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For the past six years a bacterial infection has been the cause of large losses of adult, spawning, American shad (Alosa sapidissima) in the Coos, Millicoma and Smith Rivers of Oregon. There was a sizable commercial fishery for shad in these rivers and losses of fish due to this infection represented an important economic loss to the industry. This study was undertaken to determine the causative agent of the disease and describe the pathology of the disease.

Fifteen strains of bacteria were isolated from diseased shad in the Coos, Millicoma, and Smith Rivers. Cultural and morphological studies, physiological reactions, animal infection, serological tests, and determination of the mole percent guanine + cytosine showed that the causative agent belonged in the genus Aeromonas. Since the shad disease isolates could cause "red leg"

disease in frogs and "red mouth" disease in trout, they were named Aeromonas hydrophila.

The disease was shown to be a bacterial hemorrhagic septicemia. Externally, the diseased shad had large hemorrhagic areas on their sides and reddening of the head and fins. Very little pathology could be seen internally. The causative organism was recovered from the spleen, liver, kidney, heart, blood and external lesions of the diseased animals.

A review of the literature revealed that there was confusion in the nomenclature of the three motile species of bacteria included in the genus Aeromonas. Many authors felt that the three species (A. liquefaciens, A. hydrophila, A. punctata) were one distinct species. Comparative tests performed on the shad disease isolates and known cultures of Aeromonas failed to reveal major differences between these organisms.

Attempts were made to separate the three species on the basis of the mole percent guanine + cytosine and thermal denaturation temperature (Tm) of the bacterial DNA. Three shad disease isolates had Tm values of 94.3, 94.6, and 94.2°C with corresponding mole percent guanine + cytosine values of 60.9, 61.7, and 60.7. An isolate of Aeromonas liquefaciens obtained from the Communicable Disease Center (CDC) in Atlanta, Georgia had a Tm of 94.1°C and a mole percent guanine + cytosine of 60.3.

Aeromonas hydrophila (CDC) had a Tm of 95.4°C and a mole percent guanine + cytosine of 63.4 while A. punctata (CDC) had a Tm of 93.5°C and a mole percent guanine + cytosine value of 59.0. The mole percent guanine + cytosine results indicated that the shad disease bacterium was closely related to A. liquefaciens. The three known cultures of Aeromonas each had a distinct mole percent guanine + cytosine value.

A Bacterial Disease of the American Shad (*Alosa sapidissima*)

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A BACTERIAL DISEASE OF THE AMERICAN SHAD (ALOSA SAPIDISSIMA)

INTRODUCTION

For the past six years a bacterial infection causing large losses of adult, migrating shad (Alosa sapidissima) had been observed in the Coos, Millicoma, and Smith Rivers of Oregon.[1] This disease was also known to occur in the Coquille and Umpqua Rivers.[2] The epizootic generally appeared in late May or early June with the advent of increased water temperatures. The disease was characterized externally by large hemorrhagic lesions and extreme reddening of the head and fins. Internally the animals appeared normal except in extreme cases where the musculature became hemorrhagic and the body cavity filled with blood and tissue fluids. It has been determined that this disease was caused by the bacterium, Aeromonas hydrophila (Breed, Murray, and Smith, 1957) and that the disease was a bacterial hemorrhagic septicemia.

This study was undertaken to determine the nature of the causative organism and to describe the gross pathology of infected

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[1] Personal communication, Dr. J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, Oregon.

[2] Personal communication, Mr. Edwin Cummings, Biologist, Fish Commission of Oregon, Charleston, Oregon.

animals. There was a sizable commercial fishery for shad in southwest Oregon rivers, and it was thought that death of fish due to this disease represented an important economic loss to the industry.

The study started in June, 1966 and terminated in July, 1967. Bacteria were isolated from infected shad taken from the Coos, Millicoma, and Smith Rivers during the epizootics of 1966 and 1967. Biochemical and morphological features of the unknown pathogenic bacterium were studied to determine proper classification. Serological work with isolates from shad and known Aeromonas isolates was conducted to observe any gross antigenic similarities. Transmission and infection experiments employing salmon, trout, frogs, and mice were performed to determine the pathogenicity of the bacteria from diseased shad. As a further aid in classifying the bacterium, the percent guanine + cytosine of the deoxyribonucleic acid was determined and compared with the percent guanine + cytosine of known members of the genus Aeromonas. Antibiotic sensitivity studies were performed to determine which types of antibiotics were inhibitory to the bacteria isolated from infected shad.

HISTORICAL REVIEW

History of Bacteria Now Classified as *Aeromonas hydrophila*, *Aeromonas liquefaciens* and *Aeromonas punctata*

There has been much confusion over the nomenclature of the *Aeromonas* group due to faulty or inadequate description of these bacteria and lack of agreement concerning which characteristics should be employed to establish this genus and the species contained therein.

Since many investigators gave bacteria now included in this group different names it was difficult to obtain a chronological presentation of the literature. Instead this review will describe the motile species that were proposed which are now included in the genus *Aeromonas*.

Aeromonas hydrophila

Bacillus hydrophilus fuscus (Sanarelli, 1891). In their review of the *Aeromonas* group, Ewing, Hugh and Johnson (1961) stated that Sanarelli in 1891 reported the isolation of a bacterium from diseased frogs. He named this bacterium *Bacillus hydrophilus fuscus*, and described it as pathogenic for both warm and cold-blooded animals. He further indicated that it was gram negative, liquefied gelatin and coagulated serum, produced gas from glycerol,

and grew well above 30°C. Ewing et al. (1961) also stated that Russell in 1898 isolated Bacillus hydrophilus fuscus from frogs with the disease, "red leg". Russell gave a good description of the bacterium indicating that it was polarly flagellated and produced gas from glucose. Chester (1901) in his Manual of Determinative Bacteriology shortened the name of this bacterium to Bacillus hydrophilus (Sanarelli). In 1923 Weldon and Levine placed Bacillus hydrophilus in the sub-genus Proteus because it produced acid and gas from mannitol and glucose and liquefied gelatin. They erroneously stated that this bacterium was motile by means of peritrichous flagella. Bergey (1923) listed Bacillus hydrophilus fuscus as Proteus hydrophilus in his key. Rustigian and Stuart (1941) showed that all members of the genus Proteus except P. hydrophilus, P. icthyosomas and P. bombycis were able to hydrolyze urea. They concluded P. hydrophilus should not be a member of the genus Proteus. Kulp and Borden (1942) showed that P. hydrophilus did not possess peritrichous flagella but were actually polarly flagellated. Speck and Stark showed that P. hydrophilus had produced a much higher CO_2 to H_2 ratio than other members of the genus Proteus. Guthrie and Hitchner (1943) also stated that P. hydrophilus was polarly flagellated. They suggested that P. hydrophilus was actually identical to Pseudomonas punctata (Zimmerman). Kluyver and Van Niel (1936) proposed the forming of the genus Aeromonas which they

described as a group of organisms which were polarly flagellated, gram negative rods, capable of producing 2,3 butylene glycol. In 1943, Stanier stated that because of its outstanding fermentative abilities P. hydrophilus should be removed from the genus Proteus and put in an entirely new genus, Aeromonas. Stanier and Adams (1944) showed that the end products of glucose fermentation by P. hydrophilus was 2,3 butylene glycol. In the sixth edition of Bergey's Manual (Breed, Murray, and Hitchens, 1948), this organism was called Pseudomonas hydrophilus and was described as having one polar flagella. In the seventh edition of Bergey's Manual (Breed, Murray, and Smith, 1957), the organism originally called Bacillus hydrophilus fuscus was renamed Aeromonas hydrophila.

Proteus melanovogenes (Miles and Halnan, 1937). Miles and Halnan (1937) described a bacterium very similar to Proteus hydrophilus but differing in the fact that it had one polar flagella. At that time it was thought that P. hydrophilus was peritrichous. They isolated the organism from eggs of the domestic chicken which exhibited a condition referred to as black rot. They put this organism in the Proteus group because of its hemolytic and proteolytic activity and named it Proteus melanovogenes. However in 1951, Miles and Miles concluded that Proteus melanovogenes was actually a member of the Aeromonas group and synonymous with Aeromonas hydrophila.

Vibrio jamaicensis (Caselitz, 1955). In their reviews, Eddy (1960) and Ewing, et al. (1961) stated that in 1954 Hill, Caselitz and Moody isolated a monotrichous, gram negative bacteria from an acute case of metastatic myositis in man. It was later described in 1955 by Caselitz and named Vibrio jamaicensis. In 1958 Caselitz reviewed the systematic position of this organism and decided it was actually a member of the genus Aeromonas.

Aeromonas liquefaciens

Aerobacter liquefaciens (Beijerinck, 1900). According to Eddy (1962) and Ewing et al. (1961) Beijerinck in 1900 isolated an organism from water which was polarly flagellated, produced gas from carbohydrates and liquefied gelatin. He called this organism Aerobacter liquefaciens. Kluyver and Van Niel (1936) proposed the genus Aeromonas. They suggested that the type species should be Aeromonas liquefaciens, synonym Aerobacter liquefaciens (Beijerinck). In 1951 Miles and Miles concluded that Aerobacter liquefaciens Beijerinck was probably synonymous with Proteus hydrophilus and certainly belonged to the same genus. In the seventh edition of Bergey's Manual (Breed, Murray, and Smith, 1957), Aerobacter liquefaciens was included as Aeromonas liquefaciens.

Pseudomonas fermentans (von Wolzogen Kuehr, 1932). Eddy (1960) stated that in 1932 von Wolzogen Kuehr isolated a fermentative bacteria from the feces of Chironomus plumosis larvae. He named this organism Pseudomonas fermentans because of its polar flagella. In the fourth edition of Bergey's Manual (Bergey, 1934), this bacterium was placed in the genus Flavobacterium and listed as Flavobacterium fermentans. It was not included in the fifth or sixth editions of Bergey's Manual. Miles and Miles (1951) stated that Pseudomonas fermentans should be classified as a member of the genus Aeromonas. According to Eddy (1962) one of von Wolzogen Kuehr's original strains (L. 417) was called Aeromonas liquefaciens in the seventh edition of Bergey's Manual (Breed, et al., 1957).

Bacillus icthyosmius (Hammer 1917). According to Ewing et al. (1961) and Eddy (1960), Hammer in 1917 described a bacterium in milk that had a fishy odor and named it Bacillus icthyosmius. In the first edition of Bergey's Manual (Bergey, 1923) this organism was classified as Escherichia icthyosmia. In 1934 Bergey classified this organism as Proteus icthyosmus. Rustigian and Stuart (1941) stated that P. icthyosmus should not be a member of the genus Proteus because it did not hydrolyze urea. The sixth edition of Bergey's Manual (Breed, et al., 1948) classified this organism as Pseudomonas icthyosmia. Miles and Miles (1951) said that

P. icthyosmia was actually synonymous with P. hydrophilus. It was not included in the seventh edition of Bergey's Manual (Breed, et al., 1957) and was now regarded as a strain of Aeromonas liquefaciens.

Aeromonas punctata

Bacillus punctatas (Zimmerman, 1890). Eddy (1960) and Ewing et al. (1961) stated that Zimmerman in 1890 described an organism isolated from Chemnitz tap water which he called Bacillus punctatas. In 1896 Lehmann and Neumann (Eddy, 1960) found this organism to be very common in the River Main. They called this organism Bacterium punctatum. Chester (1901) classified this organism as Bacterium punctatum. Bergey (1934) classified this organism as Achromobacter punctatum. According to Ewing (1961), Schaeperclaus employed the name Pseudomonas punctata for an organism he considered identical to Achromobacter punctatum. Breed (1948) in the sixth edition of Bergey's Manual classified this organism as Pseudomonas punctata. Guthrie and Hitchner (1943) suggested that this organism was actually identical to Pseudomonas hydrophilus (Aeromonas hydrophila). Miles and Miles (1951) were of the opinion that Bacillus punctatus Zimmerman was insufficiently described and had no status in priority of nomenclature. They considered B. punctatus as identical to P. hydrophilus

(Aeromonas hydrophila). However in the seventh edition of Bergey's Manual (Breed, et al., 1957), this organism was classified as Aeromonas punctata.

There was considerable disagreement concerning whether the three species now classified as A. liquefaciens, A. hydrophila and A. punctata were actually distinct or really just one species. Also there was disagreement as to what should be the type species if these three were actually varieties of one species.

Eddy (1960) concluded that Aeromonas hydrophila, Aeromonas liquefaciens and Aeromonas punctata were actually members of one distinct species. He concluded that the specific epithet should be Aeromonas liquefaciens.

Ewing et al. (1961) also concluded that these three species names actually applied to only one distinct species of bacterium. They concluded that the description of Bacillus punctatas Zimmerman was unrecognizable because it could have been a description of a bacterium belonging to the genera Pseudomonas, Serratia, or Proteus. They concluded that Sanarelli gave the first adequate description of a bacterium in the genus Aeromonas and therefore the name Aeromonas hydrophila should be the specific epithet of that group. It was further concluded that the description of the bacterium Aerobacter liquefaciens Beijerinck in 1900 was adequate to include it in the genus Aeromonas but that it was synonymous with Aeromonas

hydrophila.

Liu (1961) showed that these three species of Aeromonas were extremely similar in their antigenic makeup. He showed that the differences among the species were not any greater than differences among individual isolates of Aeromonas hydrophila.

Eddy (1962) reconsidered his previous statements and said that the specific epithet of the Aeromonas group should be Aeromonas punctata. He indicated the first valid description of this group of organisms was given by Lehmann and Neumann in 1896 when they further described Zimmerman's organism. Eddy states Sanarelli's bacterium was inadequately described and not given a legitimate epithet until 1901.

Eddy and Carpenter (1964) used the Adansonian computer analysis to show that strains labeled A. hydrophila, A. liquefaciens and A. punctata were actually the same species which they called A. punctata.

To further complicate the nomenclature of this group, Ross (1962) isolated a pigment (water soluble) producing motile organism which he reported to be A. liquefaciens. This organism appeared to be a link between the motile members of the genus Aeromonas and the non-motile species A. salmonicida.

As can be seen there was much confusion as to the nomenclature of the Aeromonas group. The matter will probably be resolved

in the eighth edition of Bergey's Manual.

Pathogenicity of the Motile Organisms of the Genus Aeromonas

Members of the genus Aeromonas have been shown to be pathogenic for both warm and cold-blooded animals. Recent evidence also suggested that these organisms could be pathogenic for humans.

According to Camin (1948), Sanarelli in 1891 in his description of Bacillus hydrophilus fuscus, now called Aeromonas hydrophila, showed that this organism was pathogenic for frogs, toads, salamanders, lizards, sunfish, fresh water eels, guinea pigs, rabbits, dogs, cats, chickens, and pigeons.

In his review of the genus Aeromonas, Eddy (1960) stated that Russell in 1898 isolated B. hydrophilus fuscus (Aeromonas hydrophila) from diseased frogs. The disease was characterized by a distinct reddening of the legs of the frogs. In 1905, Emerson and Norris again isolated this organism from frogs which had the disease "red leg".

In 1940 Snieszko (1940) described a disease in carp which were infected by Pseudomonas punctata, now known as Aeromonas punctata. Symptoms of the disease were deep ulcers penetrating the skin and muscle tissue. The ulcers were filled with pus containing bacteria. Snieszko also showed that immunity to the bacterium

could be built up by the carp. Griffin (1953) points out that Schaeperclaus and Mann also isolated Pseudomonas punctata from carp suffering from abdominal dropsy.

Fish (1934) noted that Pseudomonas hydrophilus (Aeromonas hydrophila) was the cause of an ulcer disease of fingerling brook, rainbow, blackspotted, and lake trout at the Cortland, New York trout hatchery. Reed and Toner (1942) showed that Pseudomonas hydrophilus was the cause of ulcer disease of hatchery reared brook trout (Salvelinus fontinalis) and the red sore disease of Northern pike (Esox lucius). Margolis (1951) described a hemorrhagic septicemia in northern pike in Quebec, Canada. The disease was characterized by lesions varying from slight hemorrhages in the skin to deep areas of hemorrhage several centimeters in diameter. He stated that the disease was caused by Pseudomonas hydrophilus (Aeromonas hydrophila). Seaman (1951) noted a disease of five to six inch rainbow trout at the Denver State Hatchery in 1950. Symptoms included liquefaction of the intestine and hemorrhage of the oral cavity. He suggested the causative organism was very similar to Pseudomonas hydrophilus (Aeromonas hydrophila). Wagner and Perkins (1952) stated that Pseudomonas hydrophilus (Aeromonas hydrophila) was the cause of "red mouth" disease of rainbow trout. The disease was characterized by an inflammation of the oral cavity of the trout and an erosion of the

anal orifice.

Lewis and Bender (1960) showed that a disease in Golden Shiners (Notemigonus crysaleucus) was caused by a member of the genus Aeromonas, possibly Aeromonas liquefaciens. The disease was characterized by saddle shaped lesions near the dorsal fin. The organism was readily isolated from the lesions and body cavity and the kidney of the diseased fish. In a later paper (Lewis and Bender, 1961) the same authors stated that factors contributing to spread of the disease were presence of large numbers of fish and mechanical damage to fish.

Rock and Nelson (1965) described an epizootic caused by Aeromonas liquefaciens in populations of channel catfish (Ictalurus punctatas), black bullhead (Ictalurus melas) and stonecat (Noturus flavus). Shortly after the catfish infection, a massive kill of gizzard shad (Dorosoma cepedianum) occurred. Symptoms of the disease in catfish included necrotic areas in the head region and blindness. The gizzard shad had hemorrhagic areas at the base of the pectoral fins.

Dias et al. (1965) described a disease caused by the bacterium Aeromonas punctata in the puntius fish of India.

Haley, Davis, and Hyde (1967) reported losses of american shad (Alosa sapidissima) in the San Juaquin River due to infection by Aeromonas liquefaciens.

As stated earlier, many warm-blooded animals and animals other than fish have been shown to be susceptible to Aeromonas infections. Camin (1948) indicated that Aeromonas hydrophila is the cause of a hemorrhagic septicemia in snakes, such as common water snakes, blacksnakes, southern banded water snakes, blue-racers, garter snakes, pilot black snakes, hog nosed snakes and brown water snakes. The disease has been shown to be transmitted from snake to snake by a snake mite (Ophionyssus serpentium).

Recently more evidence has accumulated which shows that members of the genus Aeromonas may be pathogenic for man. Bullock (1964) noted that Caselitz and Gunther established the pathogenicity of strains of A. liquefaciens taken from pus and lesions of humans. Bullock also stated that Martinez, Guzzman-Urego and Caselitz isolated pure cultures of A. liquefaciens from stool samples of infants ill with enteritis. Rosner (1964) reported that Aeromonas hydrophila was identified as the organism causing a severe gastro-enteritis in a ten-year-old girl. The symptoms of the disease included severe abdominal pains, fever and bloody stools. Her serum showed a high concentration of antibodies against the causative agent. Meeks (1963) reported that Aeromonas liquefaciens was isolated from the blood and ascitic fluid of a patient with Laennec's cirrhosis. She also stated that Aeromonas liquefaciens was isolated from the stools of a patient with severe

diarrhea and from the blood of a patient with cancer of the larynx and Laennec's cirrhosis.

Gerald Gilardi (1967) reported the isolation of Aeromonas punctata (hydrophilus, liquefaciens) from human sources. Two isolates of one strain were cultured from the sputum of a patient with aspiration pneumonia, and two isolates of a second strain were obtained from fecal samples of a five-year-old male with gastro-enteritis.

MATERIALS AND METHODS

The results of studies on the shad disease bacterium (SDB) were obtained using established microbiological laboratory techniques. All incubations, unless otherwise stated, were carried out at 37°C for 24 hours.

The unknown organism was isolated from 15 diseased shad collected from the Coos, Millicoma, and Smith Rivers of Oregon. One culture of Aeromonas liquefaciens was obtained from Mr. G. L. Bullock, U. S. Fish and Wildlife Service, Eastern Fish Disease Laboratory, Kearneysville, West Virginia. Cultures of Aeromonas punctata, Aeromonas liquefaciens, and Aeromonas hydrophila were obtained from Dr. Ewing, U. S. Public Health Service, Communicable Disease Center, Atlanta, Georgia. Both known and unknown organisms were subjected to the morphological, cultural, and physiological tests described below to determine the genus and species of the strains isolated from diseased shad and to indicate how they compared with the known organisms.

Staining Reactions and Morphology

Heat fixed preparations of bacteria were stained using Kopeloff's modification of the gram stain (Society of American Bacteriologists, 1957) and examined under the light microscope to

determine general morphology and size. The organism was also stained to determine if it was acid-fast (Ziehl-Neelsen Method), encapsulated (Anthony's Method with Tyler's Modification) and produced spores (Dorner Method) (Society of American Bacteriologists, 1957).

Flagellation of the bacterium was determined by electron microscopy. The electron micrograph was taken employing an RCA EMU 20 electron microscope at a magnification of approximately 12,000 diameters. To obtain the micrograph, the bacterial culture was suspended in a drop of double distilled water, then collected and dried on a carbon coated grid. The cells were negatively stained using a 1% solution of phosphotungstic acid at pH 6.5.

Cultural Characteristics

The streak plate method was used to study the cultural characteristics of the bacteria on solid media. The following Difco[3] media were employed for these tests:

1. Nutrient Agar
2. Blood Agar Base with 5% sheep blood added
3. Furunculosis Agar

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[3]Difco Laboratories, Inc., Detroit, Michigan

4. Eosin Methylene Blue Agar
5. Brilliant Green Bile Agar
6. Trypticase Soy Agar
7. Mueller-Hinton Agar

Growth on potato slopes was determined by streaking the bacteria on slices of potato which had been sterilized by autoclaving for 20 minutes at 121°C.

Physiological Characterization

Selected physiological properties of the organisms were studied in order to determine their proper taxonomic position. The effect of various temperatures, pH values, and sodium chloride concentrations on growth were examined as were metabolic reactions of the bacteria on or in selected types of media.

Optimum Temperature for Growth

To determine the optimum temperature for growth of the bacteria, cells were inoculated into 10 ml tubes of furunculosis broth[4] and incubated at 9, 18, 23, 30, and 35°C. Cell growth was determined by measuring the optical densities of the cultures at time intervals of 6, 8, 10, 12, 16 and 24 hours with a Bausch

[4]The formula for furunculosis broth is: 10 grams tryptone, 5 grams yeast extract, 2.5 grams sodium chloride per liter of distilled water at a final pH of 7.0.

and Lomb Spectronic 20 set at 475 mμ. No bacterial counts were made.

Sodium Chloride Tolerance

To establish sodium chloride tolerance the bacteria were inoculated into culture flasks containing 100 ml of furunculosis broth containing varying amounts of NaCl. Flasks were examined for visible cell growth after 24 and 48 hours. The concentrations of sodium chloride employed in this experiment were 0.0, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, and 10.0%. This experiment was done in duplicate.

pH Tolerance

To study the effect of pH on growth, the bacteria were inoculated into flasks containing 100 ml of furunculosis broth which had been adjusted to the proper pH value with 1.0 N HCl or 1.0 N NaOH by means of a Corning pH meter. Each set of two flasks (the experiment was run in duplicate) contained media adjusted to one of the following pH values: 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. Cultures were examined for visible growth after 24 and 48 hours' incubation.

Anaerobic Growth

To determine if the SDB isolates were capable of growing under anaerobic conditions, prepared cultures were placed in an anaerobic incubator (N_2 only) at 37°C for 24 hours. A second set of cultures were prepared in the same manner and incubated in a standard 37°C cabinet.

Fermentation of Carbohydrate

The carbohydrate fermentation broth used for these experiments was made according to the following formula:

Ingredient	Grams per liter distilled water
Tryptone	10.0
Bacto-Beef Extract	3.0
Bromcresol Purple	0.016
Carbohydrate	5.0

The carbohydrates which were added to the above base for fermentation studies were: glucose, maltose, sucrose, lactose, levulose, galactose, rhamnose, dulcitol, arabinose, glycerol, raffinose, salicin, xylose, inulin, mannitol, trehalose, and sorbitol.

The inoculated tubes were examined for production of acid and gas. A change in color of the indicator from purple to yellow

indicated that acid was produced from the carbohydrate, and gas production was indicated if the liquid in small Durham fermentation tubes was displaced by gas bubbles.

Reactions in Litmus Milk

Tubes containing litmus milk (Difco, 1953) were inoculated with the test organism and observed for the following reactions: acidity (indicated by a pink coloration of the milk), alkalinity (indicated by a purple coloration of the milk), coagulation, reduction, and proteolysis.

Hydrolysis of Starch

Starch agar used in this experiment had the following formula:

Ingredient	Grams per liter distilled water
Soluble Starch	10.0
Beef Extract	3.0
Peptone	5.0
Agar	15.0

To test for starch hydrolysis, the organism was streaked across a starch agar plate and allowed to grow for 24 hours. The plate was then flooded with a 1.0% iodine solution. Starch hydrolysis was indicated by a clear area surrounding the bacterial growth.

Hydrogen Sulfide Production

Production of hydrogen sulfide was determined using culture tubes containing lead acetate agar (Difco, 1953). Darkening of the media indicated formation of hydrogen sulfide due to the production of lead sulfide from the reaction of H_2S and lead acetate.

Motility Tests

Culture tubes containing 8 ml of motility medium (10.0 grams tryptone, 5.0 grams sodium chloride, and 5.0 grams of agar per liter of distilled water) were inoculated with cultures of the bacterium to be tested. The spread of cell growth away from the initial position of the stab indicated motility.

Catalase Reaction

Possession of the enzyme catalase by the bacteria was tested for by growing a streak culture on a furunculosis agar plate. After 24 hours' incubation, a few drops of a 3.0% hydrogen peroxide solution were placed on the streak. The appearance of bubbles was considered a positive test.

Voges-Proskauer Reaction

The ability of the organism to produce acetylmethylcarbinol

from the test medium was examined by inoculating tubes of MR-VP media (Difco, 1953) and incubating the cultures 48 hours at 37°C. Two ml of the culture were poured into a clean test tube then 0.5 ml of alpha naphthol solution and 0.5 ml of 40% KOH containing 0.3% creatinine were added. The tubes were shaken and allowed to stand for 30 minutes. The presence of a pink to red color indicated a positive reaction (Seeley and Vandermark, 1962).

The Methyl Red Test

The methyl red test for acid production was determined with the remainder of the culture used in the Voges-Proskauer reaction by addition of a few drops of an alcoholic solution of methyl red. A yellow color of the indicator was considered negative while a bright red color was considered positive.

Gelatin Liquefaction

The ability of the bacteria to hydrolyze gelatin was tested by inoculating tubes of nutrient gelatin (Difco, 1953) with the test bacteria. Cultures were incubated for 48 hours at 37°C, then chilled for 30 minutes at 5°C to solidify unhydrolyzed gelatin. If the gelatin did not solidify a positive reaction was indicated.

Indole Production

The ability of the bacterium to produce indole from tryptophan was tested by inoculating culture tubes of tryptone broth (20 grams of tryptone per liter of distilled water). To about 6 ml of the culture, 3 ml of Kovac's Reagent was added and the solution was well mixed. Two layers were then seen and a reddening of the top layer was considered a positive test.

Ammonia Production

Culture tubes containing 4% peptone broth were inoculated with the desired bacteria and incubated at 37°C for 48 hours. A drop of Nessler's Reagent was placed on a spot plate and a drop of culture media was added. A yellow color indicated the production of ammonia.

Reduction of Nitrate to Nitrite

Tubes containing nitrate broth (3.0 grams beef extract, 5.0 grams peptone and 1.0 gram of potassium nitrate per liter of distilled water) were inoculated with the bacterium to be tested. After incubation a few drops of the following reagent were added: 8.0 grams of sulfanilic acid dissolved in one liter of 5 N glacial acetic acid. Next a few drops of the following were added: 5.0 grams of

alpha-naphtholamine in 1 liter of 5 N acetic acid. A distinct pink or red color indicated the presence of nitrite.

Utilization of Citrate

To determine if the bacterium could grow in a medium containing citrate as the sole carbon source, tubes of Koser Citrate Broth (Difco, 1953) were inoculated with the test organism and incubated at 37°C for 48 hours. The presence of visible growth was considered a positive test.

Decomposition of Urea

To determine if the bacterium possesses the enzyme urease, tubes containing urea broth (Difco, 1953) were inoculated with the test organism and incubated at 37°C for 48 hours. A positive test was indicated by a change in color of the indicator contained within the broth from yellow to red. This change showed that urea had been decomposed to NH_3 and CO_2.

Digestion of Loeffler's Serum

The ability of the organism to digest Loeffler's Serum (Difco, 1953) was tested by streaking slants of the inspissated, coagulated serum. After 24 hours the slants were observed for growth and digestion.

Production of Black Rot in Chicken Eggs

To determine if the bacterium could produce black rot, fresh chicken eggs were injected with a 24 hour culture of the bacterium. One-tenth ml of a heavy saline suspension of bacteria was injected into each egg. The eggs were examined after five days for the presence of black rot.

Cytochrome Oxidase Test

To determine if the organism possessed the enzyme cytochrome oxidase, a loopful of the test bacterium was streaked on a PathoTec cytochrome oxidase identification strip. A darkening of the strip indicated a positive test.

Production of 2,3-Butanediol

The medium used for this test was:

Ingredient	Grams per liter distilled water
Nutrient Broth	8.0
Dibasic Potassium Phosphate	5.0

To test for the production of 2,3-butanediol, 1.0 ml of 2.3% periodic acid was added to 4.5 ml of the test broth in which the test organism was grown for 24 hours at 37°C. The tubes were shaken

and allowed to stand at room temperature for one-half hour, and then 1.5 ml of piperazine solution and 0.5 ml of 4.0% sodium nitroprusside were added. Development of a blue color indicated a positive test (Bullock, 1961).

Antibiotic Sensitivity

To determine the sensitivity of the organism to antibiotics, tests were conducted using Difco antibiotic impregnated sensi-discs. Two hundred ml of furunculosis agar were inoculated with 3.0 ml of a broth suspension containing the organism to be tested and plates were poured from this mixture. After the agar had hardened, the sensi-discs were placed on the agar surface and the cultures incubated at 37°C for 24 hours. An area without growth around the disc was considered evidence that the bacteria were sensitive to the antibiotic. Antibiotics used in this experiment included chloromycetin, erythromycin, kanamycin, neomycin, novobiocin, penicillin, streptomycin, and tetracycline.

Generation Time

The generation time of SDB no. 17 was determined by the following method: 0.2 ml of a 15-hour furunculosis broth culture of SDB no. 17 was inoculated into a flask containing 150 ml of furunculosis broth and incubated with shaking at 37°C. An initial

sample and hourly samples for the next seven hours were taken and plated out on furunculosis agar at dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8}. The plates were counted after 24 hours.

Survival of Bacteria in Sea Water

To determine the survival rate of the unknown organisms in sea water, two 100 ml samples of filter sterilized sea water were inoculated with 0.1 ml of a sea water washed culture of organism no. 17 and held for eight days at 18°C. Each day 1.0 ml of each duplicate sample was removed and a standard plate count was made on furunculosis agar at dilutions of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}.

Serological Methods

To determine the proper concentration of inocula of bacteria for the following experiments, the bacteria were suspended in physiological saline and the percent transmission (%T) read on a Bausch and Lomb Spectronic 20 at 475 mμ. Percent transmission is the amount of light able to pass through the solution. The greater the optical density of the solution tested, the lower the transmission of light through it.

To determine if isolates were antigenically similar agglutination tests were conducted. A large, approximately 10 pound rabbit

received two 1.5 ml injections, two weeks apart, of a mixture of equal volumes of a 20% T suspension of live SDB no. 17 (0.75 ml) and Freunds Complete Adjuvant (0.75 ml). Injections were given intramuscularly in the hip of the rabbit. After one month the rabbit was bled from the ear and the blood collected in a sterile centrifuge tube. The blood was then allowed to stand overnight and then spun down to separate out the serum. The serum was removed by pipetting and placed in a sterile serum bottle. The antibody titer of the serum was determined using the standard tube agglutination method with SDB no. 17 as the test antigen (Carpenter, 1965). To determine antigenic similarities, all shad disease isolates and known species of Aeromonas were reacted with the serum using the slide agglutination method (Carpenter, 1965). For these tests a standard saline control was used. As a second control all isolates were tested with a serum harvested from an uninjected rabbit. Definite clumping of the bacteria was considered a positive reaction.

Animal Experimentation Methods

A series of experiments were performed to determine the pathogenicity of the shad disease bacterium. The experimental animals involved were frogs, salmon, trout, and mice. Each of these animals were given injections of the shad disease bacterium.

Bacterial suspensions used were standardized in a spectrophotometer as previously described. The experiments involving salmon and trout were conducted using four gallon tanks into which 53°F water was continuously flowing.

Salmon Experiment Number 1

Three groups of juvenile coho salmon (Oncorhynchus kisutch, Walbaum) ten per group, were used in this experiment. Each salmon in the first lot was given a 0.1 ml intramuscular injection of a 20% T (8.0×10^8 bacteria/ml) suspension of SDB no. 17. Each salmon in the second lot was given a 0.1 ml intramuscular injection of a 40% T (4.7×10^7 bacteria/ml) suspension of SDB no. 17. The third group of salmon, the control group, were each injected with 0.1 ml of sterile saline. The fish were examined after 48 hours to determine the percentage of fish killed by the bacteria. Cultures taken from the kidney, liver, heart, spleen and musculature were inoculated onto furunculosis agar.

Salmon Experiment Number 2

Two groups of juvenile coho salmon, ten per group, were used in this experiment. The first group was anesthetized for five minutes with methyl pentynol (one ounce per gallon) and each of the salmon was scratched with a sterile needle over a one-half inch area

on the side of the fish. Each fish was then rubbed over the scarified area with a cotton swab soaked in a heavy broth suspension of SDB no. 17. The second group, the control animals, were scarified as above but rubbed with a swab containing sterile furunculosis broth. After 48 hours, one week and two weeks, the fish were examined for symptoms of disease.

Frog Experiment

Four groups of frogs were used to determine if the shad disease bacterium and known bacteria were pathogenic for frogs. The frogs were held in small aquaria at room temperature. Group one (two frogs) was given a one-tenth ml injection of a 20% T saline suspension of Aeromonas hydrophila (CDC) intramuscularly in the leg of the frog. The frogs in group two (2) were given a 0.1 ml injection of a 20% T saline suspension of Aeromonas liquefaciens (CDC) intramuscularly in the leg. Group three contained four frogs each of which was given a 0.1 ml injection of a 20% T saline suspension of the shad disease organism intramuscularly in the leg. Group four contained two frogs which were given 0.1 ml injections of sterile saline intramuscularly in the leg and retained as the control group.

These frogs were examined after 24 hours, 48 hours and one week for the appearance of infection. Bacteria were cultured from

the muscle tissue of the dead frogs on furunculosis agar. Resulting cultures were examined.

Rainbow Trout Experiment

The test animals were divided into three groups consisting of five rainbow trout (Salmo gairdneri, Richardson) in each group. Each fish in group 1 was given a 0.1 ml intramuscular injection of a 20% T saline suspension of SDB no. 17. Each animal in the second group was given a 0.1 ml intramuscular injection of a 40% T saline suspension of SDB no. 17. The trout in the third group were given a 0.1 ml intramuscular injection of sterile saline and were used as control animals. The fish were examined after 24 hours for pathology. Cultures from the kidney of each fish were made on furunculosis agar. Trout used in this experiment ranged from four to six inches in length.

Mouse Inoculation Experiment

Forty female albino mice weighing 28-29 grams each were used in this experiment. The mice were divided into four groups of ten each. Mice in group one were each given a 0.1 ml intraperitoneal injection of a 25% T saline suspension of the shad disease bacteria. Each mouse in the second group was given a 0.1 ml intraperitoneal injection of a 50% T saline suspension of the shad disease organism,

and each mouse in the third group was given a 0.1 ml intraperitoneal injection of a 75% T saline suspension of the shad disease organism. The fourth group of mice were used as controls, and each mouse in this group was given a 0.1 ml intraperitoneal injection of sterile saline.

Plate counts from each dilution of bacteria were done in triplicate and the average number of bacteria injected was determined.

The mice were observed after one, two, and seven day intervals for mortality. Dead mice were examined for pathology and cultures were made from the kidney, liver and peritoneal fluid of each animal.

Determination of the Mole Percent Guanine + Cytosine of the Bacterial Deoxyribonucleic Acid (DNA)

The method used for DNA isolation was a modified form of the procedure published by Marmur (1961). Two to three grams of cells were harvested from three liters of furunculosis broth after being grown at room temperature on a shaker for 15 to 24 hours. These cells were suspended in 50 ml of a pH 8.0 saline-EDTA (0.15 M NaCl + 0.1 M ethylene diaminetetra-acetate) solution and washed once. They were then resuspended in 25 ml of saline-EDTA. Three ml of a 25% solution of sodium lauryl sulfate were added to each of two 12.5 ml suspensions of cells and the mixtures were heated for

ten minutes at 60°C. The resulting lysed cells were cooled to room temperature in a 250 ml Erlenmeyer flask. The sodium ion concentration was adjusted to 1.0 M by adding 5.0 M sodium perchlorate. An equal volume of chloroform-isoamyl alcohol (24:1) was added and the mixture was shaken at room temperature for 30 minutes. This emulsion was next centrifuged at 10,000 r.p.m. for five minutes. The top layer of the separated emulsion was then removed. This layer, which contained the crude nucleic acid, was precipitated using two volumes of cold 95% ethanol. The nucleic acid was collected from the alcohol-nucleic acid interface by stirring with a glass rod. The nucleic acid was dissolved in 9.0 ml of dilute saline citrate (0.015 M NaCl plus 0.0015 M sodium citrate). The sodium ion concentration was then brought to a standard concentration of 0.15 M NaCl and 0.015 M saline citrate by the addition of 9.0 ml of concentrated saline citrate (1.5 M NaCl + 0.15 M sodium citrate). This solution was then subjected to a series of deproteinizations by shaking with chloroform-isoamyl alcohol. When very little protein could be seen at the nucleic acid-chloroform interface, the nucleic acid was precipitated a second time with cold 95% ethanol. The precipitated nucleic acid was again disolved in dilute saline citrate and then brought to standard saline citrate concentration as above. Ribonucleic acid was removed from the sample by treatment with ribonuclease (0.2% ribonuclease in 0.15 M NaCl, pH 5.0) at 37°C

for 30 minutes. The ribonuclease was added to a final concentration of 50 μg/ml. After 30 minutes the DNA was subjected to another series of deproteinizations to remove traces of protein. The DNA was then precipitated a third time and suspended in ten ml of standard saline citrate, and stored until used.

To determine the thermal denaturation temperature (Tm) of the deoxyribonucleic acid, abosrbance of the DNA was recorded on a Guilford Multiple Sample Absorbance Recorder from 25°C until the melt was completed (about 100°C). The absorbance was read at 260 mμ. The data was then corrected for thermal expansion and plotted to determine the melting temperature. From the melting temperature, the percent guanine + cytosine was determined using the formula, melting temperature (Tm) = 69.3 + 0.41 (percent guanine + cytosine) (Marmur and Doty, 1962).

RESULTS

Pathology

External Appearance of Diseased Shad

Diseased shad had external lesions (hemorrhagic areas) on the sides and near the base of the tail. The hemorrhagic areas were soft, red and raised and in advanced cases were ulcerative. General necrosis was noted in the area of the lesion. Diseased fish showed reddening in the head area and on the pectoral and pelvic fins. Figure 1 compares a normal fish with a diseased shad. The diseased fish had lesions on its side and darkening of the head region and the fins. Figure 2 shows large hemorrhagic lesion commonly observed on diseased shad.

Internal Appearance of Diseased Shad

The internal organs of diseased shad did not show any visible pathology although the disease-producing bacterium could be isolated from the kidney, liver, spleen, heart, and blood of the infected animals. In severely infected shad, the lining of the peritoneal cavity showed large hemorrhagic areas and the body cavity was filled with blood and tissue fluids.

Figure 1. Comparison of Diseased (Lower) and Normal (Top) Shad. Diseased Shad Shows Large Hemorrhagic Area and Darkening of Head and Fins.

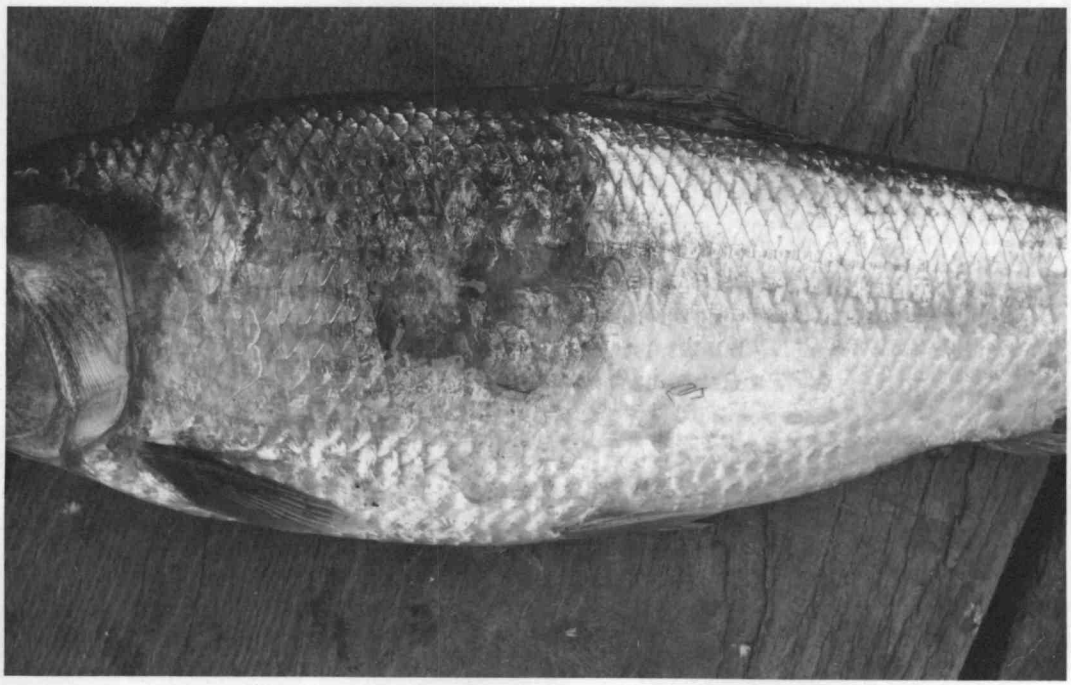

Figure 2. Characteristic Large Hemorrhagic Area on Side of Diseased Shad.

Bacteriological Examination of Diseased Adult Shad

In order to determine the causative agent of the disease, bacteriological cultures were prepared on furunculosis agar from the spleen, liver, kidney, heart, blood and external lesions of fifteen diseased shad taken from the Smith, Coos and Millicoma Rivers during June of 1966. In all cases a pure culture of the shad disease bacterium was obtained.

Correlation of Water Temperature and Time of Year with the Percent of Infected Shad

The results shown in Table 1 and Figure 3 suggest that shad which spawn at the end of the spawning period are more apt to be infected with the disease. A combination of prolonged exposure to high water temperatures and increased maturation probably make the shad more susceptible to disease. In the beginning of the shad migration very few diseased fish could be found. On May 12 no diseased fish were observed and on May 29 only 1.8% of the fish examined were diseased. Toward the end of the spawning migration a higher percentage of diseased shad were found. On June 8, 32% of the fish examined were infected, and on June 17, 68%.

Fish examined during this week were collected with gill nets. External lesions were considered evidence of infection and no further attempt was made to diagnose the disease. External pathology was

very characteristic and could be observed with ease. Daily mean water temperature data was obtained from Mr. Edwin Cummings, Oregon State Fish Commission, Charleston, Oregon.

Table 1. Development of Epizootic in Shad Population.

Date Sampled	No. Fish Examined	No. Infected Fish	Percent Infected Fish
May 12	11	0	0
May 29	53	1	1.8
June 8	25	8	32
June 17	25	17	68

Morphology and Cultural Reactions of Shad Disease Bacterium

The shad bacterium was a short rod with a single polar flagellum (Figure 4). In this photomicrograph the polar flagellum appeared to consist of an outer sheath and a hollow inner core.

This organism was gram negative, nonsporeforming and non-acid-fast. Its average size was 0.4 microns in width by 1.5 microns in length, pleomorphic forms up to 8 microns in length were found in older cultures.

This bacterium grew well on a variety of types of solid media. On furunculosis agar the colonies were round, flat, smooth, glistening, cream colored and butyrous. Eosin methylene blue agar

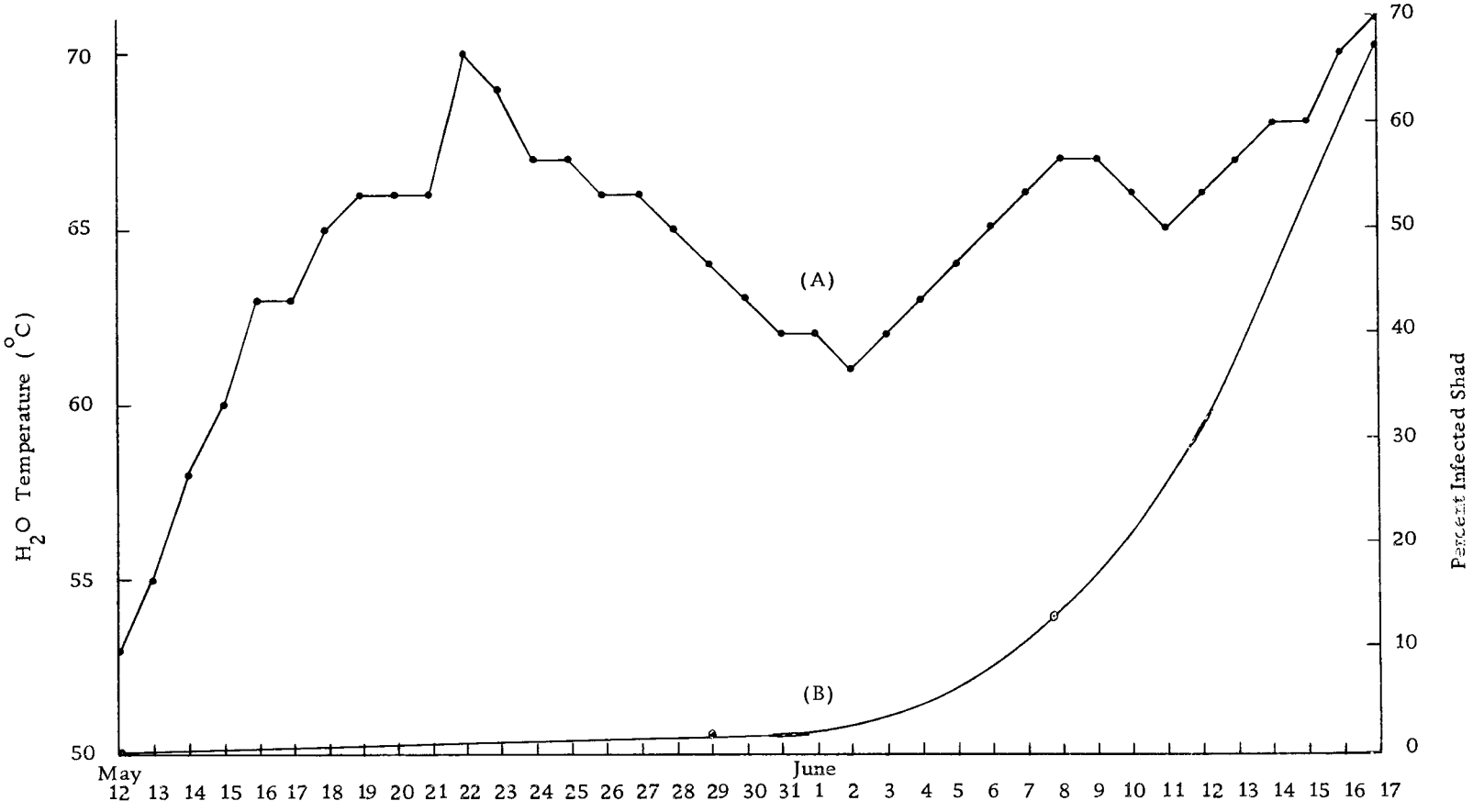

Figure 3. Curves Showing Development of Epizootic in Shad Population. Curve A Shows Daily Average Water Temperature of Millicoma River and Curve B Shows Percent Diseased Fish on Day Indicated.

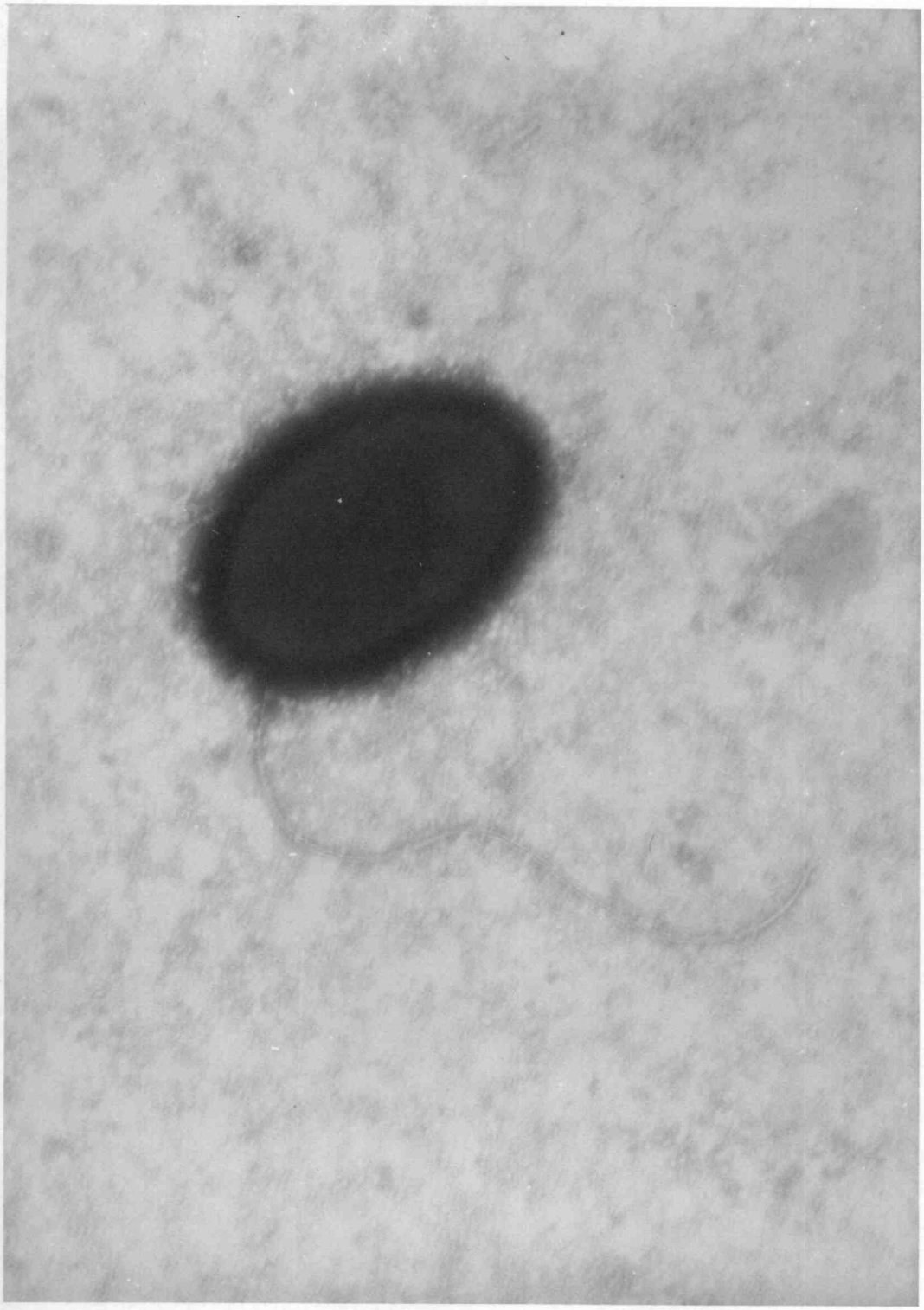

Figure 4. Photomicrograph (Approximately 12,000 x) Showing Shad Disease Bacterium. Organism has Polar Flagellum Which Appears to have a Hollow Inner Core.

produced colonies which were small, round, smooth and violet colored. Brilliant Green Agar colonies were small, round, smooth, glistening and green. Colonies on blood agar were small, white, round and surrounded by a zone of beta hemolysis. On Nutrient Agar the growth was scanty and the colonies were circular, glistening, elevated and cream colored. Trypticase Soy Agar gave abundant growth and the colonies were round, glistening, raised and cream colored. Growth was scanty on Mueller-Hinton agar and the colonies were glistening, raised, cream colored with an entire edge.

To determine the optimum incubation temperature of the bacterium isolated from shad, isolates no. 1 and 2 were inoculated into tubes of furunculosis broth and grown for 24 hours at temperatures of 9, 18, 23, 30 and 35°C. At time intervals of 6, 8, 10, 12, 16 and 24 hours the optical density of each culture was measured. Depending on the change in optical density, the cultures were given a growth rating between 1 - 4. The results of this experiment are shown in Table 2. The best growth obtained for the organism was at 30 and 35°C. Good growth was obtained at 23°C for both isolates while only fair growth was noted at 18°C. At 9° very scanty growth was observed for both isolates.

These bacteria also grew well in selected broth media. In furunculosis broth and nutrient broth there was abundant growth with no pellicle formation.

Table 2. Turbidity Produced in Furunculosis Broth at Different Incubation Temperatures by Two Isolates of the Bacterium Isolated from Shad.

Incubation Temperature (^{o}C)	Isolate No. 1	Isolate No. 2
9^{o}	1	1
18^{o}	2	2
23^{o}	3	3
30^{o}	4	4
35^{o}	4	4

1 = scanty growth (0 - 0.19 O.D.)

2 = fair growth (0.2 - 0.39 O.D.)

3 = good growth (0.4 - 0.59 O.D.)

4 = excellent growth (0.6 O.D. and above)

On a potato slant the growth was abundant, smooth, glistening and light brown in color.

Physiological Reactions of the Shad Disease Organism and of Organisms Classified As *Aeromonas liquefaciens*, *Aeromonas hydrophila* and *Aeromonas punctata*

Preliminary results of physiological tests indicated that the shad disease organism was a member of the genus *Aeromonas*. Known *Aeromonas* cultures were obtained from the Communicable Disease Center at Atlanta, Georgia and the Eastern Fish Disease

Laboratory at Kearneysville, West Virginia for the purpose of comparison with the shad bacterium.

Table 3 shows the reactions of all the bacterial isolates obtained from shad from the Millicoma, Coos and Smith Rivers. Almost all isolates gave identical results in these tests. The only difference can be seen in the results for maximum salt concentration, Loeffler's serum, and the production of 2,3 butanediol. Isolates nos. 2, 8, 12, 13, 14, 15, 16 and 17 could only tolerate 3.0% NaCl while the others were able to grow in a medium containing 3.5% NaCl. Organisms nos. 2, 4, 5, 8, 9, 10 digested Loeffler's serum while the others were able to produce only growth. Only isolates no. 3 and no. 16 were able to produce 2, 3 butanediol.

Table 3 shows that these isolates were able to ferment a variety of sugars with the production of acid and gas. These sugars included glucose, maltose, sucrose, fructose, galactose, rhamnose, arabinose, glycerol, salicin, mannose, mannitol and trehalose. Sugars which were not fermented included lactose, dulcitol, raffinose, sorbitol, xylose and inulin.

This table shows all the isolates were physiologically active. They had the enzyme catalase, reduced nitrate to nitrite, produced indole from tryptophan, hydrolyzed starch and gelatin, had the enzyme cytochrome oxidase, and grew and digested Loeffler's serum. They could not hydrolyze urea. The isolates were not

Table 3. Biochemical Reactions of Fifteen Isolates from Diseased Shad

	Millicoma and Coos River Isolates										Smith River Isolates				
	Strain Number										Strain Number				
Test	1	2	3	4	5	6	7	8	9	10	12	14	15	16	17
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maximum salt tolerance	3.5%	3.0	3.5	3.5	3.5	3.5	3.5	3.0	3.5	3.5	3.0	3.0	3.0	3.0	3.0
Litmus milk	peptonization, reduction, coagulation all the same														
Production of H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate - Nitrite	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NH_3 Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Maltose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Sucrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Galactose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Rhamnose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG

Continued on next page

Table 3. Continued.

	Millicoma and Coos River Isolates										Smith River Isolates				
	Strain Number										Strain Number				
Test	1	2	3	4	5	6	7	8	9	10	12	14	15	16	17
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Glycerol	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Mannose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Mannitol	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Susceptibility to Vibriostat 0/129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loefflers Serum	G	GD	G	GD	GD	G	G	GD	GD	GD	GD	G	G	GD	GD
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of 2, 3 Butanediol	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
Trehalose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes: + = positive
- = negative
AG = acid + gas
G = growth
GD = growth + digestion

susceptible to vibriostat 0/129 indicating that they were not members of the genus Vibrio.

To determine if the shad disease bacterium was a facultative anaerobe, SDB no. 17 was used in a series of physiological tests which were performed both aerobically and anaerobically. In all tests, except inulin fermentation where aerobically acid and gas were produced and anaerobically only acid was produced, identical results were obtained in both the aerobic and anaerobic experiments. Therefore, it was concluded that the shad disease organism was a facultative anaerobe.

The next step in the investigation was to determine if known members of the genus Aeromonas gave physiological reactions comparable to the shad disease organism, which was presumed to be a member of this genus (Table 4). Both the shad bacterium and the known members of the genus Aeromonas produced acid and gas from the following compounds: fructose, galactose, sucrose, glucose, glycerol, mannitol, maltose, rhamnose and trehalose. None of the bacteria fermented inulin, raffinose, dulcitol or lactose. Variable results between the knowns and the unknowns were obtained with the fermentation of salicin and arabinose. The SDB isolates produced acid and gas from both. A. hydrophila (CDC) was able to produce acid and gas from arabinose. The other organisms were unable to ferment these sugars. All the organisms were positive

Table 4. Physiological Reactions of Shad Bacterium as Compared to Known Species of the Genus Aeromonas.

Test	Shad Organism No. 17	A. liquefaciens (CDC)[1]	A. liquefaciens (EFDL)[2]	A. hydrophila (CDC)[1]	A. punctata (CDC)[1]
Fructose	AG	AG	AG	AG	AG
Motility	+	+	+	+	+
Raffinose	-	-	-	-	-
Salicin	AG	-	-	-	-
Xylose	-	-	-	-	-
Litmus Milk	peptonization, coagulation, reduced				
Dulcitol	-	-	-	-	-
Lactose	-	-	-	-	-
Galactose	AG	AG	AG	AG	AG
Arabinose	AG	-	-	AG	-
Sucrose	AG	AG	AG	AG	AG
Glucose	AG	AG	AG	AG	AG
NH_3 Production	+	+	+	+	+
Nitrate to nitrite	+	+	+	+	+
Indole Production	+	+	+	+	+
Voges-Proskauer	+	-	-	-	-
Methyl Red	-	+	+	+	+
Gelatin Liquefaction	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+

Continued on next page

Table 4. Continued.

Test	Shad Organism No. 17	A. liquefaciens (CDC)[1]	A. liquefaciens (EFDL)[2]	A. hydrophila (CDC)[1]	A. punctata (CDC)[1]
Black Rot Production in Chicken Eggs	+	+	+	+	+
Maximum Salt Tolerance	3%	3.5%	3%	3%	3%
Glycerol	AG	AG	AG	AG	AG
Mannitol	AG	AG	AG	AG	AG
Urea	-	-	-	-	-
Mannose	AG	AG	AG	AG	AG
Inulin	-	-	-	-	-
Methylene Blue	reduced	not reduced	reduced	not reduced	reduced
Citrate	+	+	+	+	+
Cytochrome Oxidase	+	+	+	+	+
Loefflers Serum	GD	GD	GD	GD	GD
H_2S Production	+	+	+	+	+
Susceptibility to Vibriostat 0/129	-	-	-	-	-
Growth in 1.1×10^{-3} M KCN	+	+	+	+	+
Maltose	AG	AG	AG	AG	AG
Rhamnose	AG	AG	AG	AG	AG
2,3 Butanediol Production	-	+	+	+	+
Trehalose	AG	AG	AG	AG	AG

[1] Organisms obtained from Dr. Ewing of Communicable Disease Center at Atlanta, Georgia.

[2] Organism obtained from Mr. G. L. Bullock of Eastern Fish Disease Lab. at Kearneysville, West Virginia.

for the following tests: motility, ammonia production, reduction of nitrates to nitrites, production of indole, gelatin liquefaction, starch hydrolysis, black rot production in chicken eggs, growth where citrate was the only carbon source, cytochrome oxidase, growth and digestion of Loeffler's serum, hydrogen sulfide production and growth in the presence of 1.1×10^{-3} M KCN. None of the organisms could hydrolyze urea or were susceptible to Vibriostat 0/129. All of the organisms produced peptonization, coagulation and reduction of litmus milk. Variable results between the knowns and the shad disease bacterium occurred in the following tests: maximum tolerated salt concentration, reduction of methylene blue, 2,3, butanediol production, fermentation of salicin and arabinose, and in the Voges-Proskauer and methyl red tests.

An experiment was conducted to determine the antibiotic sensitivity of the shad disease bacterium and the known species of the genus Aeromonas (Table 5). Isolates numbers 1, 2, 3, 15 and 16 and 17 were used as the SDB test organisms; all of these isolates gave identical results. The following antibiotics were used for this experiment: chloromycetin (5, 10, 30 mcg), erythromycin (2, 5, 15 mcg), kanamycin (5, 10, 30 mcg), neomycin (5, 10, 30 mcg), novobiocin (5, 10, 30 mcg) and tetracycline (5, 10, 30 mcg). As shown in Table 5 the shad disease bacterium was found to be susceptible to the following antibiotics: chloromycetin, erythromycin (2, and 5

Table 5. Reaction of Shad Bacterium and Known Species of Aeromonas to Antibiotics.

Antibiotic (concentration)		Unknown*	A. liquefaciens (CDC)	A. liquefaciens(EFDL))	A. hydrophila(CDC)	A. punctata(CDC)
Chloromycetin	5 mcg	S	S	S	S	S
Chloromycetin	10 mcg	S	S	S	S	S
Chloromycetin	30 mcg	S	S	S	S	S
Erythromycin	2 mcg	R	S	S	S	R
Erythromycin	5 mcg	R	S	S	S	S
Erythromycin	15 mcg	S	S	S	S	S
Kanamycin	5 mcg	S	S	S	S	S
Kanamycin	10 mcg	S	S	S	S	S
Kanamycin	30 mcg	S	S	S	S	S
Neomycin	5 mcg	S	S	S	S	S
Neomycin	10 mcg	S	S	S	S	S
Neomycin	30 mcg	S	S	S	S	S
Novobiocin	5 mcg	S	R	S	R	R
Novobiocin	10 mcg	S	R	S	S	S
Novobiocin	30 mcg	S	S	S	S	S
Penicillin	2 units	R	R	R	R	R
Penicillin	5 units	R	R	R	R	R
Penicillin	10 units	R	R	R	R	R
Streptomycin	2 mcg	S	S	S	S	S
Streptomycin	5 mcg	S	S	S	S	S
Streptomycin	10 mcg	S	S	S	S	S
Tetracycline	5 mcg	S	S	S	S	S
Tetracycline	10 mcg	S	S	S	S	S
Tetracycline	30 mcg	S	S	S	S	S

R = resistant S = susceptible mcg = micrograms

*SDB No. 1, 2, 3, 15, 16, 17 used as test isolates. All gave the same results.

mcg), kanamycin, neomycin, novobiocin, streptomycin and tetracycline. The known species of Aeromonas gave differing results: A. liquefaciens (CDC) was resistant to 2 and 5 mcg of novobiocin, A. liquefaciens (EFDL) was susceptible to all concentrations of erythromycin, A. hydrophila was resistant to 5 mcg novobiocin but not erythromycin, and A. punctata was also resistant to 5 mcg of novobiocin. The SDB isolates and the known species of Aeromonas were very similar in that the only antibiotic they were completely resistant to was penicillin at all concentrations tested.

Since most bacteria which are pathogenic to fish are relatively slow growing it was interesting to note that the shad disease bacterium has an extremely fast generation time. By growing SDB no. 17 at 37°C on a rotary shaker and making plate counts each hour for seven hours, the generation time was calculated to be 19.4 minutes (Table 6). Figure 5 shows the plot of the viable count of bacteria against time in hours.

An experiment was conducted to determine if the SDB isolate no. 17 could survive in a salt water environment such as is found in the bays of southwest Oregon. Duplicate samples were inoculated in 30 parts per thousand sea water and each day a count of the surviving bacteria was made. Figure 6 shows the plot of the median value of the bacterial counts (Table 7) made daily for one week. In both samples there was a 99.9 percent decrease in the number of

Table 6. Viable Counts and Log_{10} of Viable Count Used in Determining Growth Curve.

Time	No. viable cells per ml	Log_{10} of no. cells per ml
0 time	1.32×10^6	6.121
1 hour	1.84×10^6	6.265
2 hours	2.60×10^6	6.415
3 hours	5.40×10^6	6.732
4 hours	3.6×10^7	7.556
5 hours	4.0×10^8	8.602
6 hours	1.16×10^9	9.065
7 hours	1.32×10^9	9.121

average generation time

$$GT = \frac{.301\ T}{\log_{10} b - \log_{10} a}$$

$$GT = \frac{.301\ (120\ \text{min})}{\log_{10} 4.0 \times 10^8 - \log_{10} 5.40 \times 10^6}$$

$$GT = 19.4 \text{ minutes}$$

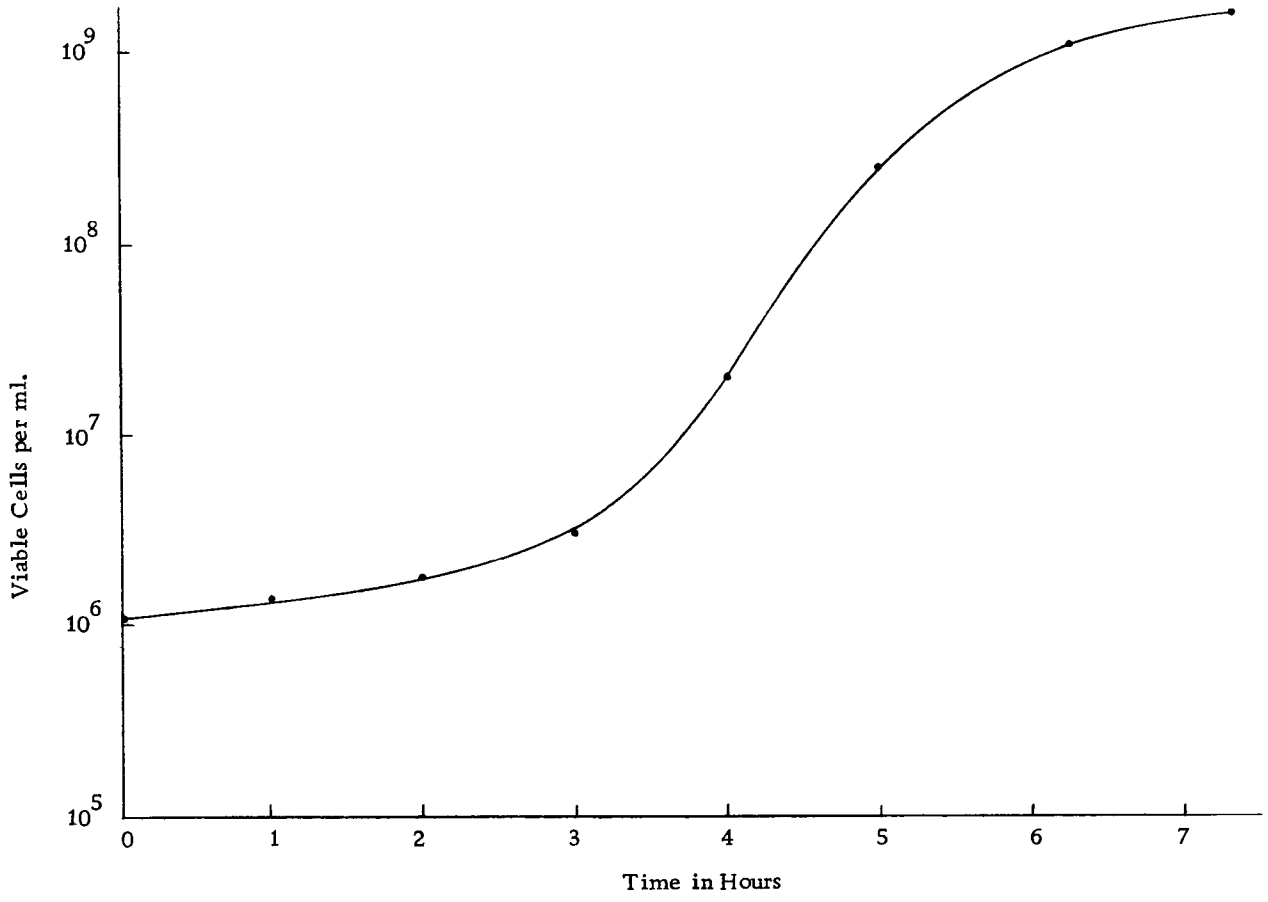

Figure 5. Growth Curve of Shad Disease Organism No. 17.

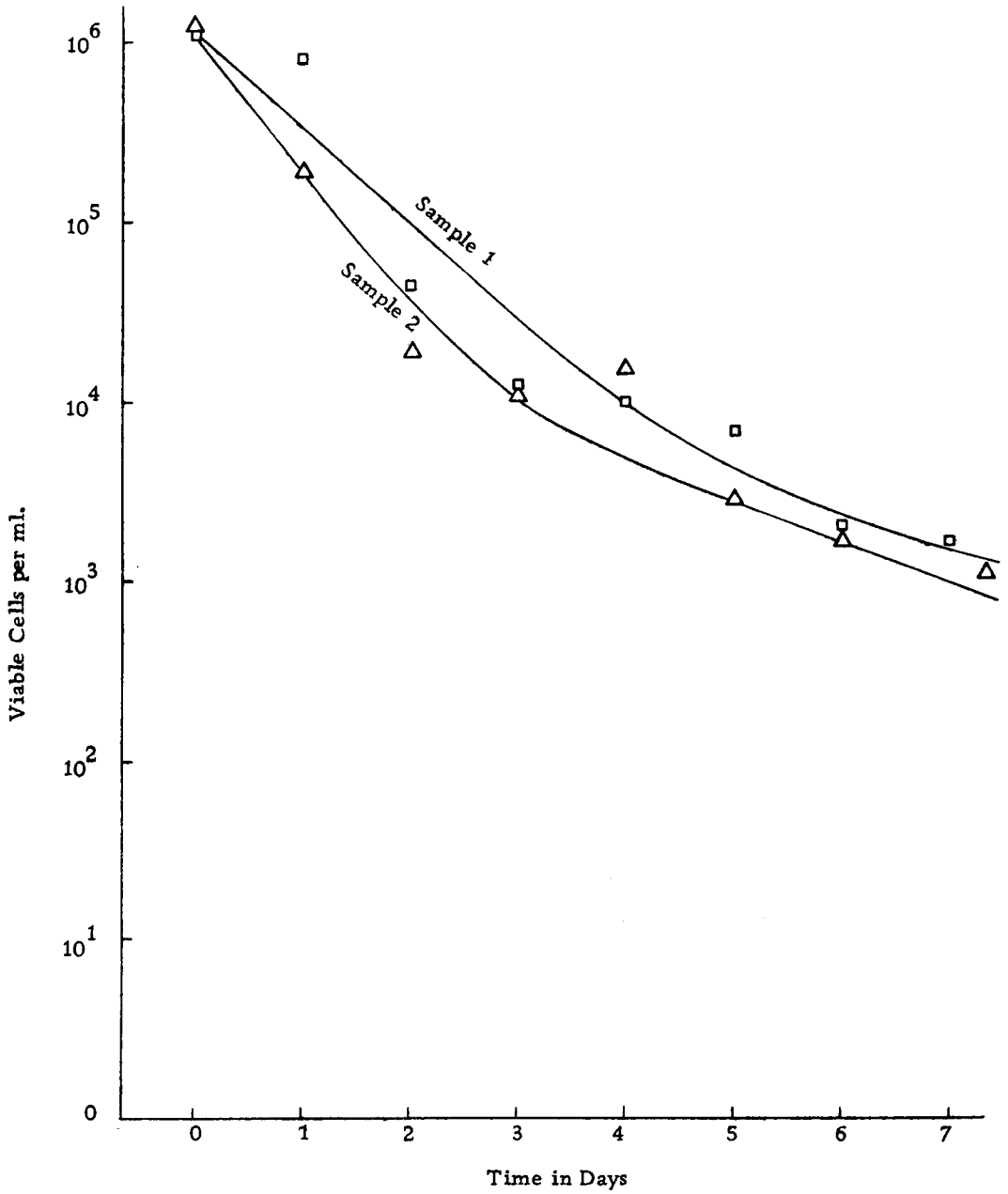

Figure 6. Survival Rate of Shad Disease Isolate No. 17 in Sea Water. Curves Show the Rapid Decrease in Number of Viable Cells of Shad Disease Bacterium Suspended in Sea Water.

bacteria after one week indicating a very poor survival rate.

Table 7. Bacterial Counts Obtained with Shad Disease Isolate No. 17 Suspended in Sea Water for One Week.

Days	Viable Cells per ml	
	Sample 1	Sample 2
0	1.57×10^6	1.67×10^6
1	9.6×10^5	3.5×10^5
2	6.3×10^4	3.02×10^4
3	1.7×10^4	1.6×10^4
4	1.0×10^4	2.05×10^4
5	8.6×10^3	5.0×10^3
6	2.43×10^3	2.27×10^3
7	2.07×10^3	1.32×10^3

Results of Experiments Comparing Thermal Denaturation Temperature (Tm) and Mole Percent Guanine + Cytosine of the DNA of the Shad Disease Bacterium and Known Members of the Genus, *Aeromonas*

As a further aid in determining the taxonomic position of the shad disease bacterium, the thermal denaturation temperature and mole percent guanine + cytosine of the DNA from three isolates of the shad bacterium and *A. liquefaciens* (CDC), *A. hydrophila* (CDC) and *A. punctata* (CDC) were determined. The DNA was isolated from the bacteria using the ethanol precipitation method of Marmur

(1959). After the DNA was isolated its melting temperature was determined on a Guilford Multiple Absorbance Recording Spectrometer. The mole percent guanine + cytosine was determined using the formula Tm - 69.3 + .41 (G + C), (Marmur and Doty, 1962).

The three isolates of the shad bacterium had melting temperatures of 94.3, 94.6, and 94.2 with corresponding mole percent G+C values of 60.9, 61.7 and 60.7 (Table 8). Aeromonas liquefaciens (CDC) had a Tm of 94.1 and a mole percent G + C of 60.3 and A. hydrophila had a Tm of 95.4 and a mole percent G + C of 63.4. A. punctata had a Tm of 93.5 and a mole percent G + C of 59.0. These results suggest that the shad bacterium appears to be closely related to A. liquefaciens and that A. liquefaciens, A. punctata, and A. hydrophila show a difference in their Tm and mole percent G + C.

Table 8. Comparison of Thermal Denaturation Temperature (Tm) and Mole Percent Guanine + Cytosine of the DNA of the Shad Bacterium and Known Species of the Genus Aeromonas.

Organism	Tm	Mole Percent Guanine + Cytosine*
Shad bacterium no. 6	94.3	60.9
Shad bacterium no. 12	94.6	61.7
Shad bacterium no. 17	94.2	60.7
A. liquefaciens (CDC)	94.1	60.3
A. punctata (CDC)	93.5	59.0
A. hydrophila (CDC)	95.4	63.4

*Mole percent G + C calculated by the formula: Tm = 69.3 + .41 (G + C). (Marmur and Doty, 1962)

Serological Results

To determine if the various isolates of the shad bacterium and the known species of the genus Aeromonas were antigenically similar, a series of slide agglutination tests were conducted. An antiserum prepared against the shad disease bacterium no. 17 was able to agglutinate SDB isolates and all known members of the genus Aeromonas. Normal serum from an uninjected rabbit was unable to agglutinate any of the test organisms. The above results would indicate that the shad bacterium and the known members of the genus Aeromonas share at least one major antigen.

Results of Animal Infection Studies Performed to Determine the Pathogenicity of the Shad Disease Bacterium

Physiological test results seemed to indicate that the shad disease organism was a member of the genus Aeromonas. Certain diseases of other animals and fish had been described as being caused by Aeromonas liquefaciens, Aeromonas hydrophila and Aeromonas punctata. The next logical step for experimentation was to determine the pathogenicity of the shad bacterium and to compare it to other members of the genus Aeromonas. To do this, SDB no. 17 was used in experiments involving salmon, trout, frogs and mice.

Ten juvenile coho salmon ranging in size from four to six inches given intramuscular injections of 0.1 ml of a 20% T

suspension of SDB no. 17 and ten salmon given 0.1 ml intramuscular injections of 40% T suspensions of SDB no. 17 were all killed by the bacterium within 48 hours. Internally, the symptoms exhibited by the animals were badly inflamed pyloric ceca and extreme kidney damage. Some fish also had hydrolyzed intestines. Pure cultures of the shad bacterium were recovered from the spleen, kidney, muscle and liver of the infected fish.

The next experiment also involved juvenile coho salmon. Ten fish were scarified on their sides with a sterile needle and then a cotton swab saturated with a heavy suspension of shad bacteria was rubbed against the scarified area. The attempt to transmit the disease by this method was unsuccessful.

The next infection studied used juvenile rainbow trout. Five trout were given a 0.1 ml intramuscular injection of a 20% T suspension of SDB no. 17 and five trout were given 0.1 ml of a 40% suspension of SDB no. 17. All trout died within 48 hours and all showed symptoms of the disease "red mouth". This disease has been attributed to Aeromonas hydrophila (Wagner and Perkins, 1952). Symptoms exhibited by the dead fish were inflamed oral mucosa, massive inflammation of all internal organs, and bleeding from the anus. Some of the fish also had liquefied livers. Pure cultures of the shad bacterium were found in the kidney of all fish examined.

The next type of animal to be injected was the frog. All four frogs given an intramuscular injection of 0.1 ml of a 20% T suspension of SDB no. 17 were killed within 48 hours. Of the known cultures used in this experiment, only A. hydrophila was able to cause the death of a frog. The dead frogs showed definite symptoms of the disease "red leg". This is a disease attributable to A. hydrophila (Eddy, 1960) and is characterized by extreme hemorrhaging of the leg muscles. Pure cultures of the bacteria could be isolated from the legs of each infected frog.

Since the shad bacterium is able to grow at 37°C, it was thought that this bacterium could possibly infect warm-blooded animals. To determine this, a study was performed using mice as the experimental animal. Three groups of mice were given intramuscular injections of differing dilutions of shad bacterium no. 17. Fifty percent of the mice injected with 1.94×10^7 bacteria died, 30% of the mice injected with 9.7×10^6 bacteria died, and 10% of the mice injected with 3.6×10^6 died. Internally the dead mice showed extreme necrosis of the abdominal wall and darkening of the spleen, liver and kidney. Pure cultures of the shad disease organism were recovered from the kidney, liver and peritoneal fluid.

DISCUSSION

Classification of the Disease Producing Agent

The results of the experiments performed on the shad disease bacterium have indicated that this bacterium belonged in the genus Aeromonas. As shown in the literature review, there was quite a bit of confusion as to whether the three motile species in this genus were actually one species and as to what should be the type species of this genus. Ewing et al. (1961) thought that the three species should actually be considered only one and that this one should bear the name Aeromonas hydrophila. C. P. Eddy (1962) agreed that the three present species should only be considered as one legitimate species bearing the name, Aeromonas punctata. Since the bacterium which caused shad disease could also cause "red leg" in frogs and "red mouth" in rainbow trout, it seemed that this bacterium should have the species name hydrophila since Aeromonas hydrophila has been reported to cause both "red leg" and "red mouth" disease. In the experiments which were conducted on the organisms classified as Aeromonas liquefaciens, Aeromonas hydrophila and Aeromonas punctata from the Communicable Disease Center in Atlanta, Georgia and the organism classified Aeromonas liquefaciens by the Eastern Fish Disease Laboratory in Kearneysville, West Virginia, very few differences could be found. The differences which did occur are

very minor and there was no consistency among these differences. For example, Aeromonas liquefaciens (CDC) did not reduce methylene blue while A. liquefaciens (EFDL) did. Also Aeromonas liquefaciens (CDC) did not produce black rot in chicken eggs while Aeromonas liquefaciens (EFDL) did. Aeromonas hydrophila (CDC) and Aeromonas punctata (CDC) also produced black rot in chicken eggs. All existing evidence would seem to suggest that there was actually only one actual species in this group. The eighth edition of Bergey's Manual when it is printed will probably show major revisions for the genus Aeromonas. For example, Ewing et al. (1961) have suggested the following revision of the genus Aeromonas. The motile, gas-producing aeromonads should be called Aeromonas hydrophila. The non-motile organisms now grouped as Aeromonas salmonicida should continue to be placed in this group. A third species which was motile but anaerogenic should be called Aeromonas shigelloidas. The shad disease organism would be placed in the A. hydrophila group. Eddy and Carpenter (1964) suggested a similar revision. They suggested that the motile, aerogenic members of the genus Aeromonas be classified as A. punctata. They further suggested that the motile, anaerogenic species be classified as A. formicans. The non-motile species of Aeromonas would still be called Aeromonas salmonicida. In this classification the shad disease bacterium would be called

Aeromonas punctata.

Some differences could be seen between the physiological test results obtained for the SDB isolates and the known cultures. For example the SDB isolates produced acid and gas from salicin, while the knowns were not able to ferment salicin. Eddy (1960) in his monograph concerning the genus Aeromonas showed in a number of Aeromonas cultures that the property of fermenting salicin was a variable one in this genus. Ewing et al. found that only 62.5% of the strains of A. hydrophila which they tested fermented salicin. The shad organism was able to produce acid and gas from arabinose while only A. hydrophila among the knowns could ferment arabinose. Of the cultures tested by Ewing (1961) only 45% could produce acid and gas from arabinose. Eddy also stated that the fermentation of arabinose was a variable test for the organisms in the genus Aeromonas. Only SDB no. 3 and no. 16 could produce 2,3 butanediol while all the knowns were positive for this test. Since this test was performed almost a year later than the other biochemical tests on the shad organism and this organism had undergone numerous transfers during this time, the organisms may have lost their ability to produce 2,3 butanediol from acetylmethylcarbinol. Schubert (1964) said that there are 2,3 butanediol negative species in the genus Aeromonas, and he called these Aeromonas punctata.

An attempt was made to clear up the confusion among the species of Aeromonas by using the mole percent guanine + cytosine and Tm (thermal denaturation temperature) as a means of separating these species. However, at the present time there seems to be confusion in the results obtained using this technique. For example Colwell and Mandel reported a mole percent guanine + cytosine for A. hydrophila of 55.7, for A. punctata 56.6 and A. liquefaciens of 59.0. Sebald and Veron (1963) reported % G + C values of 59.0 and 59.3 for A. hydrophila from the Institut Pasteur and 61.4 and 62.7 from a hydrophila culture from the National Collection of Type Cultures in London. They also reported that A. punctata from the Culture Collections of Entomogenous Bacteria in Prague had a percent G + C of 60.8. Mandel and Rownd, according to Hill (1966) reported a percent G + C for an Aeromonas hydrophila from the American Type Culture Collection of Washington, D. C. of 55.5. In this study a percent G + C of 60.3 was obtained for A. liquefaciens (CDC) and 59.0 for A. punctata (CDC) and 63.4 for A. hydrophila (CDC). The shad disease organism had an average mole percent G + C of 61.1. It would seem from these many conflicting values that at the present time this method of identification of species was not applicable to the genus Aeromonas. Limits of accuracy and precision of the method itself probably contributed to the variations of results obtained by various

experimenters.

Marmur (1961) stated that this method is accurate to within 1% G + C. A difference greater than this is therefore significant. From the mole percent G + C data obtained in the experiments on the shad organism and the known species it would seem that there were differences between all three species of Aeromonas with A. hydrophila having the highest mole percent G + C, Aeromonas liquefaciens the intermediate value and A. punctata the lowest. This would seem to be consistent with the results obtained by Sebald and Veron (1963) who found that hydrophila did have a higher percent G + C than did Aeromonas punctata. However, Colwell and Mandel (1964) did not find this to be so. This again shows confusion as to using the determination of mole percent G + C and Tm to determine the species of Aeromonas. From the results of these experiments it seemed that the shad disease organism would be Aeromonas liquefaciens. However at the present it would seem that to determine the mole percent G + C would only tell you if the bacteria you are working with was possibly in the genus Aeromonas.

The results of all the experiments run on the shad disease organism and the known species made it appear that there is very little difference among any of the three present species. As stated before, biochemically the organisms were extremely similar. Antigenically the organisms seemed to be similar. An antiserum

produced against the shad disease organism no. 17 agglutinated Aeromonas liquefaciens (CDC), Aeromonas hydrophila (CDC) and Aeromonas punctata (CDC) and all the other isolates of the shad disease bacteria. This showed that all of these species possessed at least one major antigen in common. This also was in agreement with a paper by Liu (1961) in which he showed an antiserum prepared against A. liquefaciens could agglutinate the other two motile species of Aeromonas. He also showed that differences between species were not any greater than differences between individual isolates of A. liquefaciens.

Experiments have shown that the bacterium isolated from shad is pathogenic for frogs, salmon, trout, and mice. It produced the symptoms of "red leg" disease in frogs and "red mouth" disease in trout. An infection study performed on frogs showed that A. hydrophila (CDC) could cause the disease "red leg", while A. liquefaciens (CDC) could not. The ability to infect and kill both warm and cold-blooded animals was another feature which the three species of Aeromonas shared. Also there have been reports in the last few years of all three of these species being able to cause gastroenteritis in humans. This again would tend to give credence to the possibility that the three species were actually only one since the problem of identifying the specific species of Aeromonas was so complex. In summary, it seemed that the three species of bacteria

called A. hydrophila, A. liquefaciens and A. punctata were very similar and there seemed to be no clearcut method of distinguishing among the three. It would seem that the three species were actually only one species. Since the organism now classified as A. hydrophila was originally given an illegitimate trinomial it cannot be the name of the one species if these are considered one species. Therefore, it would seem that the species name for this group should be Aeromonas punctata since it was the first motile species of Aeromonas described which was given a legitimate name. However, Ewing (1961) disagreed that this should be the name since he stated that this organism was not adequately described. The SDB isolates were definitely a member of this group and in order to go along with the convention of nomenclature now being applied they shall be called Aeromonas hydrophila since they could give "red leg" disease to frogs and "red mouth" disease to trout.

Bacterial Hemorrhagic Septicemia Occurring in Shad

The disease of shad was termed a bacterial hemorrhagic septicemia characterized by large hemorrhagic areas on the sides of the fish and a reddening of the head and fins. The internal appearance of the fish was surprisingly normal, even in fish that have severe hemorrhagic areas externally. In fish with very severe infections, there were also hemorrhagic areas on the lining of the

peritoneal cavity and the body cavity was often filled with blood. In most cases the causative bacteria, Aeromonas hydrophila, could be found in almost all of the internal organs including the liver, kidney, spleen and blood.

In the area in which the disease occurred, specifically the Smith, Umpqua, Coos, Millicoma and Coquille Rivers, this disease problem seemed to infect only shad and not any other species of fish which were found in these rivers. The infection of shad usually occurred in these rivers at the end of May and in the month of June which was the spawning season for the shad. It was also at this time that the water temperature of these rivers rose above 65°F which was about the temperature necessary for growth and multiplication of the disease-producing organism, Aeromonas hydrophila. It seems that this combination of the lowered resistance of the shad and the water temperatures favorable for bacterial growth gave impetus for the onset of the disease in shad. Since the bacteria could not survive in sea water, the shad were presumed to become infected when they enter the rivers.

Water samples showed that the Coos and Millicoma Rivers were reservoirs for large numbers of the disease producing bacterium. Since these waters were so heavily populated with this bacterium it would seem that this disease problem of shad would be an insoluble one. The only way the disease could be eliminated would

be to completely remove the causative bacteria from the five large rivers in southwestern Oregon. Since this would seem to be an impossibility, the disease of shad will probably continue indefinitely unless the source of the bacterium in the water can be found and eliminated.

SUMMARY AND CONCLUSIONS

1. The disease in shad which was investigated was a hemorrhagic septicemia characterized by large hemorrhagic lesions on the sides and reddening of the head and the fins.
2. Although the causative agent could be cultured from the spleen, kidney, liver, heart and blood of infected shad, internally the shad appeared quite normal except in advanced cases, where the body cavity may be filled with blood and other tissue fluids.
3. The bacterium responsible for the disease was a short, gram negative, motile rod, which has been identified as Aeromonas hydrophila since it could also cause "red leg" in frogs and "red mouth" in trout.
4. Isolates of the shad bacterium taken from the Smith, Millicoma and Coos Rivers were shown to be the same organism.
5. Physiological and biochemical tests conducted on the bacterium isolated from shad and known cultures of Aeromonas liquefaciens, Aeromonas punctata, and Aeromonas hydrophila failed to reveal any major differences between any of these organisms except in their mole percent guanine + cytosine ratios which were distinct for all three species.

6. Results of experimentation and a review of the literature on the genus Aeromonas indicated that the present three motile species of Aeromonas were actually only one distinct species and that the species name should be Aeromonas punctata.

7. Antiserum against the shad disease bacterium (Smith River isolate no. 17) caused agglutination of cultures of Aeromonas hydrophila, Aeromonas liquefaciens, and Aeromonas punctata as well as all other strains isolated from diseased shad.

8. The shad bacterium was pathogenic for both warm and cold-blooded animals, which was characteristic of the genus Aeromonas.

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