS AND PUBLICATIONS

None

ELECTROMAGNETIC SEPARATION OF BIOLOGICAL PARTICLES

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WORK UNIT NO. NR 103-505

CONTRACT Nonr 233(64)

OBJECTIVES

Development of electrokinetic and electromagnetic high resolution methods for separation of biological particles.

ABSTRACT

The main effort in 1967 was concerned with the completion of the development of the method of endless belt electrophoresis. In this method injected particles can be kept suspended for an indefinite period of time while subjected to electrophoretic forces. Exceedingly high resolving power can thus be obtained. The electrophoretic column is a liquid belt whose shape and motion resemble a vertically mounted belt sander. A fine streak of charged particles to be separated describes a non-circular helix under the influence of the horizontal axial electric field and of the magnetic field which is perpendicular to the fluid belt. A practical apparatus was designed which can be easily disassembled for cleaning. Exchangeable iron cores make it possible to change the thickness of the buffer belt. The membranes, previously used to separate electrode compartments from buffer compartments, have been eliminated. A more stable operation has thus been obtained in prolonged separation runs. The apparatus has been put to use for fractionation of different types of blood cells, fungi, bacteria, viruses and tissue culture cells.

PLANS FOR FUTURE

This system will now be used for characterization of microbiological particles by their electrophoretic mobilities. The method will be improved and modified on one hand for high-resolution separation of small quantities and on the other hand for large-scale separation at lower resolution. New approaches to particle separation and characterization will be explored.

CURRENT REPORTS AND PUBLICATIONS (see next page)

CURRENT REPORTS AND PUBLICATIONS

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- (b) "Preparative Electrophoresis in Liquid Columns Stabilized by Electromagnetic Rotation.Part I The Apparatus". A.Kolin, J.of Chromatography 26: 164-179,1966.
- (c) "Preparative Electrophoresis in Liquid Columns Stabilized by Electromagnetic Rotation.Part II Artifacts,Stability and Resolution". A.Kolin, J.of Chromatography 26:180-193, 1966.
- (d) "Helical Path Electrophoresis in Endless Fluid Belts". A.Kolin, Biophysical Society, Feb.1968.
- (e) "Continuous Flow Electrophoretic Separation and Characterization of Strains of Bacteria and Viruses". S.J.Luner and A.Kolin, Biophysical Society, Feb.1968.
- (f) "Electrophoresis in Endless Fluid Belts". A.Kolin, American Chemical Society, March 1968.
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- (k) "Continuous Electrophoresis in Fluid Endless Belts". A.Kolin and S.J. Luner, in preparation.
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STUDIES ON THE U. S. NAVY PARASITOLOGICAL COLLECTIONS
(ZOOGEOGRAPHY AND HOST-PARASITE RELATIONSHIPS OF HELMINTHS -)

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WORK UNIT NO. NR 103-690

CONTRACT N0014-66-C0094

OBJECTIVES

Compilation of data and information based upon a study and final evaluation of parasitological materials taken by the U. S. Navy. Although emphasis has been placed on the zoogeography and host-parasite relationships of helminth parasites, there are several investigations associated with the intestinal protozoan parasites and commensals of man as well as the hemosporidian and allied blood parasites common to lower vertebrates. Studies continue to be made on the description and identifications of ectoparasites as well as leeches which constitute a small but important segment of the U. S. Navy parasitological collections.

ABSTRACT

Processing of parasites from vertebrates examined in the Yemen (Southwest Arabia), North Borneo, Palawan (Republic of the Philippines) and Taiwan (Republic of China) and its Offshore Islands, has continued. In the current period thirteen shipments of helminths have been prepared for deposit and accessioned with the National Helminthological Collections, U. S. National Museum, Beltsville, Maryland. There has been an extensive exchange of materials and information with a number of coworkers engaged in studies on different categories of vertebrate hosts as well as on the helminth and ectoparasites obtained from these hosts.

Compilation of data and information has continued on materials accumulated in the course of investigations associated with experimental schistosomiasis, paragonimiasis and sparganosis on Taiwan.

A public presentation based upon investigations accomplished by Naval Medical Research Unit No. 3, Naval Medical Research Unit No. 2, and supported by the Office of Naval Research, was given in the annual lecture before the Society of Sigma Xi, University of Oklahoma, Norman.
Subject: "In Pursuit of Parasites."

PLANS FOR FUTURE

A continuation of efforts to compile data and information on the parasitological materials taken from vertebrates examined in North Borneo, Palawan, and Taiwan. Reports will be prepared on investigations completed in the laboratory on Schistosoma, Paragonimus and Spirometra.

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- (c) J. T. Self and R. E. Kuntz (1967), "Host-parasite relations in some pentastomida." J. Parasitol. 53(1):202-206
- (d) J. H. Fischthal and R. E. Kuntz (1967), "Annotated record of some previously described digenetic trematodes of amphibians and reptiles from the Philippines, Korea and Matsu Island." Proc. Helm. Soc. Wash. 34(1):104-113
- (e) B. J. Myers and R. E. Kuntz (1967), "Nematode parasites of fishes taken on Taiwan (Formosa) and its Offshore Islands." Can. J. Zool. 45:237-241
- (f) R. E. Kuntz (1967), "Intestinal protozoa and helminths in Chinese evacuees from the Burma-Thailand border." Mil. Med. 132(3):210-215
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- (h) R. E. Kuntz (1967), "Survey of intestinal parasites of school children in Kee-Lung Shih; Hua-Lien, Tao-Yuan, Nan-Tou, Chang-Hua and Ping-Tung Hsiens, Taiwan." J. Formosan Med. Assn. 66(6):293-299
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- (j) J. H. Fischthal and R. E. Kuntz (1967), "Digenetic trematodes of amphibians and reptiles from Fiji, New Hebrides and British Solomon Islands." Proc. Helm. Soc. Wash. 34(2):244-251
- (k) R. E. Kuntz and B. J. Myers (1967), "Primate cysticercosis: Taenia hydatigena in Kenya vervets (Cercopithecus aethiops Linnaeus, 1758) and Taiwan macaques (Macaca cyclopis Swinhoe, 1864)." Primates 8(1):84
- (l) G. D. Schmidt and R. E. Kuntz (1967), "Notes on the life cycle of Polymorphus (Profilicollis) formosus sp.n., and records of Arhythmorhynchus hispidus Van Cleave, 1925 (Acanthocephala) from Taiwan." J. Parasitol. 53(4):805-809
- (m) R. E. Kuntz and B. J. Myers, "Helminths of vertebrates and leeches taken by the U. S. Naval Mission to Yemen, Southwest Arabia." (Submitted).
- (n) B. J. Myers and R. E. Kuntz, "Nematode parasites of amphibians taken on Taiwan." (Submitted).

STUDY AND IMPROVEMENT OF PSEUDOMONAS AERUGINOSA VACCINES

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WORK UNIT NO. NR 502-697

CONTRACT N00014-67-C-0258

OBJECTIVES

(a) To assay viable and non viable monovalent vaccines, (b) to assay non viable polyvalent vaccines, (c) to study some antigenic characteristics of the polyvalent vaccines.

ABSTRACT

All P. aeruginosa strains used in our studies, isolated from human patients, were of the smooth, green-pigmented, cytochrome oxidase-positive type. All strains were grown on Nutrient Agar (Difco) at 37 C for 24 hr. Virulence was maintained by weekly passage through mice. For each type of vaccine, mice (weighing 15 to 20 g) were inoculated subcutaneously with three 0.2 ml doses at two-day intervals. Except as otherwise stated all vaccines were non toxic. Immunity was assessed by determining the capacity of the vaccines to protect mice against 1 LD₁₀₀ administered forty days after the last immunizing dose. The LD₁₀₀ consisted in 0.2 ml of a saline bacterial suspension which was fatal within 24 to 48 hr to 100 % of the control mice when administered intraperitoneally. Sera obtained from immunized or control animals were tested by a method similar to that of Huddleson for brucellosis. Antigens were prepared suspending live Pseudomonas in physiological saline and the density adjusted to equal that of the Mc Farland N° 10 standard.

Five methods of killing bacteria were used: (a) ether, (b) chloroform, (c) ultraviolet rays, (d) formaldehyde and (e) phenol.

(a) and (b) - 10 % of ether or chloroform was added to Pseudomonas saline suspensions, which were incubated at 37 C for 30 min. These vaccines were partially efficient (approximately 40 % of the mice survived the challenge dose).

(c) A suspension of cells in saline was irradiated with an ultraviolet lamp the minimum time required to kill all bacteria. 90 % of vaccinated mice survived 1 LD₁₀₀ of the same strain used for vaccine preparation.

(d) It consisted in a suspension of Pseudomonas in 0.3 % formaldehyde saline that was incubated at 37 C for 24 hr. 100 % of the mice were protected by this vaccine.

Viable vaccines - Mice were vaccinated with sublethal dose vaccines. The percentage of survivors ranged from 60 to 0.

Attenuated vaccines- These vaccines were obtained from three-month old cultures or by ageing suspensions from new cultures. The first type gave poor results and the last was very toxic.

Therefore, excepting the UV- and formaldehyde-treated vaccines, none of the above cited types compares favorably with phenol-treated vaccines, which protected 100 % of the mice.

(e) Non viable polyvalent vaccines- These non-toxic vaccines were prepared by suspending together nine strains of *Ps. aeruginosa* in physiological saline with 0.5 % of phenol added, at a concentration of 10 mg / ml. 80 % to 90 % of vaccinated mice survived 1 LD₁₀₀ of a strain included in the vaccine. When the strain used for the challenge had not been included in the vaccine the percentage of survivors descended to 20. Vaccination evoked a detectable serum antibody response in all animals.

In another set of tests, the polyvalent-vaccinated mice were re-inoculated one month later with a booster dose of : (a) the same vaccine; (b) 1 LD₁₀₀ of a strain included in the vaccine; (c) 1 sublethal dose of a strain included in the vaccine. The percentage of survivors was: (a) 6; (b) 97; (c) 80, when the challenge strain was one not included in the vaccine. Simultaneous agglutination tests show that all vaccines provoked a high serum agglutination response against strains included or not in the vaccination schedule.

In view of the good results obtained with the polyvalent vaccine we have now started work on the preparation and purification of polyvalent hyperimmune sera.

PLANS FOR FUTURE

(a) Serotherapy (b) enzymatic and sonic lysis of viable and non viable monovalent and polyvalent vaccines, and study of the antigenic substances which confer antipseudomonas protection (c) electrophoretic purification of the vaccines and chromatographic study of the fractions after hydrolysis (d) immunization tests in burned and infected animals (e) immunization tests in infected human patients.

CURRENT REPORTS AND PUBLICATIONS

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(b) R. J. Jones et al. (1966) , "Antiserum and antibodies in the prophylaxis of burns against *P. aeruginosa*." *Brit. J. Plast. Surg.*, 19, 43-57.

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(d) H. F. Laborde and C. L. de Fajardo (1967) , "Pseudomonas vaccine. II. Appraisal of different purification methods." *J. Bacteriol.*, 93, 508-509.

(e) J. Loiseleur (1965) , "Thermovaccination and serotherapy of experimental burns in rats." *Ann. Inst. Pasteur (Paris)* , 109, 118-134.

IMMUNITY AND PATHOGENESIS IN THE MYCOSES

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

Extension of studies on a killed spherule vaccine for coccidioidomycosis with reference to (a) toxicity for humans, and (b) effectivity in atypical infections. Influence of positive air ions on experimental coccidioidomycosis of mice.

ABSTRACT

In earlier reports, it was shown that mice and monkeys could be immunized against lethal challenge doses of Coccidioides immitis. In the present study, animals were challenged intranasally with arthrospores from seven heterologous strains; two were typical of the species, and five were atypical with respect to cultural characteristics and morphology. The vaccinated animals were well protected against challenge doses that were lethal to a majority of the control animals, regardless of the strain of fungus employed. The infection ratios among surviving vaccinated and control animals were comparable, but demonstrable lesions were generally smaller and less numerous in the vaccinated groups. It is suggested that these strains are at least immunogenically similar, although not necessarily identical, and that a vaccine prepared from a single strain of C. immitis would be practical for an immunization program.

Delayed hypersensitivity to coccidioidin was conferred on human volunteers by intramuscular injection of two to four doses of killed C. immitis spherule vaccine. The volunteers were still sensitive after an 18-month observation period, although the intensity of the reaction had waned in most. One of two volunteers, who was sensitive before vaccination, developed complement-fixing antibodies but neither of these subjects, nor six who were nonsensitive prior to vaccination, developed precipitins. Vaccine dosages varying between 1.2 and 2.7 mg produced neither untoward tissue damage at the inoculation site nor a generalized reaction in seven of the eight volunteers, but one subject, who had a history of allergy, reacted to the second of two doses (1.1 mg each) with urticaria. In further tests carried out in 59 male volunteers, the skin response was variable. Two lots of vaccine were tested; the first lot had clearly more irritant effect at the site of injection than the second. At doses below 5 mg, the second lot of vaccine was generally well tolerated though occasional mild tenderness and slight induration developed. Infrequently, these responses were more marked. At doses of 5 mg or greater, local discomfort,

swelling and induration were consistently produced. These reactions usually subsided in seven to ten days. Induction of delayed hypersensitivity was irregular and, when present, dermal reactions were weak even with 1:10 coccidioidin. Serologic response after vaccination was clearly elicited in only three subjects, was transient and unrelated to degree of dermal hypersensitivity.

The course of pulmonary coccidioidomycosis in mice was adversely affected by continuous exposure of the animals to concentrations of small positive cluster air ions ranging between 3 and 4×10^5 ions/cc. Within the first seven days the infected mice exhibited signs ordinarily not observed: marked listlessness, ruffled coat, weight loss and evident malaise. They died between the 12th and the 17th day. Significant numbers of control animals did not die until the 18th day. By the 30th day a late increase in deaths among ion-treated mice brought the cumulative mortality rate to 55% in contrast with a rate of 30% in the controls. In a repeat experiment with a strain of unusually high pathogenicity, the final difference in mortality was only 15%.

Fungal numbers in lungs, livers and spleens of control and ion-treated infected animals did not substantiate our working hypothesis that positive air ions might depress the host defense and facilitate fungal proliferation and dissemination. We have speculated that the 5-hydroxytryptamine theory of air ion action may explain the results observed in these experiments.

PLANS FOR FUTURE

(a) Study of methods to detect vaccination serologically; (b) further testing in human beings; (c) new antibiotics in the deep and superficial mycoses.

CURRENT REPORTS AND PUBLICATIONS

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(b) H. B. Levine and C. E. Smith (1967), "The reactions of eight volunteers injected with Coccidioides immitis spherule vaccine; first human trials." In Coccidioidomycosis, Ed. by Libero Ajello, Univ. of Arizona Press, Tucson.

(c) D. Pappagianis, H. B. Levine and C. E. Smith (1967), "Further studies on killed spherule vaccine: Reactions of 59 prisoner volunteers." In Coccidioidomycosis, Ed. by Libero Ajello, Univ. of Arizona Press, Tucson.

(d) Y. M. Kong and H. B. Levine (1967), "Loss and recovery of virulence of arthrospores and spherule-endospores of Coccidioides immitis in live vaccines." In Coccidioidomycosis, Ed. by Libero Ajello, Univ. of Arizona Press, Tucson.

(e) M. Huppert, H. B. Levine, S. H. Sun and E. T. Peterson (1967), "Resistance of mice to typical and atypical strains of Coccidioides immitis." *J. Bacteriol.*, 94, 924-927.

(f) H. B. Levine (1967), "The aerobiologic route of infection in fungal disease." In Experimental Aerobiology (textbook) Ed. by R. L. Dimmick. John Wiley & Sons, N. Y. Manuscript in press.

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STUDIES ON FLEAS AND TICKS OF KNOWN OR POTENTIAL
MEDICAL IMPORTANCE IN NEPAL

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WORK UNIT NO. NR 302-777

CONTRACT N00014-68-A-0101-0001

OBJECTIVES

To provide material and data for continuing studies in the taxonomy and bionomics of ectoparasites and their hosts from Nepal presently in progress by the principal investigator and the United States Naval Medical Research Unit Number 3, Cairo, Egypt, U.A.R.

ABSTRACT

Since November, 1966, a collector has been in Nepal gathering arthropod ectoparasites and their hosts as well as data pertaining to the prevalence of arthropod-borne diseases in the country. Emphasis has been placed on the ectoparasites of wild birds and mammals, domestic animals and arthropods associated with humans and human habitations.

Various specialists have been engaged to provide determinations of the ectoparasites and hosts. To date more than 1069 collections have been made. These segregate as follows: Mallophaga, 77; Anoplura, 136; Hemiptera, 12; Diptera, 34; Siphonaptera, 220; Ticks, 264+ and Mites, 306. All collections thus far received, excluding Siphonaptera, have been dispatched for identification.

It is premature to associate these ectoparasites with arthropod-borne diseases in Nepal at this time. However, plague has recently been reported in western Nepal and the presence of vectors of other diseases has been demonstrated.

PLANS FOR FUTURE

This project is being continued with the following goals: (a) expansion of the collecting program to the more remote and inaccessible portions of the country, (b) coordination of the program with studies of arthropod-borne diseases in Nepal, (c) development of a rearing program (especially for ticks) with the Nepalese Ministry of Health and (d) analysis of the zoogeographical and epidemiological relationships of the ectoparasites and their hosts with diseases of medical and veterinary importance.

FACTORS INFLUENCING THE ENZYMIC CONSTITUTION OF BACTERIA

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WORK UNIT NO. NR 103-555

CONTRACT Nonr-3609(00)

OBJECTIVES

To study factors which influence the enzymic activity and/or constitution of bacterial cells, with particular emphasis on nutritional factors.

ABSTRACT

During the past year emphasis was placed on studies of the physiology and metabolism of biotin in microorganisms.

A. Control of Biotin Transport in *Saccharomyces cerevisiae*.

We have reported the results of experiments demonstrating that the level of biotin controls the transport of this vitamin into cells of *S. cerevisiae*. When such cells were grown at 30 C in Hertz medium containing an optimal level of biotin (2×10^{-4} $\mu\text{g/ml}$), they possessed a transport system permitting the accumulation of large quantities of the vitamin when placed in a glucose-phosphate-biotin reaction mixture. However, when cells were grown in media containing excess biotin (200×10^{-4} $\mu\text{g/ml}$), their ability to take up the vitamin was reduced greatly. Recent studies have shown that when avidin (0.045 units/ml) was added to a culture growing in excess biotin, the cells regained their ability to take up large quantities of biotin. Boiled avidin had no effect. If cycloheximide (5 $\mu\text{g/ml}$) was added to cells growing in excess biotin prior to the addition of avidin, there was very little increase in the amount of biotin taken up by the cells. Furthermore, treatment of biotin excess grown cells with avidin under nongrowth conditions did not relieve the inhibition of biotin transport. It appears that growth in the presence of excess biotin represses the synthesis of some component(s) of the biotin transport system, and that this component(s) is resynthesized when external biotin is removed.

B. Effect of Glucose on Biotin-Vitamer Synthesis by Thermophiles.

During studies on biotin metabolism of *Bacillus stearothermophilus* (NCA 2184) it was observed that the presence of glucose in the culture medium affected markedly the appearance of biotin-vitamer in the medium. This effect had not been noted previously with other microorganisms. Moreover, no vitamer synthesis was detectable unless the basal medium was supplemented with pimelic

acid (10 µg/ml). In the absence of pimelic acid, glucose depressed growth while in the presence of pimelic acid glucose stimulated growth. Biotin-vitimers appeared in the medium of cells grown without glucose shortly after the initiation of growth. However, examination of cell-free supernatant fluids of glucose grown cells revealed a lag period prior to the appearance of biotin-vitimers assayable by Saccharomyces cerevisiae but inactive for Lactobacillus plantarum. True biotin if present was excreted in trace amounts. Vitamer formation occurred only after depletion of glucose suggesting that glucose or products of glucose degradation affect the synthesis or excretion of biotin-vitimers by this organism. Chromatography of cell-free culture media revealed that the major vitamers released were the same in glucose-free or glucose supplemented media.

C. Biotin Degradation by a Pseudomonad.

Studies have been initiated in an attempt to understand the metabolism of the biotin degrading system present in a Pseudomonad and first reported by Wright and coworkers from Cornell.

CURRENT REPORTS AND PUBLICATIONS

(a) J. R. Waller and H. C. Lichstein, "Cell permeability: a factor in the biotin-oleate relationship in Lactobacillus arabinosus. II. Effect of oleic acid and other surfactants on free biotin uptake". J. Bacteriol., 93, 151-155 (1967).

(b) T. O. Rogers and H. C. Lichstein, "Factors affecting biotin transport in Saccharomyces cerevisiae". Bacteriol. Proc., p. 156 (1967).

(c) J. Birnbaum, C. H. Pai, and H. C. Lichstein, "Biosynthesis of biotin in microorganisms. V. Control of vitamer production". J. Bacteriol. in press (Dec., 1967).

(d) C. H. Pai and H. C. Lichstein, "Biosynthesis of biotin in microorganisms. VI. Further evidence for desthiobiotin as a precursor in Escherichia coli." J. Bacteriol. in press (Dec., 1967).

IMMUNOLOGICAL, ENVIRONMENTAL AND EPIDEMIOLOGICAL STUDIES OF
NEISSERIA MENINGITIDIS AT MCRD SAN DIEGO

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WORK UNIT NO. NR. 63206352

CONTRACT M4114-1010

OBJECTIVES

(a) To attempt recovery of Neisseria meningitidis from the air of congested areas of the recruit training center. (b) Determination of nasopharyngeal and oral carriage rates among one recruit platoon periodically during their training period. (c) Weekly serological sampling of recruits to determine antibody levels for N. meningitidis in relation to such chronological phases of recruit training as studied above. (d) Study of clinical cases at the US Naval Hospitals, Oak Knoll, San Diego, and Camp Pendleton.

ABSTRACT

The in-field phase of this study which followed two platoons (approximately 120 men) during nine weeks of basic training has been completed. Air samples taken in areas of the recruit camp at places of maximal personnel congestion and at times of greatest activity showed that Neisseria sp. could be recovered from the atmosphere. Gram-negative cocci or diplococci were isolated more than 300 times from 1,000 colonies selected for study from the 800 plates of Thayer-Martin media used. Mold contamination and overgrowth by motile air contaminants made characterization of many other prime suspect cultures impossible. Epidemiological questionnaires were of five basic types designed to delve into the man's background and his present state of health. They were designed to check symptomatology recording methods against each other to determine validity of data and determine the extent of bias built into the method of questioning. Graphs for respiratory complaints gleaned from recruits by any method of questioning showed plots reminiscent of those obtained elsewhere for "herd infection" and "herd immunity" reactions.

PLANS FOR FUTURE

(a) Explore further the role of environmental factors associated with the transmission or nontransmission of N. meningitidis. (b) Study specific and nonspecific immune responses to exposure or infection by N. meningitidis. (c) Seek bacteriophages specific for N. meningitidis from strains isolated from carriers discovered during the course of this study. (d) Work with aerosol recovery from known carriers in this and nearby facilities. (e) Perform further laboratory work on certain selected specimens and cultures obtained from atmosphere samples during the study. (f) Make resultant data available to other naval medical research units for collaboration.

CURRENT REPORTS AND PUBLICATIONS

(a) Progress Report of 15/12/66 submitted on DD form 1498 for BuMed
Approved Project 63206352, M4114-1010.

(b) Progress Report of 15/3/67 submitted on DD form 1498 for BuMed
Approved Project 63206352, M4114-1010.

(c) Progress Report of 30/6/67 submitted on DD form 1498 for BuMed
Approved Project 63206352, M4114-1010.

BIOCHEMICAL AND TRANSFORMATION STUDIES OF PLEUROPNEUMONIAE-LIKE
ORGANISMS AND OF L-FORMS FROM GROUP A STREPTOCOCCI

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WORK UNIT NO. NR 103-576

CONTRACT Nonr-4378 (00)

OBJECTIVES

(a) To interpret our recent lipid findings in terms of their possible significance in metabolic alterations during or following inhibition of bacterial cell wall biosynthesis in a resulting L form from a group A streptococcus, (b) the configurational characterization of a glycolipid found accumulating in coccal L form membranes and (c) to determine the destructive capabilities of these L forms in vivo.

ABSTRACT

(a) Most of our recent lipid findings have been presented as a synopsis in an attempt to interpret their possible significance in metabolic alterations during or following inhibition of bacterial cell wall biosynthesis. Since this laboratory has been concerned with the study of comparative biochemical changes of a stable L form with its parent S. pyogenes, we anticipated that the use of this cell wall-less form might prove an excellent antithetical approach to the study of bacterial cell wall biosynthesis. It has been concluded that these collective differences between S. pyogenes and its L form, discussed in (1), may be the result of inhibition of cell wall formation and/or an attempt by the L form to compensate for this defect by strengthening or altering its membrane. These alterations may be expressed, biochemically, in the octadecenoic acid positional isomeric, glyco- and phospholipid redistributions and in the elevated fatty acid and lipid content noted in the L forms membrane. Morphologically, they may be reflected by the apparent lack of an orderly cellular division process in this L form derived from S. pyogenes.

(b) As mentioned previously, L forms from the group A streptococci have been found to accumulate a glycolipid, diglucosyldiglyceride, in their membranes (see 1). Currently, the significance of this type-lipid in microorganisms remains unknown. Recent survey studies, however, have found such glycolipids to be more widely distributed in bacteria than hitherto suspected. The configurational characterization of this lipid from coccal L forms has been determined by employing the usual chromatographic, enzymatic and chemical degradative procedures. Aside from its fatty acid content, which was detailed previously, this glycoside has been found to be 1-(0- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-0- α -D-glucopyranosyl)-2,3-diglyceride.

(c) Most preliminary studies, in collaboration with Dr. I. Ginsburg, Hadassah Medical School, Jerusalem, Israel have been concluded. Utilizing L form whole cells and membranes prepared in these laboratories, it was observed that injection of such preparations into the rabbit resulted in areas of myocardial necrosis.

PLANS FOR FUTURE

Results of past efforts will be assimilated into three concentrated approaches: (a) examination of TDP-rhamnose (a proposed cell wall precursor) biosynthesis in a stable L form from S. pyogenes and the presence of this nucleotide-containing rhamnose in this group A coccus, (b) expand current collaborative in vivo studies in attempts to correlate streptococcal L form structural components with tissue damage and (c) structural characterization of isolated membranes from mycoplasmas and bacterial L forms.

CURRENT REPORTS AND PUBLICATIONS

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2. Panos, C., Cohen, M. and Fagan, G. "Antibiotic Inhibition and Binding Studies with a Group A Streptococcal L-Form" J. gen. Microbiol., 46, 299 (1967).

Chapters (Accepted for Publication)

- a. C. Panos, "Biochemistry of Streptococcal L-Forms" - in Present State of Research of the Group A Streptococci, International Children's Centre, Paris, R. Caravano, ed., 1968.
- b. C. Panos, "Comparative Biochemistry of Membranes from S. pyogenes and Derived Stable L-Forms" in Microbial Protoplasts, Spheroplasts and L-Forms, Williams and Wilkins Co., Baltimore, L.B. Guze, ed., 1968.
- c. C. Panos, "Chemical and Physiological Aspects of the Bacterial L Phase Variant" - in The Mycoplasmatales and the L Phase of Bacteria, Appleton-Century-Crofts Co., New York, L. Hayflick, ed., 1968.

BIOLOGY OF THE NEISSERIA

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WORK UNIT NO. NK 103-700

CONTRACT Nonr-595(24)

OBJECTIVES

To study various biological characteristics of Neisseria species, including (a) relationships between species as revealed by evidence of transformation, (b) metabolic characteristics of in vivo cultured (tissue culture) gonococci, and (c) susceptibility of Neisseria to lytic agents.

ABSTRACT

Lysine and Tryptophan nutritional auxotrophs of Neisseria catarrhalis were obtained by a nitrous acid procedure. Attempts to transform a lysine mutant to prototrophy were successful. Factors contributing to the development of high levels of transformability (10%) are: (1) growing cells in the presence of trypticase soy broth + yeast extract and 1.5% calf serum; (2) presence of lysine in the transforming medium; (3) vigorous shaking during DNA exposure; and (4) temperature of 37° C. Transformants were then determined by conventional methods.

HeLa cells have continued to be employed as an in vivo growth system for gonococci. The biphasic flask (agar with a broth over-lay) was used as the in vitro growth system. Further enzymatic tests have been performed on virulent strains grown in vivo and avirulent strains grown in vitro. The following activities were tested for: lecithinase, lipase-esterase enzymes, neuraminidase, phosphatases, hyaluronidase, hemolysins, and DNase.

The endotoxin was extracted from virulent and avirulent strains and was found not to differ significantly in levels between the two strains.

Growth curve experiments were performed on virulent cells in the in vivo situation, and avirulent cells in the in vitro situation.

The bacteriolytic substance produced by Myxococcus xanthus, which preferentially lysis the Neisseria, has been further purified and characterized as a true exoenzyme. A 220-fold purification was obtained by a procedure involving carbowax treatment, acetone precipitation, ammonium sulfate fractionation, and DEAE cellulose exposure. The purified enzyme retained proteolytic and bacteriolytic activity. These properties could not be separated by Sephadex gel filtration. The temperature optimum, pH optimum, heat stability, and effect of cell

wall concentration have been determined. Amino acid analysis of the cell walls of Neisseria are in progress to determine the mode of action of the enzyme.

A comparison of the susceptibility of the cell walls and whole cells of the Neisseria and the Moraxella to the Myxococcus factor has been initiated in an attempt to determine the taxonomic status of the Neisseria to these Gram negative organisms which resemble them in many ways.

PLANS FOR FUTURE

Studies will be continued (1) to further define optimal conditions for transformation and establish evidence of relatedness among strains through transformation; (2) continue studies on in vivo and in vitro growth of gonococci; and (3) to characterize the lytic effect of the Myxococcus factor on Neisseria and related species.

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PHYSIOLOGY OF IMMUNOLOGICAL PHENOMENA IN
GERMFREE ANIMALS

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WORK UNIT NO. NR 103-600

CONTRACT Nonr 1(23)(15)

OBJECTIVES

To study the physiology of the immune reaction in germfree rodents.

ABSTRACT

Investigation of immune response in germfree rodents has been conducted using the agar plaque technique for detecting single antibody producing cells. Basic studies concerned with establishing the level of normally occurring plaque forming cells (PFC) in unstimulated mice and rats, has demonstrated fewer such cells in the germfree rodent as compared to the conventional animals. The type of diet and the conditions used to maintain the germfree animals also appear to influence the level of normal PFC. Despite this lower level of normal PFC, there does not appear to be any qualitative deficiency in the immune mechanism of germfree mice as compared to conventional mice of the same strain, when sheep erythrocytes are used as antigen. The quantitative aspects of the immune response, at least as far as IgM antibody is concerned, are the same in both groups of animals. However, the appearance of cells producing antibody of 7S classes occurs sooner in germfree animals.

A mouse strain with congenital, persistent infection with lymphocytic choriomeningitis (LCM) virus has been established in the "germ-free" state. All mice are viremic, asymptomatic, resistant to challenge inoculation, and antibody-free. All organs have infiltrations with lymphoid cells and the kidneys show stages of glomerulonephritis. This virus-induced immunopathology is associated with high levels of serum globulin (70%) as determined by acrylamide gel electrophoresis and IgM as determined by Ouchterlony technique.

Immunological tolerance in germfree rats is associated with changes in spleen and lymph nodes: germinal zones are absent, the red pulp contains many small lymphocytes, and the lymph nodes contain plasma cells. Rats with rapidly growing chemically-induced tumors show similar changes.

PLANS FOR FUTURE

These interesting aspects are presently being investigated further to determine in which manner the three subclasses of 7S antibody producing cells are appearing. Also the mechanism by which such cells do arise is completely unknown and is being investigated primarily at the level of nucleic acid requirements.

The character of tolerance associated with the congenital LCM infection is being further investigated as regards: characteristics of globulin, ultrastructural nature of the glomerular lesion with ferritin-tagged antibody, methods of interrupting the congenital passage of virus.

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**EPIDEMIOLOGICAL STUDIES OF ARBOVIRAL ACTIVITY IN
THE CARIBBEAN AREA**

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CONTRACT ONR:443

OBJECTIVES

(a) In order to determine the patterns of arbovirus activity and movement in the Caribbean Area (b) to study the large Cuban Exile population in Florida for antibodies in their sera to 3 arboviruses, (c) to determine their serological anamnestic response following 17D yellow fever vaccination and (d) correlate the presence and titer of their antibodies with personal histories of each Cuban including places and times of residences in Cuba and the U. S. A.

ABSTRACT

Epidemiological studies of arboviral activity in the Caribbean Area are being carried out by an Infectious Diseases Viral Research Unit of the University of Miami School of Medicine. This first phase is a basic epidemiological study of Cuban Exiles arriving in the U. S. A. before, as well as after, the large dengue epidemic which was known to occur in 1963-1964 in at least three Caribbean Islands (Jamaica, Puerto Rico, Antigua), perhaps in others. In order to study the flow of arboviruses through the Caribbean Island Areas, the serological profiles of Caribbean residents are being determined before and after 17D yellow fever vaccination. This means of detecting past exposure to Group B arboviruses is one being developed and evaluated in this laboratory and has recently been described (Pond, Ehrenkranz, and Danauskas, J. Immunol. 98, 673, 1967).

In order to achieve the objectives of this particular phase of the program, studies are being carried out on sera from up to 1,000 Cuban Exiles over eight years of age in the Miami area. The organization of this population under study is unique since it consists of Cubans who for the most part have been arriving in the Miami Area since 1956 and indeed up to the present time in 1967. For an understanding of the population being studied it should be noted that of the 136,244 Cuban Exiles in Florida in 1967 as of April, 122,628 of them were residing in Dade County (Miami).

Pertinent information was obtained from each Cuban refugee being included in this study, a preliminary sample ("pre") of blood obtained, each vaccinated with 17D yellow fever vaccine virus, and an additional blood specimen ("post") obtained three weeks or more following the vaccina-

tion. The concentration of haemoglobin (Hb in mgm percent) of each exile was also determined at the time of first bleeding however, the results of these tests were not related to the purpose of this study except as an incentive to participate. Both "pre" and "post" sera are now being tested for hemagglutination inhibiting (HI) antibodies to four Group B arboviruses, namely St. Louis encephalitis (SLE), West Nile (WN), yellow fever (YF), and dengue viruses. In addition the "pre" sera are being tested for HI antibodies to the Group A viruses of Venezuelan equine encephalitis (VEE), Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), and Chikungunya.

The data compiled and the correlations being drawn pertain to distributions according to sex, age group, place of birth and residence by Cuban province (as well as outside of Cuba), time of departure from Cuba, occupation, type of local environment (urban or rural), and other factors. The HI antibody titers, according to type of virus, will be related to the abovementioned factors for each person.

PLANS FOR FUTURE

(a) To complete the tests for HI antibodies in the sera of the Cuban Exiles to the arboviruses indicated, (b) to complete the correlation analyses of these data as indicated, (c) to possibly test selected sera from these Cuban Exiles for neutralizing antibodies to the same arboviruses, and possibly (d) to extend the studies herein described as a continuing study on selected Cuban Exiles as they arrive.

CURRENT REPORTS AND PUBLICATIONS

No data from the studies summarized here have been submitted for publication as of the end of 1967.

Studies on Transformation and Bacteriophages with
Bacillus subtilis

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CONTRACT Nonr-233(67)

OBJECTIVES

To isolate and study the infectiousness of DNA from mature, temperate SP02 phage particles and from bacteria stably lysogenic for phage SP02.

ABSTRACT

Bacteriophage SP02, which was used for the ultraviolet sensitivity studies previously reported, is a temperate phage capable of stably lysogenizing the transformable strain 168 of Bacillus subtilis. This is the only phage for B. subtilis thus far reported with this property. We have demonstrated that DNA extracted from bacteria lysogenized with SP02 retains its ability to transform any bacterial marker tested, and additionally, can be used to infect competent bacterial recipients. For convenience, this latter biological activity of such DNA preparations is called "transfection with prophage DNA." The majority of competent recipients that take up, from such preparations, a segment of DNA bearing the phage genome become actively infected, and are recognized as infected bacterial centers. This phenomenon is probably closely analogous to "zygotic induction" observed during conjugation of suitable strains of E. coli. A much smaller fraction of competent bacteria exposed to such DNA preparations may stably integrate the phage genome into their chromosome and become themselves lysogenic for SP02.

Quantitative dose response studies were performed to compare the efficiency of transformation and transfection of DNA isolated from lysogenic bacteria. These show that transfection with prophage DNA is about 10-fold less frequent than transformation for any of a series of bacterial genes, over a wide range of DNA concentrations. We think this lower transfection efficiency is a consequence of the difference in sizes of the complete phage genome required for transfection and the average segment of DNA required for transformation. Dose response curves obtained with prophage DNA preparations have almost the same slope as those obtained with DNA isolated from intact phage particles.

Recent studies on "prophage DNA" preparations strongly suggest that the phage genome of lysogenic bacteria is integrated into the bacterial chromosome. This conclusion is based on studies showing that the transfecting activity of DNA extracted from lysogenic bacteria co-sediments in a centrifugal field with the transforming activity. On the other hand, the transfecting activity of DNA extracted from mature SP02 particles sediments at a slower rate, but one which is compatible with its molecular weight. The faster rate of sedimentation of the transfecting activity of prophage DNA is most easily explained by assuming that its molecular weight is increased by being inserted into a larger fragment of bacterial DNA.

PLANS FOR FUTURE

To further characterize the physical and biological nature of prophage DNA isolated from bacteria lysogenic for phage SP02.

THE MOLECULAR BASIS OF LYSOGENY

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CONTRACT Nonr-266(89)

OBJECTIVES

To study (a) the molecular basis of virus-induced neoplasias and (b) the mode of action of potential anti-tumor agents.

ABSTRACT

Studies on the mode of action of hydroxyurea were pursued further. The biological action of this drug can be viewed as consisting of two effects: (a) an immediate cessation of DNA synthesis which is reversible and (b) a lethal action which is seen when exposure of cells is prolonged in excess of 4 hours. This last effect is presumably due to a degradation of the cellular DNA. It has been established that these two effects of hydroxyurea are due to two different metabolites of the drug. The intermediate responsible for the lethal action has tentatively been identified as O-carbamoyl-hydroxylamine. Bacterial mutants resistant to either of these effects have been isolated and are being characterized.

PLANS FOR FUTURE

(a) To make use of the selective action of hydroxyurea to study the role of DNA in viral development and to ascertain the potential therapeutic usefulness of this drug as an antiviral agent and (b) to characterize each of the active intermediates of hydroxyurea and to determine their mode of action.

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FUNCTION OF SIALIDASE IN INFLUENZA VIRUS

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WORK UNIT NO. NR 103-527

CONTRACT Nonr 3723(00)

OBJECTIVES

(a) To investigate the role(s) of sialidase during influenza virus multiplication, (b) to study the biological properties of sialidase and related surface antigens.

ABSTRACT

Fowl plague virus (FPV), an influenza A virus, was previously used as a model to show that the role of sialidase was associated with the release phase of FPV multiplication cycle. Experiments on kinetics of virus multiplication revealed that virus was not released into the chick embryo tissue culture medium when highly specific sialidase antiserum was present. It was also noted that virus replication as measured by plaque forming units (PFU), hemagglutination titers (HA), and sialidase activity, was consistently lower in the experimental cells than in the FPV control cells. The decrease in the synthesis of viral components could be interpreted as an indication of virus maturation influenced by enzyme antiserum. This possibility was investigated by electron microscope studies.

In the FPV chick embryo tissue culture system, typical morphological changes were observed on the surface of infected cells which were incubated in the presence and absence of enzyme antiserum. In another system consisting of X7 strain of influenza virus and a line of conjunctival cells (clone 1-5C-4), filamentous particles were seen on the surface of cells infected and incubated with sialidase antiserum incorporated in the culture medium. Virus specific cellular alterations were not at all evident in the X7 virus infected control cells.

Cell associated egg infectivity (EID_{50}) to hemagglutination (HA) ratio of X7 virus was a thousand-fold lower in the experimental cells than in the virus control. In other words, cell associated X7 virus was relatively more "incomplete" in the experimental cells than the virus in control cells. It should be noted that in the 1-5C-4 cell line X7 virus system, under the same experimental conditions as the FPV chick embryo tissue culture system, an "abortive" cycle of infection takes place. The results from the X7 virus 1-5C-4 cell line system suggests that perhaps viral maturation was influenced by specific X7 virus enzyme antiserum.

Since sialidase antiserum has no apparent effect on the early phase of virus multiplication in vitro, it is conceivable that the functional significance of sialidase may also be involved in destroying the

glycoprotein "barrier" lining the epithelial cells in the respiratory tract of animals. Thus, experiments on the effect of virus and of enzyme antisera on the mouse infectivity were carried out. Approximately ten-fold reduction in the mouse infectivity (MI_{50}) of mouse adapted A2 Taiwan virus was effected by X7 virus enzyme antiserum. This is in contrast to greater than 10,000-fold neutralization of infectivity resulting from the action of virus specific antiserum. These results suggest that the low level of virus neutralization by X7 enzyme antiserum can be attributed to the inhibition of the release of virus. It should be noted that X7 virus is a recombinant derived from A₀ and A2 strains of influenza. X7 virus has the hemagglutinin of A₀ and the enzyme of A2 virus.

Highly specific enzyme antiserum did not neutralize the neurotoxicity of A2 virus in mice or the cytotoxicity of A2 virus in HeLa cells, but the antiserum enhanced the cytotoxicity of A2 virus in HeLa cells. The enhanced cytotoxicity might explain the appearance of filamentous particles emerging solely from 1-5C-4 cells infected with X7 virus and which were treated with specific enzyme antiserum.

PLANS FOR FUTURE

(a) To investigate further the role(s) of sialidase during influenza virus multiplication and/or infections, (b) to determine the role of lysosomes during myxovirus multiplication.

CURRENT REPORTS AND PUBLICATIONS

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CLINICAL RESEARCH ON THE COMBINED ACTION OF ANTI-
BACTERIAL AGENTS AND POLYAMINES ON INFECTION

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CONTRACT NONR 551 (47)

OBJECTIVES

The in vitro and in vivo evaluation of atabrine in the prevention of emergence of bacterial resistance to antibiotics in patients with urinary tract infections.

ABSTRACT

The purpose of this study is to evaluate groups of patients with more than 100,000 organisms/cc. of urine (catheterized specimen), but without obstructive uropathy, as evaluated by an intravenous pyelogram. Pathogens isolated from patients' urines are subjected to tube dilution sensitivity tests to five antibiotics (Colymycin, Furadantin, NegGram, Ampicillin, and Keflin) with and without Atabrine. An antibiotic is administered to the patients according to the results of the tube dilution study. Every other patient is given Atabrine in addition to the antibiotic. Both are administered for twenty-one days. The patients have catheterized urine specimens collected for cultures and sensitivities each month. Thus far, results from the tube dilution antibiotic sensitivity studies on the bacterial isolates from the urines of sixty-one patients indicate that, in the majority of instances, the minimum inhibitory dose of antibiotic (for each antibiotic listed above) can be decreased by the presence of Atabrine. Atabrine itself was shown to have no bacteriocidal action. In addition, in the case of each antibiotic there were several bacteria whose growth was inhibited only by a combination of Atabrine and the antibiotic. The results of the in vivo data thus far show that of twelve patients treated with antibiotic alone, five had a recurrence of the same organism after treatment. Of seventeen patients given an antibiotic in combination with Atabrine, there were three who had recurrence. One of these patients was found to have a surgical lesion as the cause of the persistent urinary tract infection, one patient was

lost to follow-up, and one patient is being presently studied.

Several patients in our clinic with chronic urinary tract infections have been cured for several months after being given as therapy Atabrine and an antibiotic selected by the tube dilution sensitivity method.

PLANS FOR FUTURE

To continue present study until a larger series is obtained.

IN VITRO BIOLOGICAL AND PHYSIOCHEMICAL STUDIES
OF SELECTED VIRAL AGENTS

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) Characterization of the behavior of selected mammalian viruses as it pertains to the general problem of viral multiplication; (b) propagation in tissue culture and purification of adequate quantities of selected viruses; (c) determination of chemical and physical properties by chemical, immunological, and biophysical techniques; (d) relationship between physicochemical properties and strain differences and virulence.

ABSTRACT

The selected viruses previously employed as models in these studies included vesicular stomatitis virus (VSV), vesicular exanthema of swine virus (VESV), poliovirus, and reovirus. Work with VESV and poliovirus has been discontinued, and current research emphasis centers around VSV.

Previous studies with VSV showed that autointerference was due to a particle morphologically similar to, but shorter than, the infectious virion. These short (S) particles were produced by infected cells, and were physicochemically separable from the long (L) rods. L and S particles are produced in all cell systems tested, and by various strains of VSV; they are produced at 25 C as well as at 37 C. Sucrose gradient centrifugation techniques have been employed in preliminary studies on RNA extracted from infected cells and from virus preparations. A large amount of new RNA, partially separable into two or more components, is synthesized in infected cells. These components are of lower molecular weight than viral RNA. The RNA components of L and S particles are readily obtained from virus concentrates and are separable in sucrose density gradients. Technical problems in separation of viral RNA from cellular material have delayed full exploration of the relationships among the various RNA components and the mechanisms of VSV replication and autointerference.

Infectious RNA has not been obtained from VSV. Treatment of crude preparations of VSV with the detergent sodium dodecyl sulfate has yielded low levels of infectivity with properties differing from both intact virus and free RNA. These properties include partial resistance to ribonuclease, and ultracentrifugal sedimentation and buoyant density characteristics.

The standard plaque assay technique for VESV, which employs agar as an overlay, was not applicable to all antigenic types of the virus. A new technique using methyl cellulose in the overlay was developed for assay of VESV in pig kidney (PK) cells; its applicability to a number of types, and its accuracy were tested.

VESV was previously believed to have a very limited host range. A number of the antigenic types were found to grow in a line of African

green monkey kidney (Vero) cells. The virus grew to a high titer in Vero cells and produced typical cytopathic effects. Passage of VESV in Vero cells appeared to select for large plaque variants, a characteristic previously reported to be correlated with in vivo virulence.

Cytopathic effects of VESV in PK cells were studied by cytochemical and fluorescent antibody techniques. Early rounding of cells and cytoplasmic bubbling were commonly observed, and there was a correlation between appearance of specific antigen and of basophilic RNA staining. Ultrastructural changes in PK cells were studied by electron microscopy of thin sections. Cytoplasmic changes were observed early in infection, followed by nuclear degeneration. Aggregates (viroplasmic foci) were observed in the cytoplasm at the time of maximal virus production, and crystalline arrays of virus particles were seen later.

VESV was compared with poliovirus, Newcastle disease virus (NDV), and VSV with respect to thermal stability in the presence of cations. As expected, poliovirus was stable in the presence of high concentrations of certain cations, while NDV and VSV were not. Unexpectedly, VESV behaved more like the complex RNA viruses, NDV and VSV, than like poliovirus, to which VESV appears to be structurally more similar. It appears that the cationic stabilization test may not be generally applicable to the small RNA virus group known as the picornaviruses.

PLANS FOR FUTURE

(a) Further studies on the biochemical nature of VSV, and particularly of the small particles and their mechanism of action in autointerference, with emphasis on the role of RNA; (b) electron micrographic studies of VSV-infected cells, emphasizing the small particles and autointerference; (c) further characterization of the subviral infectious units from VSV; (d) investigation of physicochemical and genetic nature of surviving virus, following ultraviolet exposure.

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INTERACTION OF ANIMAL VIRUSES WITH
POLYMORPHONUCLEAR LEUCOCYTES AND MONOCYTES

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CONTRACT Nonr 3127 (00)

OBJECTIVES

To study replication of vesicular stomatitis virus and its small plaque mutants in CEF and HeLa cells.

ABSTRACT

Host induced modification of plaque type, occurring as a rare mutator-like event, has been observed with six stable small plaque mutants of vesicular stomatitis virus. The host modified virus occurs during growth in HeLa but not during growth in L or CEF monolayers. Of all the mutants, p-la and its host modified form, the H-60 type, have been studied the most extensively. Mutant p-la forms plaques 2 mm in diameter on CEF monolayers after 48 hours of incubation, and about 0.5 mm on L and HeLa monolayers after 84 hours of incubation. In comparison, the H-60 type forms plaques 2 mm in diameter on the three hosts after corresponding incubation periods. p-la grows at a comparable rate on L and HeLa monolayers, reaching maximum titers of about 10^4 PFU/ml. The H-60 type grows at a faster rate than p-la in L and HeLa monolayers, with maximum titers of about 10^6 PFU/ml. The similar growth rates in L and HeLa monolayers of p-la and of the H-60 types, show that the modified form of p-la did not pre-exist in the virus population and was not simply selected for by HeLa cells. The H-60 type arises in HeLa cells adapted to grow in either Hackett-McClain medium or MEM. The modification does not arise as a result of some component in the growth medium, since the same medium is used for L and HeLa cells. The phenotype of the H-60 variant is characterized as unstable, reverting to p-la with a high frequency during growth in CEF, L or HeLa cells. The H-60 modification and its reversion to p-la appear to occur in one step, since no "in between" forms has been found.

A study was carried out on comparative intracellular development of p-la and W+ in L and HeLa cells using fluorescein- and ferritin-conjugated antibodies. The results are incomplete at this writing and will be discussed in the next report.

PLANS FOR FUTURE

Continue studies dealing with intracellular development of p-1a and W+ in L and HeLa cells, using fluorescein- and ferritin-conjugated antibodies.

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BIOCHEMICAL INVESTIGATIONS ON
BLOOD PARASITISM

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WORK UNIT NO. NR 103-650

CONTRACT Nonr-1842(07)

OBJECTIVES

To test the hypothesis that the quality and quantity of free amino acids are controlled by the host, and such control is lacking in blood parasites. Furthermore, the host balance of these metabolites is essential for parasite growth, and is the basis of parasite dependence upon the host.

ABSTRACT

Three facets of the hypothesis concerning regulation of amino acid production, uptake and catabolism have been studied.

Amino acid production from CO₂ fixation has already been described. The main stable products of CO₂ fixation for the parasite and parasitized cells are glutamic acid, aspartic acid and alanine, whereas for the uninfected cells they are the organic acids, malic and citric acid. The alteration of the metabolism of the parasitized red blood cell is almost entirely due to the metabolism of the parasite itself. Correlated with this were studies on the accumulation of amino acids from the ambient fluid. Three patterns of amino acid uptake were found when red blood cells were suspended in glucose-saline medium containing a single amino acid at a concentration of 1mM. The amino acid accumulation of glutamic acid, lysine, arginine, taurine, histidine, methionine, glycine, valine and leucine is the same for normal duck erythrocytes and malaria-infected erythrocytes. In the case of alanine, serine and threonine the accumulation is less for malaria-infected cells when compared to uninfected erythrocytes, and for proline the converse is true. Erythrocyte-free malaria parasites (*Plasmodium lophurae*) accumulate only glutamic acid, arginine and lysine whereas all other amino acids tested enter by simple diffusion. Such results suggest that these malaria parasites have limited ability to accumulate amino acids, and that their cell membrane may be 'leaky'. If such is indeed the case it would fit the suggestion that these organisms lack the regulatory mechanisms characteristic of free-living organisms, and require the regulatory processes of the host for maintenance and perpetration of their kind.

The second area of investigation has been the relation of glucose catabolism to amino acid production. Preliminary results show that the principal products of glucose metabolism in the normal duck erythrocyte are lactic acid, succinic acid, alanine, glutamic acid and aspartic acid. Similar products are found in the malaria-infected cell and in erythrocyte-free parasites. However, in the case of the normal erythrocyte the organic acids are the major end products, whereas in parasites and infected cells the amino acids are dominant. These results further confirm our previous studies on CO_2 fixation. In addition, using glucose-1- C^{14} and glucose-6- C^{14} it was found that the normal erythrocyte produced very little C^{14}O_2 from glucose-6- C^{14} . Similar results were obtained with malaria-infected cells. The rates of C^{14}O_2 release for normal cells with glucose-1- C^{14} was 0.0177, and for infected cells 0.221; for C^{14}O_2 release with glucose-6- C^{14} it was 0.023 for normal and 0.09 for infected. Erythrocyte-free *P. lophurae* metabolized glucose-1- C^{14} at a rate similar to that of the uninfected erythrocyte. These results suggest that *P. lophurae* has either an incomplete tri-carboxylic acid cycle, or is of extremely low activity.

The third facet of our investigations on amino acid metabolism has involved a detailed study of glutamic dehydrogenase in the hope that it would provide a clue to the role of glutamic acid. The kinetics of product formation from C^{14}O_2 suggested that α -ketoglutarate may have been an immediate precursor of glutamate. Two reactions which could be involved: 1) α -ketoglutarate + aspartate $\rightleftharpoons$ glutamate + oxaloacetate (glutamic-oxaloacetic transaminase, GOT) and 2) α -ketoglutarate + NH_3 + NAD (NADP) $\rightleftharpoons$ glutamate + NADH(NADPH) (glutamic dehydrogenase, GDH). GOT activity was detected in both normal and malaria-infected erythrocytes. Neither NAD or NADP glutamic dehydrogenase was detected in normal red cells, however an NADP GDH was isolated from infected erythrocytes. Starch gel electrophoresis indicated a single NADP-dependent band of GDH activity. The enzyme was separated from GOT and malic dehydrogenase by gel filtration with Sephadex G-200. Estimations of K_m 's for NH_4^+ and α -ketoglutarate were 4.45 and 1.43mM respectively. These data suggest that glutamate could be synthesized by the transaminase or dehydrogenase reactions.

PLANS FOR FUTURE

(a) Detailed studies of glucose-1- C^{14} metabolism (b) the relationship of amino acid uptake, especially proline and glutamic acid, to carbohydrate metabolism.

CURRENT REPORTS AND PUBLICATIONS

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STRUCTURE AND BIOLOGICAL ACTIVITY OF THE STREPTOCOCCI

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CONTRACT Nonr(G)-0001-64

OBJECTIVES

(a) To evaluate and relate the chemical nature of structural units of the streptococcal cell to their antigenicity, (b) enzymatic activity, and (c) ability to produce streptococcal infection in man and animals.

ABSTRACT

A. The use of 0-stearoyl derivatives of streptococcal cell wall group carbohydrate antigens to determine antibodies in human and animal sera

Stearic acid esters of the group A and E antigen have been prepared containing 5-8 per cent ester by weight. Such modified antigens give a positive precipitation reaction, although more antigen is required at the equivalence point and the quantity of antibody nitrogen precipitated is about one-half that of the control.

The stearoyl antigens are able to sensitize red blood cells so that agglutination occurs in the presence of antiserum. Rabbit anti-A and anti-E sera, sera from children with group A respiratory disease, and sera from swine either infected with or immunized against group E streptococci, have been tested. Significant levels of antibodies have been determined. The use of 2 antigens, each of different chemical structure from serologically distinct species but of the same bacterial genus, has established the specificity of the reaction.

B. Mechanism of resistance of streptococci to antibiotics

Mutants of group H streptococci to oxamycin (D-cycloserine) and its analogue, O-carbonyl-D-serine, were isolated (after spontaneous mutation) by continuous culture in the presence of the antibiotics. These mutants possessed a 10-20 fold increase in resistance to the antibiotics when compared on the basis of minimum inhibitory concentration.

Several enzymes concerned in the synthesis of cell wall by streptococci are known to be inhibited by cycloserine (alanine racemase and D-alanine-D-alanine synthetase) and carbamyl-serine (the racemase only). It was considered worthwhile to determine the activity of these

enzymes in the streptococcal mutants which were able to grow in the presence of the antibiotic. The activity of the racemase enzyme was found to be increased 8-fold in both antibiotic-resistant mutants as compared to the non-resistant parent streptococcus. The activity of the synthetase was increased 5-fold in the mutant resistant to carbamyl-serine. These results demonstrated for the first time that a bacterial cell can 'bypass' an antibiotic by synthesizing increased levels of the enzymes that normally would be inhibited by the antibiotic.

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COMPARATIVE PHYSIOLOGY OF PLEUROPNEUMONIA-LIKE
ORGANISMS AND L-TYPE ORGANISMS

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CONTRACT Nonr 4898(01)

OBJECTIVES

Examine physiological and biochemical characteristics of Mycoplasma and bacterial L-forms which might aid in their differentiation.

ABSTRACT

Previous results indicated that Mycoplasmal membranes, in particular, *M. laidlawii*, disaggregate into subunits of equal mass upon treatment with anionic detergents. In addition, several lines of evidence favored lipid-protein interactions as the major cohesive force holding the membrane subunits together. More recent evidence indicates that radionuclide ions bound to the reaggregated membrane cannot be removed by chelating agents. Furthermore, chelating agents are not bound by the reaggregated membrane. Therefore, it appears that cations bound in the membrane are not available to chelating agents.

Additional evidence supporting lipid-protein interactions in the membrane was the finding that partially delipidized membrane particles are solubilized in part by negatively charged amphoteric substances, such as diphosphatidyl glycerol, phosphatidyl glycerol, sodium lauryl sulfate, mono-glucosyl diglyceride and diglucosyl diglyceride, but not by positively charged amphoteric substances, e.g., phosphatidyl ethanolamine, phosphatidyl choline, cetyl pyridinium bromide. The delipidized protein particles are completely insoluble in water. All of these solubility characteristics can be altered and improved by acetylation, succinylation and benzylation of the lipid poor protein. Following solubilization of delipidized membrane particles with S^{35} labeled sodium lauryl sulfate, dialysis results in almost complete loss of label in the protein, while only incomplete removal of S^{35} occurs with lipid containing membrane particles.

Subjection of membrane particles to electrophoresis on preparative polyacrylamide gel yields two distinct proteins, one of which has a sedimentation constant of 2.5 similar to the starting material. Like treatment of membrane particles

freed of excess detergent yields at least three ultracentrifugally distinct proteins, i.e., 1.74s, 1.11s and 2.85s.

A model compatible with our current knowledge of the physical, chemical and enzymic properties of the mycoplasmal membrane has been constructed to further the design of future experiments.

PLANS FOR FUTURE

(a) Further assess the charge and solubility characteristics of membrane particles (b) continue fractionation attempts to separate enzymic activities (c) determine the physical and chemical properties of different species of membrane subunits.

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AGE, STRESS AND VIRAL INFECTION
IN GERMFREE ANIMALS

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WORK UNIT NO. NR 302-610

CONTRACT No. --1623(14)

OBJECTIVES

(a) To study age-related resistance in pathogenesis of mouse hepatitis,
(b) To study the role of the RES in interferon tolerance, (c) To produce
a time-lapse movie on polykaryon formation in peritoneal mouse macrophage
cultures infected with mouse hepatitis virus.

ABSTRACT

(a) The effects of cortisone on titers of mouse hepatitis virus and on levels of interferon were studied among 3- and 5-week-old germfree and conventional mice. The following observations were made: 1) Cortisone increased mortalities significantly for all groups of mice. Cortisone-treated mice (3 weeks of age) had a higher mortality than conventional counterparts. Mortalities were similar among cortisone-treated germfree and conventional mice (5 weeks of age). 2) Cortisone had no significant effect on liver virus titers of germfree and conventional mice when measured 1 day and 4 days post inoculation. On the 6th day, virus titers of cortisone-treated mice remained elevated, while titers of untreated mice declined. Thus, cortisone appeared to prolong the period of virus production and enhanced final yields of virus. 3) Cortisone treatment reduced interferon levels of liver and spleen for all groups of mice, except 3-week-old germfree mice. Animals in this group displayed relatively low levels of interferon in the absence of cortisone. It is concluded that under conditions of cortisone-induced stress, 3-week-old germfree mice were more susceptible than conventional counterparts. Increased mortality following combined cortisone-virus challenge was associated with reduction in interferon levels and enhanced final yields of virus. The effect of cortisone on the virus-Kupffer cell relationship was considered in addition to the time-course of neutralizing antibody production. Our results are consistent with the view that physiologic amounts of antibody do not alter the course of infection once established and that antibody response is not likely a factor in age-related resistance.

(b) Massive doses of vaccinia virus (VV) caused a state of partial "interferon tolerance" or hyporeactivity. After pretreatment with VV, both germfree and conventional mice were rendered hyporeactive to an otherwise effective dose of Newcastle disease virus (NDV). Heat-killed VV did not have this property. Thorotrast and cortisone, agents which suppress many functions of the RES, also, caused a state of interferon

refactoriness. VV, thorotrast, and cortisone, but not heat-killed VV, suppressed carbon clearance. It is suggested that the RES is involved in the "interferon tolerance" phenomenon. In addition, we noted that germfree animals showed a greater interferon response when compared with their conventional counterparts. The presence of the normal microbial flora of conventional animals may, therefore, indirectly influence interferon production; for example, by absorption of microbial products through the intestinal wall. In this manner, a specific microbial flora could influence the course of subsequent virus infection.

(c) We have produced, as yet unedited, a 16 mm time-lapse movie showing polykaryon formation among mouse peritoneal macrophages infected with mouse hepatitis. The phenomenon may be applicable to membrane properties involving self recognition.

PLANS FOR FUTURE

(a) To continue study of pathogenesis of mouse hepatitis in germfree and specific microbial-associated mice. (b) To consider the mechanism of action of interferon. (c) To determine the role of intestinal microorganisms and their products on the immune response of mice to subsequent challenge with virus.

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BACTERIAL GROWTH WITHOUT NET PROTEIN SYNTHESIS

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WORK UNIT NO. NR 103-039

CONTRACT Nonr-3806(3)

OBJECTIVES

This project deals with postexponential growth and the nature of the so-called stationary growth phase of bacteria. Exponential growth can cease for different reasons, and different depletions of the biochemical milieu result in different patterns of postexponential growth and different types of "resting" cells (*S. faecalis*). These types differ prominently in the relative amounts of cellular wall and membrane substance, and we seek to understand the underlying biochemical mechanisms.

ABSTRACT

In continuation of the work reported last year, a procedure has been developed for the separation of CoA and ACP in *S. faecalis*. It is based on extraction of cells with 0.01 M HCl or a pH 2.1 buffer. Experiments with cells grown at low levels of C-labeled pantothenate (which contain essentially all of the label in the form of ACP) showed that ACP of *S. faecalis* has a well-defined solubility minimum at pH 2.1. To facilitate the study of the fate of pantothenate, CoA and ACP under different growth conditions, we have developed a buffered system of scintillation counting which permits C¹⁴ counts in cells, culture supernatants or alkaline cell extracts in a constant counting environment, thereby making multiple determinations of counting efficiencies unnecessary. By using this method on cultures grown with C¹⁴ labeled pantothenate under different conditions we found, (a) that during normal exponential growth cellular ACP is about 22% and cellular CoA about 78% of incorporated pantothenate, (b) that during exponential growth cellular CoA turns over at the rate of about 20% per generation time, while ACP is stable, (c) that during the growth of exponential cells transferred to a pantothenate-free medium most of the cellular CoA is rapidly converted to ACP, while some of the label (about 13% per generation) is lost to the medium, and (d) that during exponential growth in the presence of limiting pantothenate levels (which culminates in lysis) cellular ACP is conserved at the expense of CoA, so that at the time of incipient lysis cellular CoA approaches zero, while the ACP level has changed little.

PLANS FOR FUTURE

This is a terminal report.

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THE TOXINS OF CLOSTRIDIUM TETANI
AND CLOSTRIDIUM SORDELLII

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WORK UNIT NO. NR 103-474

CONTRACT N62558-4781

OBJECTIVES

To study (a) the mode of action of tetanus toxin, and (b) the biochemical basis of the pathogenicity of Cl. sordellii.

ABSTRACT

Tetanus. (a) We have confirmed and extended previous studies on the fixation of tetanus toxin by cerebroside/ganglioside complexes. Water-insoluble complexes of ganglioside with cerebroside fix the toxin at low concentrations (a few LD₅₀/ml) of toxin. A complex containing 25% ganglioside with cerebroside is 50 times better at fixing toxin than complexes containing either 2% or 50% ganglioside. A complex containing 25% of a mixture of gangliosides G_{III} and G_{IV} is 12 times better at fixing toxin than a similar complex with gangliosides G_I and G_{II}. Complexes of ganglioside with sphingomyelin and lecithin fix toxin to a slight extent, while complexes with tripalmitin and cholesterol do not fix toxin. The complex of cerebroside and ganglioside containing 25% ganglioside does not fix strychnine, serotonin, botulinum toxin or plasma albumin. At high concentrations of reactants ganglioside alone fixes strychnine and serotonin. It can therefore be seen that the fixation of tetanus toxin by cerebroside/ganglioside is more specific for tetanus toxin than is the fixation by ganglioside alone. The complex is therefore more likely to be included in the receptor for tetanus toxin in nervous tissue. (b) The possible prophylactic and therapeutic value of ganglioside and of cerebroside/ganglioside complexes in experimental tetanus in mice has been investigated. It has been found that mice can be protected against tetanus (whether produced by injection of toxin or of live Clostridium tetani) by ganglioside and, more effectively per unit weight ganglioside, by cerebroside/ganglioside complex. (c) Further studies have been made of the effect of sodium glutamate on the production of toxin by Cl. tetani. Immuno-electrophoretic investigation of culture filtrates has shown that the suppression of toxin production is accompanied by changes in some other antigens. (d) Since the fixation of tetanus toxin by ganglioside is concerned with the sialic acid residues of the ganglioside it seemed possible that the toxin might perhaps also interact with enzymes containing

sialic acid. There has been no effect observed on the enzymes so far tested. (e) Much time and energy has been expended in attempting to obtain consistent sporulation and toxinogenesis in Cl. tetani. It was hoped to study the relationship, if any, between these processes.

Sordellii. During a search for lipolytic enzymes which might be responsible for the pathological action of Cl. sordellii, it was found that the organism consistently produced a lysolecithinase. In order to investigate whether there was any correlation between lysolecithinase activity and the pathological action of Cl. sordellii two approaches were used:- firstly, the organism was grown for 4, 8 and 12 hr. and the various activities measured in culture supernatants, sonic extracts and saline extracts of the cells at each of these three times. It was hoped to see whether there was any correlation, in time of production or extractability, between the activities. Secondly, the enzyme was put through a 6-fold purification step and the pathological activities of the fractions with low and high lysolecithinase specific activities compared.

The enzyme is not responsible for the lethality, oedema-producing or haemorrhagic actions of extracts of the organism, and these activities are further shown probably to be independent of each other. The lysolecithinase, is activated by ether, calcium ions and ammonium sulphate and inhibited by EDTA, magnesium and manganese.

PLANS FOR FUTURE

(a) To continue studies on the role of the fixation of tetanus toxin by complexes containing ganglioside in the pharmacological action of the toxin. (b) To continue studies of the peripheral action of tetanus toxin in lower vertebrates. (c) To continue with our investigations of the toxins of Cl. sordellii.

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RESEARCH ON MENINGITIS VACCINE

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CONTRACT N00014-67-C-0144

OBJECTIVES

1. To study the procedures for cultivation, harvesting, and preparation of *Neissera meningitidis* group B, Strain L-1. 2. To acquire specific antisera pools for in-vitro immunological characterization of purified fractions prepared from cell walls of the bacteria. 3. To characterize and quantitatively estimate the deoxysugar in the cell wall preparations. 4. To determine the susceptibility of various primates to the group B, L-1 strain.

ABSTRACT

Aliquots of the standardized suspensions of viable *Neissera meningitidis* cells were fractionated, employing physical disruption of the cells, to prepare reproducible cell wall preparations. Following the standardization of the culturing, harvesting, standardization and fractionation techniques, large volume preparations of both viable cell suspensions and cell wall preparations were routinely produced for the immunological and biochemical characterization studies, as well as for the virulence testing of the organisms in primates.

Typical antibody response curves in New Zealand albino rabbits were obtained in all of these experiments, and a linear dose-response relationship was observed. These studies indicated that humoral antibody formation was demonstrable by the third day postinjection, and maximal antibody elucidation was achieved by the fourteenth day postinjection. A single, intravenous injection of 500 micrograms per rabbit (the smallest quantity administered to date) has been shown sufficient to produce a significant antibody response. Parallel studies, in which formalinized whole cells were administered to rabbits, indicated that a series of nine intravenous or intraperitoneal injections over a twenty-one day period (totalling 1.0×10^9 cells) were required to produce the same level of antibody formation as was achieved by the single intravenous administration of as little as 1000 micrograms of the cell wall preparations.

These studies have also resulted in the acquisition of specific antisera pools, which will be employed in the evaluation of the in vitro immunological characterization of the cell walls, the purified fractions prepared from the cell walls, and in in vivo passive-protection type assay procedures.

Alkaline hydrolysis of the cell wall preparation cleaves an acid-resistant linkage with concomitant liberation of a deoxysugar, bearing trans-vicinal hydro-oxyl groups, which is susceptible to periodate-oxidation. For purposes of characterization and quantitative estimation, hydrolytic techniques are being employed in conjunction with a sensitive colorimetric assay which has allowed detection of the deoxysugar at concentrations as low as 20 micrograms of cell wall preparation per ml.

By intraperitoneal inoculation of the squirrel monkeys, one kills about 30% with 10^6 organisms, about half with 10^7 bacteria, the same proportion with 10^8 and there seems to be no distinct increase with one billion organisms. With intracranial inoculation, 6 of 7 squirrel monkeys died when 10^7 organisms were used per animal.

With owl monkeys, almost all animals die at 10^8 and 10^7 organisms.

PRODUCTION OF LARGE QUANTITIES OF COLIPHAGE T₃ IN HIGH TITER

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

To devise a method for producing large quantities of coliphage T₃ with a concentration of at least 10¹⁰ phage particles per ml.

ABSTRACT

The production of bacteriophage particles in high titer using a liquid medium requires a high degree of aeration. This aeration, especially during lysis of phage infected cells, results in copious production of foam which is difficult to control. The use of antifoam agents has been found to interfere with phage production.

A new method of aeration and foam control has been developed wherein the foam or air above the liquid is continuously withdrawn, entrained in the intake side of a self-priming pump along with part of the process liquid, mixed, and reintroduced into the bulk of the process liquid.

PLANS FOR FUTURE

No future plans are contemplated.

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(c) Aeration and foam control in sparged fermentation. Patent application introduced through ONR patent officer, San Francisco.

AEROBIOLOGY

This research involves the effects of physical, chemical, and biological factors on the behavior of disease producing microorganisms in the aerosolized state. Individual tasks concern themselves with the effects of the surrounding atmosphere on production, stability, movement and decay of aerosolized agents. Much of the present capability in the world of aerobiology resides in the Naval Biological Laboratory, Oakland, California, where this relatively new discipline has developed over the last eighteen years.

PHYSICAL METHODS IN DETECTION OF AIRBORNE MICROORGANISMS

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ASSISTED BY

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

- (a) To evaluate the feasibility of using Closed Circuit Television (CCTV) Scanning Systems and ultraviolet light microspectrophotometry (UVM) as a means of detecting the presence of airborne bacteria,
- (b) to determine the extent of normally occurring airborne particulates which comprise the background signal when observing a collected sample,
- (c) to compare UVM with other detection techniques, such as "Partichrome",
- (d) to evaluate classification methods used on particle morphology.

ABSTRACT

We have confirmed (as previously reported) that bacteria absorb light at 260 and 280 mμ and are virtually transparent at 313 mμ or longer wavelength. While the absolute absorption at these wavelengths varies, the absorption ratio of $\frac{280}{313}$ or $\frac{260}{313}$ is greater than 1.

Use of the equipment to determine the absorption ratios of bacteria stained with crystal violet yielded results similar to those obtained by others. A reappraisal of our microscopy methods emphasized the requirement for complete homogeneous immersion of the optics. When this was used, Green/Blue ratios of over 5.5 were realized.

Modifications of commercial video system scanning techniques have been carried out. A section of the TV raster is intermittently scanned at multiple field intervals and the resultant video signal analyzed for pulse height. Improvement of slightly greater than 9 dB has been observed compared to the non-interrupted scan.

In further instrument development, a 200 - 500 watt Hg or Hg-Xe lamp offers promise for UV illumination and a beam splitter (being designed for this use by an optical firm) will direct the short wavelength region (<290 mμ) into one TV camera and the longer region (>310 mμ) into another camera. Operation of both cameras from one synchronizing signal generator allows direct comparison of video beam amplitude at any point in the field being scanned.

PLANS FOR FUTURE

(a) Comparison of "Pink RL" staining techniques with UVM, (b) obtain beam splitter accessory and assess dual TV camera system for direct comparison in two wavelength regions of UVM detection of microbial cells, (c) apply simple data processing techniques to the video scan to eliminate particle images which do not meet the criteria for microbial cells.

CURRENT REPORTS AND PUBLICATIONS

(a) H. Wolochow, M. A. Chatigny, and Doris Clinger (1967), "Ultraviolet microspectrophotometry", Forty-first Tech. Prog. Rep., Nav. Biol. Lab., Rep. ENG-1, 47-50.

(b) H. Wolochow, M. A. Chatigny and Doris Clinger (1967), "Ultraviolet microspectrophotometry", Forty-second Tech. Prog. Rep., Nav. Biol. Lab., Rep. ENG-2, IX.

(c) H. Wolochow, M. A. Chatigny, and Doris Clinger (1967), "Discrimination between microbial cells and other small particles by ultraviolet microscopy." Ann. New York Academy of Sciences (submitted for publication).

INSTRUMENTATION IN AEROBIOLOGY

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To develop laboratory apparatus with the capability for providing close simulation of the outdoor environment, (b) development of air sampling instrumentation suitable for collection of low level aerosols, (c) development of techniques for hyperbaric aerobiology studies.

ABSTRACT

Current studies include preparation of design of test equipment for the study of aerosols in large volume programmed dynamic environments. Theoretical analyses of thermal characteristics of dynamic environment chamber systems (maximum temp change 5° F per minute) have been completed and RFP's for construction have been prepared. Control systems are being analyzed and studies on application of direct digital control providing program memory are in progress.

Concurrent efforts in development of a large volume air sampler yield indication that a sampling device based on growth of particles by condensation of moisture appears feasible but is subject to widely variable efficiency due apparently to climatic conditions. Collection efficiencies vary from 10% to 95% with particles from volume of approximately 50 cfm standard air being collected into ca 1 to 5 ml of water. Studies using fluorescent particles, microorganisms (SM) yield comparable data.

PLANS FOR FUTURE

(a) Continue development of large volume air sampler resolving variability problems, (b) continue development of apparatus for study of aerosols in programmed environments, (c) initiate assembly of prototype equipment for studies of aerosols in hyperbaric environments.

CURRENT REPORTS AND PUBLICATIONS

None.

AEROSOL TRACER TECHNOLOGY STUDIES

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To obtain a fluorescent dye which is biologically compatible with a selected number of test organisms. (b) To have a capability of detecting these fluorescent particles in the size range of greater than 0.5 μ diameter at an effective sampling rate of 12 liters per minute.

ABSTRACT

One of the areas of basic aerobiological research currently being studied and instrumented at the Naval Biological Laboratory is the survival of bacterial and viral aerosols under simulated natural aerosol holding conditions. In this study we are attempting to simulate the biological behavior of aerosols undergoing adiabatic shifts as well as frontal mixing.

Coupled with this problem of biological survival is the determination of physical dilution within air masses. Without an exact knowledge of physical aerosol dilution within an air mass, the determination of biological survival is not possible. A fluorescent tracer technique has been developed in order to measure physical aerosol concentration independent of viable survival. Adding Calcofluor White BGT, a water-insoluble fluorescent colloid which is available in a 0.05- to 0.30- μ diameter range, to cultures of Escherichia coli, Pasteurella tularensis (LVS) and Serratia marcescens as 0.1% solids, little or no measurable change in subsequent viable aerosol persistence is observed. It was also observed that Calcofluor White BGT is stable under simulated sunlight for periods in excess of one day-night cycle, thus providing the capability of quantitative field aerobiology for extended time periods.

The current NBL Microaerofluorometer has an improved selectivity of 50-fold over the original unit, thus providing the capability of working at fluorescent tracer levels of one particle per liter within an urban environment which can contain 10,000 countable particles per liter.

PLANS FOR FUTURE

(a) It is planned to introduce Calcofluor White BGT as a fluorescent tracer into all of our laboratory studies to establish a base reference for future parallel laboratory-field studies. (b) The extension of the developed fluorescent technique into field technology will be emphasized.

CURRENT REPORTS AND PUBLICATIONS

(a) L. J. Goldberg and M. J. Simmons (1967), "Fluorescent technique for tracking airborne microorganisms. VI. An improved microaerofluorometer." Forty-first Tech. Prog. Rep., Nav. Biol. Lab., Rep. AB-2.

(b) I. J. Ford and L. J. Goldberg (1967), "Fluorescent technique for tracking airborne microorganisms. VII. Aerosol studies of Calcofluor White ST and BGT in solution or suspension with Bacillus globigii, Escherichia coli, Pasteurella tularensis (LVS) and Serratia marcescens." Forty-second Tech. Prog. Rep., Nav. Biol. Lab., Rep. AB-1.

FACTORS AFFECTING BEHAVIOR OF AIRBORNE MICROORGANISMS

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To determine whether abrupt changes in relative humidity of aerosols have a similar effect on survival of various species of microbial cells, which have significant differences in either their phenotype or genotype; (b) to develop suitable methods for studying factors affecting behavior of selected strains of bacterial and viral agents in aerosols for purposes of understanding why some cells survive in an air environment where others die; and (c) to investigate factors affecting stability of bacteriophage in various extracellular environments.

ABSTRACT

The general behavior of particles in air atmospheres is predictable and may be accounted for in accordance with laws governing physics and chemistry of disperse systems and possible physicochemical laws of colloidal chemistry, but assessment of behavior of living cells within these particles is extremely difficult to predict because of the uncertainty of what action, if any, cells take in response to them. The concept that airborne bacteria are biologically responsive to atmospheric conditions prevailing in natural or artificially produced aerosols and that even small changes of moisture in the air atmosphere evokes measurable biological responses has been proposed. Data in support of this view have been described in previous reports from this Laboratory. We have shown that bacteria equilibrated to low humidity ranges are inactivated by suddenly increasing relative humidity (RH). This sorbed death phenomenon has been shown to be correlated with physiological activity of airborne cells. We also found that airborne cells which are physiologically active are more sensitive to the deleterious effects of RH than cells which had been held under suboptimal growth conditions immediately prior to aerosolization. Shifting RH from high to midrange does not always change the decay rates but does cause a sudden overall reduction (>90%) in numbers of viable cells.

The stability of bacteriophage during storage and in air has been investigated. An entirely new mechanism of reactivation of airborne bacteriophage has been identified. When the phages of Pasteurella pestis and Escherichia coli (T3) were suspended in aerosols at low humidity levels, relatively few phage particles remained infective after collection in all-glass impingers. Rehumidification of the air sample in a Dynamic Aerosol Transport Apparatus, or just prior to collection as advocated by Maltman (J. R. Maltman and D. H. Lunt, Suffield Technical Note No. 201, 23 August 1967), resulted in a dramatic increase in numbers of infective particles recovered. An apparent reactivation of about 4 logs of either virus was frequently observed. Preliminary studies indicated that this reactivation Phenomenon occurred with an animal virus as well.

We have investigated the aerosol stability of several microorganisms which have received little attention in respect to survival in the airborne state, even though they are prevalent in air and are of importance to man economically and from a public health viewpoint. Selected strains of Mycoplasma and Meningococcus have been characterized for survival at varied RH. Airborne Mycoplasma pneumoniae and Mycoplasma gallisepticum, for instance, were found to have good stability at either high or low humidity levels and poor stability in the midrange. Groups B and C Neisseria meningitidis showed better stability at very low and midrange humidities than at about 25% RH and high humidity. Apparently each species of microorganism has its own characteristic pattern for survival in air, which is very much dependent on both the pre- and post-aerosol treatments as well as on the atmospheric conditions. The phenomenon of sorbed death similar to that seen previously with other bacteria was also observed in studies on shifting RH with Mycoplasma and meningococci.

Studies of the aerosol stability of EMC group viruses have been extended to include their infectious nucleic acids. Like the bacteriophages, these viruses show the property of reactivation by rehydration prior to impinger collection.

PLANS FOR FUTURE

To investigate further the physicochemical and physiological properties of microorganisms in aerosols. We are particularly interested in determining the role of water and other environmental factors on behavior of selected strains of bacterial and viral agents. The basic findings are of significance not only in the field of public health but also in extending our limited knowledge concerning mechanisms of death of airborne organisms.

CURRENT REPORTS AND PUBLICATIONS

(a) N. A. Vedros, M. T. Hatch, J. Ng and G. Culver (1967), "Aerosol studies on Neisseria meningitidis." Forty-first Tech. Prog. Rep., Nav. Biol. Lab. Rep. BA-4.

(b) J. L. Franklin, J. Hresko and M. T. Hatch (1967), "Studies on selective media for the aerosol recovery of Neisseria meningitidis." Forty-first Tech. Prog. Rep., Nav. Biol. Lab., Rep. BA-6.

(c) M. T. Hatch (1967), "Effect of relative humidity on inactivation and reactivation of Pasteurella pestis bacteriophage in aerosols." Forty-first Tech. Prog. Rep., Nav. Biol. Lab., Rep. AB-3.

(d) D. N. Wright, G. D. Bailey and M. T. Hatch (1967), "Survival of airborne mycoplasma as affected by relative humidity." Forty-second Tech. Prog. Rep. Nav. Biol. Lab., Rep. BA-4.

(e) J. C. Warren and M. T. Hatch (1967), "Studies on the survival of bacterial viruses in the extracellular environment. I. Viability of T3 coliphage during storage and in aerosols." Forty-second Tech. Prog. Rep., Nav. Biol. Lab., Rep. AB-3.

AEROSOL BEHAVIOR OF NEISSERIA MENINGITIDIS IN ANIMALS

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

To study the course of infection of laboratory animals exposed to aerosols of Neisseria meningitidis.

ABSTRACT

A survey of several strains showed that the Group B strain, LOWE-O of N. meningitidis had high intraperitoneal virulence for mice. When animals were exposed to aerosols of this strain, counts of about 10^5 organisms per ml of 10% lung tissue suspension were obtained after one hour. Within 48 hr the lungs were cleared and no meningococci could be found. When mice were treated with hydrocortisone at the level of 400 mg/kg, the clearing of the lungs did not occur until seven days later. Among the latter animals occasionally N. meningitidis was recovered from spleens.

When broth cultures of N. meningitidis were instilled intranasally into anaesthetized mice, counts of 10^6 or 10^7 organisms per ml were obtained in 10% lung tissue suspensions. These infections paralleled those seen after aerosol exposure and the courses of infection in hydrocortisone-treated mice were similar.

Variable results have been seen when infected mice were held in atmospheres of 10% or 15% CO_2 in air for periods of time varying from two to seven days. Some animals have shown high lung counts with meningococci spilling over into the blood stream as shown by the presence of the latter in the spleens. However, no mice have died of the infection.

PLANS FOR FUTURE

A search is being made for ways to shock infected mice in order to breach the defensive mechanism so that the large number of microorganisms in the lungs can break out and initiate a sustained infective process which will overwhelm the host.

CURRENT REPORTS AND PUBLICATIONS

None

STUDY OF POTENTIAL HAZARDS OCCURRING DURING
DENTAL OPERATIVE PROCEDURES

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WORK UNIT NO. NR 61245012

CONTRACT MR 005.12-6040

OBJECTIVES

(a) To determine the extent which conventional dental procedures contribute to dissemination of microorganisms and toxic products into the air for further transmission to dental patients, dental officers, and auxiliary help.

(b) To develop corrective methods for reduction or elimination of health hazards associated with such dissemination or transfer.

ABSTRACT

Various types of aerobiological equipment are being used to determine causative initiation, duration and characterization of aerosols generated within the dental operatory by procedures which utilize high and slow speed rotary equipment, oral evacuators, cuspidors, compressed air nozzles, forcible water sprays, and other commonly used equipment. The Anderson stage sampler is being used for particle sizing of the dental procedure-initiated aerosols to determine if conditions exist for penetration of this particulate matter into the lung alveolar spaces. Liquid vehicle impingers located at varied distances away from the operative field are being used to determine the extent of the cloud formed. Slit samplers are used to scribe upon microbial agar a time-tracing of the dental operatory air. Finally, a log is kept noting the time and type of dental procedure performed in order to seek its relationships with the data collected from the time-tracing, the particulate sizing, and aerosol propagation. It has been possible to recover exclusive oral inhabitants such as Streptococcus salivarius, Streptococcus mitis, neisseria sp. and an "L-form" variant of Pseudomonas from aerosols disseminated during dental treatment, and also, to relate the time of aerosol propagation with dental procedures being performed even though it has been found equivocal to determine extent and duration of the "dental cloud". The particle sizing has been noteworthy because of repeated demonstrations of microbes, tooth debris and amalgam residue upon the last three stages of the Anderson sieve sampler, and therefore, capable of penetration directly into the alveoli of the lungs.

PLANS FOR FUTURE

(a) Work with more varied selective media in order to follow "marker" oral microbes as they are disseminated by dental operative procedures.

(b) Such characterizations of dental aerosols will be sought for obtaining further knowledge of extent, duration and particle size with a view towards suppression of dental aerosols.

(c) Accompany a trip to MCRD, San Diego during the next episode of increased carriage of N. meningitidis, in order to gather more data concerned with dissemination and control of this dangerous nasopharyngeal inhabitant by means of rubber dam and face mask utilization during operative dentistry procedures.

(d) Attempt closer liaison with the U.S. Public Health Service Dental Health Center, San Francisco, in order to utilize the relatively sterile environment of their experimental dental operatory to seek better definition of the extent, duration and particle size of dental aerosols.

(e) Effect air hygiene studies in a "typical" small Naval dental clinic at Naval Supply Center, Oakland, because this clinic has just recently been placed under cognizance of NAMRU#1's Officer in Charge of Dental Research.

(f) Initiate animal experimentation for the study of potential for lung pathology from inhalation of dental aerosols.

CURRENT REPORTS AND PUBLICATIONS

(a) R. E. Micik, R. L. Miller, G. Ryge, and M. A. Mazzarella (1967), "Microbial Aerosols Discharged from the Oral Cavity of Dental Patients", Paper 243, 45th General Meeting of the International Association for Dental Research, March 1967, Washington, D.C.

DEVELOPMENT AND EVALUATION OF AIR CONTAMINATION TECHNIQUES

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ASSISTED BY

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WORK UNIT NO. NR 302-001

CONTRACT Nonr-222(73)

OBJECTIVES

(a) To evaluate simplified techniques for testing personnel protective devices, (b) to develop improved testing procedures, (c) to develop and evaluate new protective systems.

ABSTRACT

Current efforts are in evaluation of the oropharyngeal wash techniques as a means of testing respiratory protection devices. Preliminary tests, and programs, involve the use of the Navy Department MK V mask worn by Navy personnel in the course of damage control training. Protected subjects are exposed to aerosols of spores of B. subtilis var niger. Results to date indicate that this technique can yield mask penetration data in general agreement with that acquired from other more cumbersome test procedures. Mask penetration ratios on the order of 1 part in 10^5 have been consistently observed.

PLANS FOR FUTURE

(a) To continue evaluation of ND MK V mask system and quantitate test procedure further using selected mask and personnel combinations, (b) to evaluate techniques for physical protection of occupied spaces, (c) to determine magnitudes of particle deposition in occupied spaces from challenge aerosols, secondary aerosol generation thereafter, and magnitude of dose to human occupants.

CURRENT REPORTS AND PUBLICATIONS

None.

MICROBIAL AEROSOLS IN A SUBZERO TEMPERATURE ENVIRONMENT

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WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

To characterize behavior of microbial aerosols as a function of subzero temperatures.

ABSTRACT

Cold temperature facilities consisting of a 300-liter stainless steel rotating drum housed in an insulated chamber where temperature may be regulated and controlled from 50 to -40 C recently became available. This permitted preliminary investigations on behavior of airborne Bacillus globigii spores (BG) and a vegetative Flavobacterium species (F1) in a -30 C environment. The organisms were suspended in a crytotolerant, non-toxic mixture consisting of 1:1 ethylene glycol and gelatin phosphate, and aerosols were generated with a Wells-type atomizer at -30 C using air of 3% relative humidity (RH) previously determined at 20 C. Extrapolation from a psychrometric chart indicated 3% RH air became a saturated atmosphere in the drum at -30C. For comparison, sampling of aerosol was carried out by slit sampling and capillary impinging (AGI-30) at 22 C. Frozen and nonfrozen Difco casitone agar prepared with a crytotolerant base (Won, W. D. and Ross, H., Cryobiol., 2:88-93, 1966) were used as the slit sampling substrate and 1:1 ethylene glycol-gelatin phosphate as the impinger fluid. Results obtained over a 5-hr period of observation showed aerosol stability of BG spores was not affected by the subzero temperature. Stability characteristics were comparable with those of ambient temperature (22 C) aerosols. Typically there occurred a slightly accelerated early total decay during the first 30 min of aerosol life ($K_{30} = 0.004$) followed by a gradual exponential decay extended to 300 min ($K_{30-300} = 0.0006$). Comparative results of mixed aerosol experiments where BG was the tracer and F1 the test organism indicated aerosol stability of F1 was likewise not affected by the low temperature factor. Aerosols sampled with AGI-30 yielded identical slopes in decay curves for the tracer and test organisms. Those sampled with the slit sampler likewise yielded identical quantitative recoveries for BG on frozen and nonfrozen plates whereas, in the case of the test organisms, frozen substrates yielded lower recovery values as well as requiring an additional 24 hr for incubation than the nonfrozen counterparts. Presumably O_2 restriction limited the development of subsurface colonies, a characteristic occur-

rence on sampling on frozen substrates (Won, W. D. and Ross, H., Cryobiol. 2:88-93, 1966). F1 has been shown to be a strict aerobe extremely sensitive to O₂ requirement for maximal development (Leif, W. R. and Hebert, J., Forty-second Tech. Prog. Rep., Nav. Biol. Lab., Rep.AB - 2. 1967).

PLANS FOR FUTURE

Continue and extend studies on effect of cryotemperatures including that of relative humidity on airborne microorganisms particularly with respect to pathogens.

CURRENT REPORTS AND PUBLICATIONS

(a) Won, W. D. and H. Ross (1968), "The influence of a subzero temperature (-30 C) on microbial aerosols." Manuscript to appear in Forty-third Tech. Prog. Rep., Nav. Biol. Lab.

AEROBIOLOGY OF MYCOPLASMA

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ASSISTED BY O. Willis

WORK UNIT NO. NR 302-001

CONTRACT Nonr 222(73)

OBJECTIVES

(a) To determine the effects of temperature and humidity on aerosolized mycoplasma. (b) To determine the infectivity of mycoplasma recovered from the aerosol state.

ABSTRACT

Suspensions of Mycoplasma laidlawii and Mycoplasma gallisepticum were atomized into dynamic aerosol chambers held at 80 F with varied humidity levels. Samples were taken over a 5-hr period by use of all-glass impingers, and the number of surviving organisms was determined. The biological stability of Mycoplasma in air was dependent on relative humidity (RH) and on the species used. In general, Mycoplasma survived best in aerosols at either low or high RH, but at intermediate humidity levels, around 45%, less than 10% survived for 5 hr. In these studies, the Mycoplasma behaved as bacteria do, in that survival of mycoplasma was similar to that previously observed for bacteria and that survival was similarly affected by RH. Apparently the possession of a cell wall, per se, is not an important factor in the survival of airborne microorganisms.

PLANS FOR FUTURE

Continuation of the studies of Mycoplasma as aerosols in order to complete the outlined objectives

CURRENT REPORTS AND PUBLICATIONS

(a) D. N. Wright (1967), "Nature of penicillin-induced growth inhibition of Mycoplasma neurolyticum". J. Bacteriol., 93, 185-190.

(b) D. N. Wright, G. D. Bailey and M. T. Hatch (1968), "Survival of airborne mycoplasma as affected by relative humidity". J. Bacteriol., 95.

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