

AN ABSTRACT OF THE THESIS OF

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Title: A STUDY OF CORYNEBACTERIAL KIDNEY DISEASE OF  
SALMONID FISHES

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Abstract approved: \_\_\_\_\_

Dr. ~~J. L. Fryer~~

This study was undertaken to compile a comprehensive review of the literature and to gain a better understanding of the host-parasite relationship in corynebacterial kidney disease of salmonid fishes. An indirect fluorescent antibody technique was developed which allowed differentiation of intra- and extracellular bacteria. This technique was used in combination with phase contrast microscopy to study phagocytic interactions between salmonid cells and kidney disease bacilli. Contrary to observations made at four to five passages (Fryer, 1977), STE 137 cells (used from passages 150 to 156) did not phagocytize the bacteria. Under similar experimental conditions, the bacilli were avidly phagocytized by mouse macrophages. Potential explanations for the loss of phagocytosis in the STE 137 line were explored.

Primary monolayer cell cultures from kidney and spleen, and leucocyte cultures were established and grew well, but

failed to phagocytize the kidney disease bacillus. Differential counts of blood films from normal, hatchery-raised, 12.5 to 17.5 cm brook trout (Salvelinus fontinalis) showed:  $82.4 \pm 0.9\%$  lymphocytes,  $12.1 \pm 0.9\%$  thrombocytes,  $2.4 \pm 0.2\%$  neutrophils, and  $2.6 \pm 0.3\%$  blast forms.

The histopathology of corynebacterial kidney disease was described by organ system (kidney, liver, pancreas, gut, heart, gills, brain). The typical lesion was a distinct, localized growth of bacteria and central mass of amorphous, granular debris surrounded by a mixed, non-specific inflammatory response consisting of lymphocytes, some degenerating neutrophils, and macrophages. Very few polymorphonuclear leucocytes were observed. The lesions are distinguished from the classic focal granulomas of piscine tuberculosis or flavobacteriosis. The disease is described as a "bacteremia characterized by a systemic, diffuse...histiocytic inflammation" (Wolke, 1975).

The ultrastructure of the kidney disease bacterium and its relationship to host tissues were studied via electron microscopy. The mean length was  $0.65 \mu\text{m}$  and the mean width was  $0.39 \mu\text{m}$ . The mean cell wall thickness was  $16.1 \text{ nm}$  and the mean thickness of the cytoplasmic membrane was  $8.8 \text{ nm}$ .

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# A STUDY OF CORYNEBACTERIAL KIDNEY DISEASE OF SALMONID FISHES

## I. INTRODUCTION

Corynebacterial kidney disease (BKD) is a chronic, systemic infection which often causes high mortality among hatchery-raised salmonid fishes. The etiological agent of the disease is a slow-growing Gram positive diplobacillus with an absolute requirement for cysteine; it is classified as an unspciated Corynebacterium on the basis of present information.

Although BKD was first reported in the United States in 1935, little is known about the bacterium or its mode of pathogenesis. Workers have reported both intra- and extra-cellular bacteria in vivo, but the role of phagocytosis remains undefined. The relationship between intra- and extra-cellular growth is significant because it may hold the key to the epizootiology and chemotherapy of this disease. In addition, the pathology of this disease is poorly understood. Because BKD is chronic and debilitating, secondary infections or parasitic infestations frequently occur, complicating the pathological picture.

The purpose of this study was two-fold: (1) to compile a comprehensive review of the literature summarizing current knowledge of the kidney disease bacterium and disease processes, and (2) to gain a better understanding of the host-

parasite relationship between the infected fish and the bacterium. An indirect fluorescent antibody (IFA) technique was developed to study the intracellular growth kinetics of the kidney disease bacilli in salmonid cell cultures. Phagocytic interactions between fish cells and the bacilli were examined using the IFA technique and phase contrast microscopy. The histopathology of the disease in yearling brook trout (Salvelinus fontinalis) was described and compared with the histology of normal brook trout. In addition, the ultrastructure of the bacterium and of diseased tissues was described.



## II. REVIEW OF LITERATURE

### Corynebacterial Kidney Disease

#### Historical Perspective

The first recorded outbreak of BKD occurred in Scotland and was reported in the Second Interim Report of the Furunculosis Committee (Mackie et al., 1933). During the spring and early summer of 1930, 1931 and 1932, small necrotic lesions were found on the spleens of fourteen salmon from the Aberdeenshire Dee and one from the River Spey. Although small Gram positive bacilli could be demonstrated in material from these lesions, attempts at culturing the organism were unsuccessful. When splenic emulsions from infected fish were injected into brown trout (Salmo trutta), BKD developed and mortalities occurred in 3 to 5 weeks; therefore, it was inferred that this organism was the etiological agent of the disease.

In June, 1934, the first recorded outbreak of BKD in North America occurred in a Massachusetts fish hatchery. Belding and Merrill (1935) described the gross pathology (external and internal lesions), etiology, and epidemiology in their preliminary report.

A serious epizootic of BKD occurred in brook (Salvelinus fontinalis), rainbow (Salmo gairdneri) and brown trout populations in California during 1935 and 1936; the disease

subsequently appeared in two California fish hatcheries and two commercial hatcheries (Wales, 1977).

Earp et al. (1953) attributed high mortalities at the following Washington state fish hatcheries to BKD:

1946 - 1947 Kalama

1948 - 1949 Green River, Skagit, Deception Pass

1949 - 1950 Green River, Issaquah, Bowman's Bay

1951 - 1953 Klickitat

This was the first report of BKD in the state of Washington and the first time the disease had been observed in chinook (Oncorhynchus tshawytscha), coho (Oncorhynchus kisutch) and sockeye salmon (Oncorhynchus nerka).

In 1950, BKD was identified in young salmon from Oregon state hatcheries by T. B. McKee (Rucker et al., 1951). Since then, BKD has been detected in hatchery-reared salmonids throughout the United States, Canada, England, Scotland and recently, in France (de Kinkelin, 1974), Japan (Watanabe, 1975) and Italy (Ghittino, 1977).

BKD in wild salmonids has been presumptively diagnosed in Canada (Pippy, 1969) and the United States (Maclean and Yoder, 1970). Confirmed diagnoses, involving the isolation of the bacterium from wild fish, have been reported in the United States by Wood and Wallis (1955) and in Canada by Evelyn et al. (1973).

## Etiological Agent

### Morphology

The organism is a Gram positive diplobacillus. It is non-motile, non-capsulate, non-acid fast and non-sporulating (Smith, 1964). The dimensions reported vary, ranging from 0.3 to 0.5  $\mu\text{m}$  by 0.5 to 1.0  $\mu\text{m}$  (Ordal and Earp, 1956; presumably, stained organisms), to 1.5  $\mu\text{m}$  by 0.6 to 1.0  $\mu\text{m}$  (Smith, 1964; wet mount), to 0.5 to 0.7  $\mu\text{m}$  by 0.8 to 1.0  $\mu\text{m}$  (Bell, 1961, Gram stain). The organism frequently occurs in pairs (diplobacilli) and chains are sometimes observed in old cultures. Ordal and Earp (1956) noted that pleomorphism is common in intracellular forms in infected tissues and in some cultures. Smith (1964) reported some metachromatic granules and "Chinese letter formation," but no evidence of branching. All three of these characteristics are common among the *Corynebacteria*. The organism is classified as an unspiciated Corynebacterium on the basis of present information.

### Cultivation

Early attempts to cultivate the kidney disease bacillus on conventional media proved unsuccessful (Mackie et al., 1933; Schäperclaus, 1954). Belding and Merrill (1935) reported that several types of special enrichment media incubated under aerobic and anaerobic conditions gave

discouraging results. Most preparations failed to grow or were heavily contaminated with other bacteria. Finally, four cultures of an organism resembling the bacterium observed in kidney smears were obtained. These colonies were described as "delicate, slowly growing, slightly opalescent" and appeared on hormone agar at room temperature after 2 days. Growth was also observed on meat infusion agar, meat extract agar, defibrinated blood agar and in meat infusion broth. The organism was a Gram positive, non-acid fast, non-capsulated motile bacillus, 1 to 4 m in length with an optimum growth temperature of 22 to 26 C. The four strains isolated were serologically identical, but differed slightly in their fermentation of carbohydrates.

Results of experimental infections were disappointing. Twenty healthy brook trout were injected intramuscularly with saline suspensions of two of the four cultures isolated. Death occurred from 33 to 64 days post-injection, with an average time of death of 47 days. Two small white hard areas were present in the kidney of one trout. In two other trout, the kidney was badly congested and two more showed infection in the hind-gut. Bacilli could be demonstrated in smears from the site of inoculation in five cases and from the heart blood in one case. Cultures of organisms similar to those injected were obtained from the site of inoculation in four cases.

The organism isolated by Belding and Merrill did not produce the typical disease when inoculated into experimental fish. In addition, it differed in size from the organism observed in natural infections. More recent attempts at culture are at variance with Belding and Merrill's reports of mobility and culture requirements (with respect to suitable media and temperature range).

Belding and Merrill recognized the questionable role of the bacillus they isolated in the etiology of BKD. In their discussion and summary, they stated that BKD may be a nutrition-related metabolic disease but that the etiology was most probably bacterial, although Koch's postulates were not fulfilled.

Earp et al. (1953) were the next group of researchers to attempt cultivation of the kidney disease bacillus. Incubation temperatures of 8, 15, and 22 C, a pH range of 7.3 to 7.5 and "aerobic and anaerobic conditions (with and without partial pressures of carbon dioxide) [sic]" were routinely used in an effort to culture the organism. Dilute media with reduced agar concentrations which had been used successfully in the isolation of Flexibacter (Chondrococcus) columnaris and Cytophaga psychrophila did not support growth of the kidney disease diplobacillus. Materials such as fresh rabbit blood (10 to 20%), human blood (10 to 20%), extracts of fish tissue, soluble starch (1%), sterile beef serum (10%), glucose, maltose, sodium acetate, sodium

chloride, casein hydrolysate, cysteine, and yeast autolysate were incorporated into basal media as adjuvants without success. Attempts at isolation using enriched media commonly employed in the isolation and culture of fastidious human pathogens were also unsuccessful.

The development of microcolonies was observed when peritoneal fluid from infected fish was streaked on medium containing (a) 10% fish extract, 0.5% glucose, 0.25% yeast extract, 10% sterile beef serum, 0.9% Difco agar; or (b) meat infusion, 10% fish extract, 0.25% yeast extract, 10% sterile beef serum, 0.9% agar. After 14 days of incubation, small white areas became visible on plates which had been incubated "aerobically and anaerobically...(partial pressure carbon dioxide) [sic]" at 15 C and 20 C. When agar block impression slides prepared from these areas were fixed in situ with Bouin's fluid or osmic acid vapor and stained by Gram's method or with Victoria blue 4R, microcolonies of several hundred typical diplobacilli were observed. Although growth of the bacterium on these media was not abundant, it paved the way for rudimentary cytological studies.

Subsequently, Earp et al. found that the bacterium could be cultured in suspensions of minced chick embryo tissues. Ten to 12 day old embryos were harvested aseptically, mascerated, and suspended in an equal volume of sterile Ringer's solution. The inoculum was obtained from infected kidney tissue or intraperitoneal serous fluid in

fish exhibiting obvious distention of the body cavity. Cultures were incubated aerobically at 15 C, with growth occurring in less than a week.

Plate cultures using minced chick embryo tissue imbedded in 1% Difco agar showed growth of the diplobacilli adjacent to bits of chick embryo tissue. However, the diplobacilli did not grow on sterile filtrates from suspensions of chick embryo tissue.

During the summer of 1952, Earp et al. made renewed attempts to cultivate the kidney disease bacterium on media free from living tissues. A wide variety of culture media were inoculated and subjected to prolonged incubation of 15 C. Although several media showed some evidence of growth, the best growth appeared on Dorset's egg medium (used in the cultivation of Mycobacterium tuberculosis). After approximately 2 months of incubation at 15 C, well developed, raised colonies were observed on this medium. These colonies contained short Gram positive rods, frequently occurring in pairs, which were morphologically identical with those occurring in the lesions of diseased fish. The bacteria were transferred on Dorset's medium, and after five serial subcultures were tested for virulence in young salmon. Typical kidney disease was produced in all fish and the characteristic Gram positive diplobacilli were seen in large numbers in smears from the organs. In addition, the bacterium was reisolated from a number of fish taken either

in a moribund condition or shortly after death, thus fulfilling Koch's postulates.

However, standard Dorset's medium proved unsatisfactory for the routine isolation of the kidney disease bacterium during natural outbreaks of the disease. A modified Dorset's medium fortified with cysteine, tryptone, and yeast autolysate and subjected to greater heat treatment than usual was developed. Inocula taken from the lesions of infected fish regularly resulted in good growth of the bacterium on this modified medium. Good growth of serial subcultures was normally obtained in 3 to 4 weeks at 15 C.

In 1956, Ordal and Earp reported the results of a further search for a suitable plating medium. Growth occurred in 7 to 10 days on tryptose base blood agar with 0.05 to 0.1% cysteine incubated at 17 C. Growth was further improved by increasing the amount of human blood from 10 to 20% and adding 0.05% yeast extract. The formulation of the medium follows:

<u>Component</u>	<u>Percent</u>
tryptose	1.0
yeast extract	0.05
beef extract	0.3
cysteine hydrochloride	0.1
sodium chloride	0.5
human blood (by volume)	20.0
agar	1.5

In Evelyn's modification, fetal calf serum (10 to 20%) is substituted for the human blood. This medium (cysteine serum agar) is currently used for the routine isolation of



the kidney disease bacterium from infected fish and for maintenance of stock cultures in many laboratories.

It is not always possible to isolate the kidney disease bacillus on cysteine serum agar. During a 4 yr period, Smith (1964) isolated the organism from only 23 of a group of 53 fish suffering from kidney disease. Often, other piscine pathogens are present and kidney disease cultures are quickly overgrown by these faster growing contaminants. In other cases, the presence of the organism can be demonstrated by organ smears, serologically or by the characteristic pathology, but the organism cannot be cultured.

In 1959, Wolf and Dunbar (1959a) suggested the use of Mueller Hinton agar with the addition of 0.1% cysteine for the cultivation of the kidney disease diplobacillus. However, several investigators have questioned the efficacy of this medium (Smith, 1964).

It has been suggested that lipids may be essential for the cultivation of the kidney disease bacilli. However, lecithin, cardiolipin, and cholesterol added in separate tests to Ordal and Earp's blood agar, Ordal and Earp's basal agar (minus blood) and a mineral salt medium had no effect on growth (Ross, 1970). Pantoyl lactone added to Ordal and Earp's blood and basal agar also had no effect on growth. Efforts to isolate a growth factor from the kidney disease bacilli have been unsuccessful to date (Cisar, 1969; Ross, 1970).

Thus, it appears that a reliable medium for the isolation of the kidney disease bacillus has yet to be found. Studies are underway at the Pacific Biological Station in Nanaimo, British Columbia to elucidate the role of cysteine in the bacterium's metabolism with the hope of developing an improved culture medium (Bell, 1977).

The best description of colonial appearance is in Smith's paper, "The Occurrence and Pathology of Dee Disease," page 6 (1964):

On Ordal and Earp's agar the colonies are white, round, raised and shiny and of varying size. After prolonged incubation the growth becomes creamish-yellow but the colonies are still variable in size. The smaller ones tend to give the surface of the media a ground glass appearance. The growth has an elastic consistency.

On Mueller Hinton plus 0.1% cysteine the growth is very scanty. It is white, shiny, translucent and watery in consistency. There is not much evidence of colony formation.

On Loeffler's coagulated serum the organism produces a creamish growth with a matt surface.

On Dorset's egg medium the growth in three to four weeks at 15 C appears as a raised, smooth, shiny, yellow layer.

On Ordal and Earp's medium without agar, i.e. broth, the growth is found adhering to the walls of the container at the surface of the broth. It is creamish-yellow in colour.

### Metabolism

The organism grows very slowly at 5 C, well at 15 and 18 C and very slightly at 22 C; it does not grow at 37 C. The kidney disease bacillus does not grow on semi-solid sugar agar containing Ordal and Earp's base without blood, Hiss's serum water sugars, or peptone water sugar media (Smith, 1964). The organism grows reasonably well when gelatin is substituted for agar in Ordal and Earp's cysteine blood agar. However, liquefaction of the gelatin does not occur even after prolonged incubation of up to 6 months (Smith, 1964). Growth and proteolysis without any apparent pH change have been observed in litmus milk made up with 25% Ordal and Earp's basal broth. Catalase production can be demonstrated on Mueller-Hinton agar which, unlike blood agar, does not contain catalase. Attempts to use the filter paper plaque technique have been unsuccessful, so to date, nothing is known of the organism's enzyme system. Suspensions of the kidney disease bacterium undergo rapid auto-agglutination; this is effectively prevented when the cells are heated to 63 C for 45 min.

### Pathogenicity

Bacterial kidney disease is generally a chronic, systemic infection of both juvenile and adult salmonids, although acute outbreaks have been reported at moderate temperatures (13-18 C). It has been reported in three

species of trout by Snieszko and Griffin (1955): brook, brown and rainbow trout, and five species of salmon: Atlantic salmon (Salmo salar) by Smith (1964); chinook salmon by Wood and Wallis (1955); sockeye and coho salmon by Earp et al. (1953); pink salmon (Oncorhynchus gorbuscha) by Bell (1961). The disease is more severe in salmon, although no comparative studies with respect to species susceptibility have been made. Of the three trouts affected, BKD is most severe in brook trout, least severe in rainbow trout. Hunn (1964) found that the ratio of diseased males to diseased females (brook trout) was two to one.

Although the incubation period of BKD is unknown, it is believed that the disease develops slowly. In transmission experiments, the time from exposure to mortality has ranged from 12 days to over 3 months (Ordal and Earp, 1953; Wolf, 1966) depending on the method of exposure and the concentration of bacteria in the infective dose.

There appears to be a great deal of variation in the virulence of different strains of the kidney disease bacterium. Wolf and Dunbar (1959a) found that an eastern strain produced higher mortalities, larger lesions and had a shorter mean time from inoculation until death than a western strain when comparative studies of virulence were conducted in brook trout. Other investigators have reported variations in the size of inocula constituting an infective dose; some of these variations may be due to the number of

passages on artificial media since there is a correlation between loss of virulence and extended passage on artificial media.

If an inoculum is prepared from frozen infected organs, the virulence can be affected by the length of time the tissues have been stored. Ordal and Earp (1956) found that organisms retained their virulence for up to 4 months when infected organs were held at temperatures below 0 C. This is in agreement with Smith (1964), who stated that the kidney disease bacilli did not cause mortalities when stored for longer than 14 to 16 weeks at -17 C.

Kidney disease is remarkable in that it occurs over a wide range of water temperatures. Belding and Merrill (1935) were the first to report the seasonal occurrence of BKD and the relationship of increased mortalities to changing water temperatures. Earp et al. (1953) stated, "Water temperature is a major factor in determining the severity of the disease." They found that most outbreaks of BKD occurred during the declining water temperatures of autumn and winter (-1.1 C to 4.4 C), and that the disease produced a slow, steady death rate during periods of low water temperature. However, explosive epizootics occurred from mid-July to September when the water was warmer (12.7 C to 18.3 C). According to Snieszko and Griffin (1955), losses due to BKD began in April and May, peaked in June and gradually subsided during the summer, presumably following

a curve in rising water temperatures. Wood and Wallis' data (1955) show increased salmon mortalities during periods of declining water temperature although they do not discuss this in their paper. Wolf and Dunbar (1959b) found that two strains of the organism (one from the eastern United States, the other from the west) were more virulent at 7 C than at 12.5 C when tested in eastern brook trout under experimental conditions. Smith (1964) reported that the only recorded epizootic in Scotland occurred when water temperatures rose rapidly in early summer.

In a comprehensive study entitled "Temperature, Infectious Diseases, and the Immune Response in Salmonid Fish," Fryer et al. (1976) found that temperatures of 6.7 C to 12.2 C produced a high percentage (78% to 100%) of fatal infections in juvenile coho salmon and steelhead trout inoculated intraperitoneally with kidney disease bacilli. The mean time from inoculation until death was 22 to 34 days at 15 C, 61 to 73 days at 6.7 C, and 87 to 98 days at 3.9 C. The authors found that mortalities declined progressively at temperatures above 12.2 C. In these experiments, the relationship between water temperature and log time of death was linear; increasing temperature was associated with shorter survival and higher mortality.

In further studies, Sanders et al. (1978) reported similar results in coho and sockeye salmon and steelhead trout. However, these authors found no significant

suppression of the disease in sockeye salmon at 17.8 or 20.5 C. This was attributed to the greater susceptibility of sockeye salmon to BKD.

Recently, Wedemeyer (1976) has reported the activation of a subclinical corynebacterial kidney infection in a population of smolting coho salmon subjected to population densities of 1 lb/ft<sup>3</sup> or more.

Although little is known about the relationship between nutritional factors and BKD, some evidence indicates that the incidence of chronic disease may be related to dietary and environmental factors.

According to Wood (1968), feeding dry diets containing corn gluten increased BKD mortalities in Pacific salmon while substituting cotton seed meal for corn gluten conferred protection. Wedemeyer and Ross (1973) found that the disease incidence was approximately the same for fish fed the Abernathy dry ration made up with either corn gluten or cottonseed meal, but that the non-specific stress of infection was more severe in fish fed the corn gluten.

Woodall and LaRoche (1964) studied the iodine requirements of chinook salmon fingerlings raised in water containing 0.2 µg iodine/liter. The fish were fed a low iodide basal ration with added sodium iodide to give 0.1, 0.6, 1.1, 5.1, and 10.1 µg iodide/g dry diet for 24 weeks. There were no mortalities for nearly 6 months. However, a persistent daily mortality in the low iodide group began in mid-

January and within 6 weeks over one-half of the fish in this group died. At the termination of the experiment in late April, only 18% of the original population in this group remained. Cumulative mortalities in other groups were 24%, 22%, 5%, and 4%, respectively. Postmortem examination revealed that fish in all but group 5 (10.1  $\mu$ g iodide) were infected with BKD.

Although the significance of increased mortalities in fish fed lower levels of iodide is not fully understood, it has been hypothesized that anadromous teleosts kept in fresh water for prolonged periods of time are under increased osmotic stress which demands additional thyroid hormones (Hoar, 1952). The kidney, with its important role in osmotic regulation, may have been stressed in those fish fed insufficient iodide to meet the increased challenge to the thyroid, thus permitting colonization by the kidney disease bacteria.

Warren (1963a) noted that the severity of kidney disease is inversely related to total water hardness. He reported an increased incidence of kidney disease at hatcheries with "soft" water, i.e. a mean total hardness below 13 ppm calcium carbonate (range 4.0 to 44.9 ppm). The reason for this relationship is not clear, but Warren postulated that a parasitic vector may flourish in softer waters or that water hardness may affect the mechanism by which salmonids resist infection.



Workers at the Eastern Fish Disease Laboratory have studied further the relationship between severity of kidney disease and water chemistry. Wold (1963) fed carcasses of fish which had died from BKD to both brook trout and rainbow trout fingerlings held in tanks of "hard" spring water (2,500 ohms resistivity) and the same water diluted with deionized water to "soft" quality (25,000 ohms resistivity). Contrary to expectations, both brook trout and rainbow trout became infected and died in the hard water, but only rainbow trout died in the soft water.

A severe outbreak of BKD in yearling brook trout occurred at Leetown, West Virginia hatcheries during the winter of 1963-1964 (Wold, 1963). Since Leetown is a "hard" water station, it is evident that water hardness is not the sole limiting factor in BKD. These observations on the mineral content of water and diet and the incidence of BKD may provide new insights into the study of piscine resistance to communicable disease.

Undoubtedly, genetic factors play a large role in determining susceptibility to the disease. Suzumoto (1977) studied the relative resistances of three transferrin genotypes (AA, AC, and CC) of juvenile coho salmon to BKD. He found that the AA genotype was the most susceptible and the CC genotype the most resistant. The addition of exogenous iron did not appear to increase the pathogenicity of the bacterium. A decrease in hemoglobin, packed cell

volume, total plasma protein, plasma iron, and plasma corticoid levels was observed during the course of the disease.

It is not uncommon to find fish infected with the kidney disease bacterium and another piscine pathogen. Disease processes or organisms which have been reported in conjunction with diagnosed kidney disease include: Pseudomonas hydrophila (Rucker et al., 1951), an unidentified fluorescent pseudomonad (Rucker et al., 1951), acid fast bacteria (Wood and Wallis, 1955), furunculosis (Smith, 1964), Saprolegnia sp. (Earp et al., 1953; Wolf, 1959a), Ichthyophonus sp. (Snieszko and Griffin, 1955; Smith, 1964), infectious pancreatic necrosis (Yamamoto, 1975), Gyrodactylus sp. (Allison, 1961), Schizamoeba salmonis (Wood and Yasutake, 1956a), Nanophyetus salmincola Chapin (Wood and Yasutake, 1956a), Ceratomyxa sp. (Wood and Wallis, 1955), Acanthocephala sp. (MacLean and Yoder, 1970), Cystidicola sp. (MacLean and Yoder, 1970), and lymphosarcoma (Ehlinger, 1963).

### Epizootiology

Little is known about the epizootiology of BKD. It has been postulated that infected or "carrier" fish serve as the reservoir of infection. Brood stock in areas where BKD is enzootic are often infected with the chronic form of the disease.

Transmission via infected eggs has also been suggested, and there is an instance in the literature where BKD developed when eggs were transported from a hatchery where the disease had been endemic for many years to a hatchery where it had never been reported (Allison, 1958). In addition, there is some immunological evidence indicating egg transmission (Banowetz, 1975).

Earp et al. (1953) were the first group of workers to suggest a parasitic vector. At that time, a relationship was thought to exist between kidney disease and salmon poisoning of dogs and foxes, hence the idea of a common vector. Snieszko and Griffin (1955) noted that BKD was more frequent in hatcheries receiving water from open streams rather than from a spring. They theorized that there was a direct correlation between the incidence of BKD and the concentration of carrier-parasites. More recent studies have shown that the salmon poisoning fluke (Nanophyetus salmincola Chapin) is limited to a few river systems in the Pacific Northwest. Thus, it is either not involved as a carrier or more than one carrier exists. More work needs to be done in the area of salmonid host-parasite relationships with reference to BKD.

Unlike ulcer disease or furunculosis, there is no experimental evidence for transmission of the kidney disease organism from fish to fish through the water (Smith, 1964). Allison (1961) found no indication of the spread of the

disease when brook trout from an infected stock were released into streams and lakes where native trout were present. And, Snieszko and Griffin (1955) were unable to transmit BKD from inoculated to healthy trout via contact.

Efforts by workers to transmit BKD have met with variable success. Belding and Merrill (1935) reported 100% mortality in 18 to 25 days when healthy brook trout were injected intramuscularly with purulent material from the kidney abscesses of diseased fish. Earp et al. (1953) were able to transmit BKD to chinook, sockeye and coho salmon and to rainbow trout by injection. In addition, BKD was transmitted to chinook and fingerling sockeye salmon by two or more feedings of infected viscera from fish which had died from the disease. Snieszko and Griffin (1955) stated that attempts to transmit BKD by contact or feedings were unsuccessful. These early experiments were often inconclusive due to the difficulty of obtaining stocks free from BKD and the tendency to terminate such experiments too quickly without allowance for a long incubation period.

In 1956, Ordal and Earp reported the fulfillment of Koch's postulates. In these transmission experiments, 0.05 ml of a dilute suspension of the diplobacilli in saline were injected intraperitoneally into yearling sockeye salmon; death occurred between days 12 and 23 and days 12 and 24 in two experiments.

Wood and Wallis (1955) found that feeding the infected flesh and viscera of adult spring chinook salmon to young salmon fingerlings for a 41 to 52 day period resulted in nearly 100% transmission of the disease to the fingerlings in all trials. Although BKD occurred among control fish in one of the trials, control lots in two others were found to be free from the disease during a reasonable period of observation.

Snieszko and Griffin (1955) were not able to transmit BKD to eastern brook trout by 3 weeks of contact with infected fish followed by a single feeding of infected tissues.

Wolf and Dunbar (1959a) attempted to transmit BKD to eastern brook trout orally and by abrasion. The bacterium was fed alone, with glass splinters and with bile salts (to increase intestinal permeability by decreasing gastric hydrogen chloride secretion). The oral route proved singularly unsuccessful, but some deaths did occur after manual and auto-abrasion. However, Smith (1964) states that noticeable wounds are seldom seen on Atlantic salmon with BKD.

Because feeding and contact cannot be relied upon to uniformly infect large numbers of fish with kidney disease, workers have had to resort to the severe and highly artificial method of parenteral injection to provide fish for experimental work.

## Pathology

Clinical signs of BKD are variable and range from no external symptoms to exophthalmos, abdominal distention, swollen raised areas or hemorrhagic spots (petechiae) in the musculature, and small white to cream colored dermal blisters or "blebs," often in the region of the lateral line. Not all of these symptoms occur in each infected fish or in every outbreak of the disease.

Spieszko and Griffin (1955) were the first workers to record an extensive microscopic description of the disease. They described the tissue changes as granulomas similar to those seen in the various mycoses and tuberculosis of mammals. These workers reported typical well-defined tubercles surrounding the visceral organs and frequent giant cells.

Wolke (1975) characterized the disease histopathologically as a "systemic diffuse granulomatous (histiocytic) inflammation," differentiating it from the classic focal granulomas of fungal or parasitic etiology.

Wood and Yasutake (1956a) compared the eastern and western forms of the disease, concluding that histologically, they were nearly identical. They reported granulomatous nodules with intra- and extra-cellular bacteria, infrequent giant cells, frequent involvement of the reticulo-endothelial system and cytoplasmic inclusion bodies in liver parenchymal cells and pancreatic acinar cells. Marked endothelial cell proliferation was most commonly seen in

kidney, liver, spleen, skeletal musculature and gills, although any organ may be involved. The inflammatory cells observed were large (20  $\mu$ m), polygonal, and often contained eosinophilic granular cytoplasm with an eccentric vesicular nucleus (Wolke, 1975). He states that "the granular appearance of the inflammatory cell cytoplasm suggests that these cells are serving a phagocytic function."

Internally, the organisms appear to have a predilection for the kidney, so that this organ is most commonly involved. However, Wood and Wallis (1955) reported that the liver was the most affected organ in outbreaks among chinook salmon, while Bell (1961) reported spleen and liver more commonly involved in pink salmon. Smith (1964) stated that petechial hemorrhages of the muscles lining the peritoneum were the most characteristic lesions in Atlantic salmon.

In the early stages of kidney disease, one or more small grayish-white areas 1 to 5 mm in diameter may be observed beneath the capsule of the kidney on the ventral side. These areas usually contain a thick, white, finely granular fluid consisting of bacteria, leucocytes and cellular debris. As the disease progresses, the number and size of these abscesses increase and the kidneys appear swollen. In advanced stages of the disease, these abscesses coalesce and the entire kidney is grayish-white, enlarged and necrotic.

Wood and Yasutake (1956a) postulated that the hemato-poietic tissue of the kidney was the first organ affected since it was always in an advanced state of destruction in their specimens. They reported that in advanced cases of the disease, the posterior portions of the kidney were destroyed by necrosis and suppuration, whereas in the anterior portion, the granulomas progressed to extensive fibrotic lesions which enveloped the renal tissue.

Belding and Merrill (1935) reported that the liver in infected fish had a discolored or mottled appearance and that the surface was often studded with small, white "pin point" abscesses similar to those seen in the kidneys. Wood and Wallis (1955) observed white watery pustules filled with a turbid fluid containing large numbers of the organism, caseous necrotic areas and occasional large abscesses on the livers of infected chinook salmon.

Microscopically, Wood and Yasutake (1956a) noted granulomatous nodules in the connective tissue stroma which increased in size as the disease progressed, gradually forcing the liver tissue aside. Necrosis of the parenchymal cells appeared minor, but Gram negative cytoplasmic inclusions were present. These inclusions occasionally contained Gram positive globules and appeared morphologically identical to inclusions observed in the pancreatic acinar cells.



Belding and Merrill (1935) reported an occasional stippling of the surface of the spleen with minute abscesses and perisplenitis. Wood and Wallis (1955) and Bell (1961) observed lesions on the spleen similar to those reported for the kidney. Smith (1964) described the splenic lesions as creamish-white, varying in size from pinhead to 4 mm in diameter, ranging in number from very few to many, normally soft although they were occasionally hard and almost caseous, necrotic, and distributed throughout the spleen. Wood and Yasutake (1956a) reported that the splenic tissue was gradually replaced by proliferating granulomatous tissue until its normal structure was unrecognizable.

Congestion and swelling of the hind-gut with distention of the blood vessels was a common finding and frequently, the intestine was filled with a yellow, purulent fluid (Snieszko and Griffin, 1955). Petechial hemorrhages of the muscle lining the peritoneum (Smith, 1964) and distention of the peritoneal cavity by an accumulation of amber-colored, semi-transparent fluid containing blood cells and small gram positive diplobacilli were also observed frequently (Belding and Merrill, 1935; Snieszko and Griffin, 1955). Snieszko and Griffin (1955) noted small papillae-like lesions projecting from the outer walls of the stomach and esophagus in the early stages of the disease, while in more advanced cases, the intestines and pyloric caeca appeared to be encased in a glistening white tissue

resembling the mature testis.

Microscopically, the histopathology of the digestive tract was characterized by a marked eosinephilic inflammation of the lamina propria (Wood and Yasutake, 1956a). Normally, a layer of eosinophilic granular cells (the stratum granulosum) occurs in the submucosal layers of the stomach and intestine. These workers also noted a granulomatous inflammation in the large intestine involving subcutaneous tissues and a "solid core of proliferating fibroblastic tissue" in the mucosal folds. In later stages, an acute, extensive peritonitis with many lymphocytes and polymorphonuclear leucocytes was characteristic. The lesions spread throughout the visceral fat and pancreatic tissue and the inflammation continued to the chronic granulomatous stage. Occasionally, necrosis of the entire pancreatic system occurred.

Many workers today feel that Wood and Yasutake's description of the histopathology of corynebacterial kidney disease was complicated by the presence of visceral granuloma in the trout they examined (Bullock et al., 1971). Although these two diseases do occur simultaneously, there is not relationship between them. The lesions of BKD and visceral granuloma are similar upon gross examination, but the histopathology of the two conditions is very different. A brief description of visceral granuloma is presented here for comparative purposes.

Visceral granuloma was originally described by Young and Olafson (1944) as a neurilemmoma in brook trout from two New York hatcheries. Wood et al. (1955) described material from three Federal hatcheries as a mycosis-like granuloma on the basis of yeast-like structures in fixed material. Wood and Yasutake (1956a) discussed the gross and histologic aspects as well as possible causes of visceral granuloma. These workers felt that the disease represented an infectious process although they were unsuccessful in isolating an etiological agent. Snieszko (1961) proposed visceral granuloma as the most appropriate name since no infectious agent could be demonstrated. Visceral granuloma appears to be diet-related; Dunbar and Herman (1971) have shown a strong correlation between the incidence of the disease and the presence of cottonseed meal in the diet.

In the early stages of the disease, small papillary lesions can be felt on the dorsal surface of the stomach upon gross examination. These lesions enlarge and proliferate, and can spread to the visceral fat, giving it a testicular-like appearance. As the disease progresses, grayish-white streaks and spots appear in the kidney. These become more prominent and the kidney becomes swollen to varying degrees. The lesions consist of semifluid deposits of calcium salts with a gritty texture.

Histologically, visceral granuloma begins as an inflammatory infiltrate in the stomach wall extending into

the submucosa and along the muscle septa. This is followed by the appearance of Langerhans-type giant cells. These frequently contain concentric deposits of amorphous material which resembles the Schaumann bodies seen in diseases such as sarcoidosis. These concretions also occur extracellularly and are sometimes multilobed. Gradually, the giant cells and concretions are walled off by connective tissue, macrophages and epithelioid cells, becoming more and more tubercle-like. Several of these lesions may coalesce to form a single, large mass; eosinophils are sometimes associated with these centers. The disease follows a similar course in the kidneys, accompanied by tubular dilation and degeneration, the formation of tubular casts, and in extreme cases, the destruction of the majority of functional kidney tissue. Bacteria are not associated with the lesions of visceral granuloma.

Exophthalmos resulting from accumulation of fluid behind the eye is often one of the external symptoms associated with kidney disease. Wood and Yasutake (1956a) found that exophthalmos during the terminal stages of the disease was caused by granulomatous tissue development behind the eye.

Hendricks and Leek (1975) have found that massive postorbital granulomatous lesions resulting in exophthalmos can occur in chinook salmon before kidney disease becomes systemic. They described the postorbital lesions grossly

as a mass of grayish-white tissue with small amounts of bloody, purulent fluid in the ocular cavity. The ocular muscles were in various stages of degeneration and necrosis, and the eye was swollen or ruptured, with subsequent loss of the lens.

Histologically, the lesion presented as a chronic granuloma, with proliferating macrophages and "distinct to diffuse nodules of epithelioid tissue" predominating. Many mature macrophages as well as some neutrophils and lymphocytes were observed; some of these had phagocytized large numbers of diplobacilli.

Positive identification of the organism was made with the Ouchterlony gel diffusion test using antibody against known kidney disease antigen (Chen et al., 1974) and via examination of imprints of postorbital tissues, kidney, liver and spleen. Fresh imprints of the postorbital tissues revealed large numbers of Gram positive diplobacilli. However, bacteria could not be demonstrated in imprints from the kidney, liver and spleen of these fish, and were present only in the swollen kidneys and spleens of terminal cases.

The authors have hypothesized that the eye may serve as a route of entry for the pathogen. Apparently, the fish are capable of isolating the bacteria in the postorbital site for considerable periods of time. Thus, fish with postorbital kidney disease may serve as carriers. The

authors stressed the importance of recognizing this site of entry since in this study, antigen could not be detected in the kidneys of fish exhibiting gross exophthalmos.

Nearly all the cases observed by Belding and Merrill (1935) showed some stage of pericarditis, ranging from "slight fibrous changes to an advanced fibrinous pericarditis with plastic adhesions and purulent effusion." Pericardial effusion varied from white and turbid to a purulent fluid, causing marked distention of the pericardium in some cases. The visceral and parietal pericardium were pale and covered with small to large white plaques or a shaggy exudate. Bell (1961) observed some fish with cardiac lesions, and Wood and Yasutake (1956a) found that a massive myocarditis with destruction of the major portion of the muscle often occurred in advanced cases.

In advanced cases, histopathology may be seen in virtually all tissues and organs. Wood and Yasutake (1956a) found that the inflammatory reaction in the gills extended from the arches to the tips of the filaments. Wolke (1975) stated that the presence of reticuloendothelial cells below the epithelial lining of the gill lamellae often caused swelling in the affected lamellae. Organisms are frequently seen within the blood vessels of the terminal gill filaments, indicating the systemic nature of the disease.

The brain occasionally had rings of inflammatory cells surrounding minor blood vessels or necrotic foci, while the cerebral meninges often contained granulomatous nodules (Wood and Yasutake, 1956a). Smith (1964) noted that the swim bladder was occasionally covered with petechial hemorrhages, and Wood and Yasutake (1956a) listed it (along with the gall bladder) as the site of distinct granulomatous nodules. Snieszko and Griffin (1955) were the first to note the presence of an opaque false membrane which sometimes covered the spleen and heart, imparting a whitish appearance to these organs. Smith (1964) reported a false white membrane partially or completely covering the spleen, liver, gonads and swim bladder in some fish. The membrane was not firmly attached, but could be peeled off leaving the surface of the organ unharmed.

Histologically, the membrane varied from a thin layer of fibrin and leucocytes to a structure with three clearly discernable layers. The innermost layer consisted of fibroblasts and histiocytes, then a layer of degenerating leucocytes with macrophages and the outermost layer, fibrin and nucleated cells. Gram positive bacilli were seen in the outermost layer. The membrane is distinguished from the true diptheritic membrane caused by Corynebacterium diptheriae in that it is superficial and does not penetrate the capsule of the organ it covers.

Smith (1964) has associated the formation of the membrane in Atlantic salmon with low water temperature and chronic infection. Membranes were observed at temperatures below 6.7 C, both membranes and necrotic lesions from 6.7 C to 11.1 C, and necrotic lesions only above 11.1 C.

Yasutake (1969) has studied the histopathology of experimental kidney disease infections. In sockeye salmon fingerlings inoculated intraperitoneally with the organism, hyperplasia of the hemoblastic cells and decreased lymphocytes in the kidney were observed at the end of 1 week post-infection. Minute foci of macrophages containing phagocytized bacteria and a noticeable increase of leucocytes in the vascular system were also observed.

These same lesions developed in fish which had been fed viable organisms. However, observable tissue changes did not occur until the fifth week post-infection, and immature cells in the kidney and leucocytes were not as abundant.

The infection in the test inoculated group was systemic by the end of the fourth week, and sampling was discontinued during the sixth week. Two of the five test-fed specimens taken at the termination of the experiment (twelfth week) showed clinically observable pathological changes in the kidney. Septicemia was evident and bacteria-engorged macrophages were present throughout the circulatory system.



Hunn (1964) has studied the pathophysiological effects of bacterial kidney disease in brook trout. He found that the hematocrit was reduced by 44% and the total plasma protein dropped by 52%. Electrophoretic analysis of the plasma protein indicated that the albumin fractions were greatly reduced. This data correlates well with the pathological picture of hematopoietic tissue destruction, kidney damage and reduced liver function.

#### Detection and Diagnosis

Historically, diagnosis of BKD has been made by demonstrating the presence of small, Gram-positive diplobacilli in infected tissues. However, the accuracy of this method of identification depends upon the presence of sufficient numbers of bacteria. Thus, fish in the early stages of the disease and carriers harboring small numbers of the organism (as compared to a fulminant infection) could easily be missed. Also, other saprophytic Gram-positive bacilli having the same morphology as the kidney disease bacterium have been reported in fish tissues (Ross and Toth, 1974; Bullock et al., 1971; Bullock and Snieszko, 1969; Collins, 1970). Another complicating factor is the presence of melanin granules in the kidney. These can easily be confused with the bacterium since they are both about the same size and shape.

The development of a medium for the organism (Ordal and Earp, 1953) aided in the identification procedure. However, it is not always possible to isolate the kidney disease bacillus. Often, fish are contaminated with other micro-organisms which quickly overgrow on the medium. Occasionally, the presence of the organism can be demonstrated by other methods, but attempts at culture are unsuccessful. Also, visible colonies may take from 1 to 6 weeks to develop on initial isolation.

Chen et al. (1974) applied immunodiffusion techniques based on the presence of soluble antigen in infected tissues to the diagnosis of kidney disease. Using the Ouchterlony gel diffusion method, they found that specific precipitin lines were formed within 24 hr when kidney disease bacilli suspensions or tissue homogenates containing the bacterium (from both natural and experimental infections) were reacted with specific rabbit antiserum. No precipitin lines were formed with tissue homogenates from healthy fish or from those injected with other fish pathogens. This procedure, along with other advances in the detection and diagnosis of infectious diseases of fish, has been reviewed by Bullock and Wolf (1974) for the third U.S.-Japan meeting on aquaculture.

Since previous studies (Bullock et al., 1974) have indicated that there is only one serotype of the kidney disease bacterium in North America, antiserum against any

kidney disease bacillus culture may be used in the immunodiffusion test.

Serological diagnosis has several advantages. This method is specific for kidney disease, is rapid (results are obtained in 24 hr) and requires a minimum of equipment and skill. Although the sensitivity of the test is not known, the presence of one to two bacteria per microscopic field in an organ smear is sufficient to provide a positive immunodiffusion test.

Bullock and Stuckey (1975) have applied an indirect fluorescent antibody technique to the diagnosis of kidney disease. Material to be tested (a culture of the organism, fresh, frozen or preserved tissue from diseased fish) is flooded with goat or rabbit anti-kidney disease serum, incubated for 30 min, then rinsed in phosphate buffered saline (pH 7.2), and air dried. Then, the slide is flooded with the appropriate immunoglobulin (previously labeled with fluorescein) and incubated 30 min. Slides are then washed 10 min in carbonate-bicarbonate buffer (pH 9.3), air dried, and examined for fluorescence.

Bullock and Stuckey found that this procedure was specific for the kidney disease bacterium and had no cross-reactivity with other fish pathogens. Some background fluorescence in fish tissue was observed; however, this was reduced by using a 1:20 dilution of rhodamine.

These investigators found that fluorescent antibody techniques were far superior to the conventional Gram stain method in detecting low numbers of the kidney disease bacterium. Out of a group of 13 fish, bacteria could be demonstrated in Gram-stained kidney smears of seven, while fluorescent antibody techniques revealed the kidney disease bacterium in all 13.

The fluorescent antibody technique has the advantages of a high degree of specificity and rapid diagnosis (results in 2 hr). However, fluorescent microscopic equipment and trained personnel are not widely available to hatchery managers. Thus, detection of kidney disease bacteria by immunodiffusion appears to be the method of choice for diagnosis in most cases.

Recently, the Canadian government has published a manual explaining the application of the Fish Health Protection Regulations under the Fisheries Act of Canada (Can. Dept. of Fish. and Env., 1977). The act establishes national standards for sampling, handling, and diagnostic procedures for the detection of bacterial, viral, protozoan, and parasitic piscine diseases found in Canada. This program is designed to "prevent the spread of infectious diseases through inspection of production sources of fish stocks, and to control the movement of infected fish stocks." Satisfactory inspections must be conducted at 6 month intervals for a production source to retain its certified status.

It is hoped that control of diseases and ultimately prevention of epizootics can be achieved through this program of early detection. The organization and operation of the fish health program as well as the occurrences of fish diseases in the Pacific Region of Canada are further described by Bell and Margolis (1976).

### Treatment

Treatment has been the focus of the bulk of the research relating to bacterial kidney disease. This is undoubtedly due to the heavy economic losses the disease can cause in a hatchery and the many difficulties encountered in working with the organism. The approaches taken have varied from diet therapy to the use of a variety of drugs, some of which have proved toxic to the fish.

Davis (1934) recommended a diet of 3% cod liver oil, iodine, clam meal and other vitamin additions (green vegetables) for the treatment of kidney disease. However, Belding and Merrill (1935) reported that the diet was tried unsuccessfully in 1934, when the etiology of the disease was unknown but believed to be of a metabolic nature.

Rucker et al. (1951) were the first to report controlled chemotherapeutic drug trials. Their data showed a definite decrease in mortalities from BKD in 15 cm sockeye salmon when sulfadiazine was incorporated into the food. The drug was fed at a rate of 12 g per 100 lb of body weight

for 7 days and then the sulfadiazine was reduced to 6 g per 100 lb for days 8 through 28. Sulfa therapy was discontinued for days 29 through 43, then resumed at the 6 g level for days 44 through 59. Total loss from BKD for the 74 day period of observation was 92% and 98% in the two control ponds versus 33% and 42% in the ponds where the fish had been treated with sulfadiazine. When the sulfa-treated fish were sacrificed at the end of the experiment, only two of the 60 showed bacteria in kidney smears and cultures.

Rucker et al., pages 136-137, stated:

...heavily infected fish were not cured by the sulfadiazine treatment, but the treatment does have definite prophylactic value - sick fish do not eat and are therefore really not reached by the sulfadiazine therapy...All in all...kidney disease can be controlled with sulfadiazine therapy, as treatment suppresses the spread of the infection and possibly cures some cases...

Earp et al. (1953) injected 40 sockeye salmon yearlings (about 12.7 cm long) with a pure culture of the kidney disease bacillus grown on chick embryo tissues. Half of the fish received no treatment; the other half were fed sulfadiazine at the rate of 12 g per 100 lb of body weight for 10 days, followed by 6 g per 100 lb of body weight for days 11 through 20. Mortalities in the untreated (control) fish began on day 25; all fish in this group died by day 41. The group fed sulfadiazine had a delayed mortality period. The majority of fish died between days 60 and 70--40 to 50 days after the sulfonamide was removed from the

food. Earp et al., page 69, concluded:

The effect is bacteriostatic rather than bactericidal. It is, therefore, concluded that sulfadiazine at the levels employed will effectively control the disease but does not necessarily destroy the bacterium which is capable of inducing death after the sulfa therapy is discontinued.

Earp et al. carried out field experiments on therapy from 1946 to 1952 using sulfamerazine, sulfamethazine, sulfadiazine, and Aurofac (a growth-stimulating adjunct containing aureomycin). Therapy with aureomycin was ineffective; treatment with the sulfonamides met with varying degrees of success, depending on the dosage, length of treatment, severity and extent of the disease in a given population of fish and the species of diseased fish.

Earp et al. also studied the effect of penicillin on the viability of the kidney disease bacterium. They found that 1 unit of penicillin per ml had no effect when added to a culture of the bacillus which was then incubated for 24 hr and injected into fingerling sockeye salmon.

Wood and Wallis (1955) found that the incorporation of low levels of sulfamethazine (2 g per 100 lb of fish) effectively prevented the transmission of kidney disease when young chinook salmon were fed a diet containing the flesh and viscera of infected fish.

Snieszko and Griffin (1955) studied the efficacy of several sulfonamides (sulfanilamide, sulfadiazine, Gantrisin<sup>®</sup> and a 50-50 mixture of sulfamerazine and sulfathiazole) and

antibiotics (chloramphenicol, terramycin and aureomycin) in the treatment of eastern brook trout infected with kidney disease. Sulfonamides were given at a rate of 200 mg (9 g per 100 lb of body weight) and antibiotics at a rate of 75 mg (3.4 g per 100 lb of body weight) per kg of fish per day. Fish were fed twice daily at the rate of 7% of the weight. The water temperature was 12.5 C and the treatment was continued for 45 days.

This experiment differed from previous work in that the population of fish was artificially infected with kidney disease rather than suffering from a natural outbreak. Healthy brook trout fingerlings, weighing an average of 12 g, were randomly distributed into two experimental troughs. When attempts to produce disease via contact with infected fish or feeding a diet strongly enriched with the remains of infected fish proved unsuccessful, half the trout in each trough were injected intraperitoneally with a suspension from diseased trout. Treatments were started 2 days in advance of the inoculation in anticipation of the rapid progress of the disease.

Snieszko and Griffin found that all sulfonamides arrested the disease to approximately the same degree. However, Gantrisin<sup>®</sup> and sulfamerazine had the lowest growth retarding effect; sulfadiazine showed the most growth retardation. Of the antibiotics, only chloramphenicol delayed the progress of the infection.



They concluded that Gantrisin<sup>®</sup>, sulfamerazine and sulfamethazine were the drugs of choice at levels of 8 to 10 g per 100 lb of fish per day. They recommended that during an epizootic, therapy be continued until mortalities dropped to a very low point, with successive treatments for weekly periods each month during the kidney disease season. They also suggested that multiple therapy with several sulfonamides might prove to be more effective than therapy with a single sulfonamide.

Allison (1958) tested the following drugs or combinations on a group of 20 cm brook trout fingerlings chosen at random from a lot that had kidney disease: sulfamerazine; sulfamerazine and sulfaguanidine; sulfamerazine and sulfadiazine; sulfamerazine, sulfaguanidine and sulfadiazine. All drugs were fed at the rate of 12 g per 100 lb of fish per day, except the last combination; it was fed at the rate of 12 g of sulfamerazine, 8 g of sulfaguanidine and 8 g of sulfadiazine per 100 lb of fish per day. Drugs were fed at these levels for 4 consecutive days, then with two-thirds of the above dosages for 25 days. All groups were observed for an additional 22 days after therapy was discontinued.

Allison found that all combinations of sulfa drugs were effective but that sulfamerazine alone was just as effective as any of the combinations. Higher mortalities in the group receiving three sulfas were presumably due to

the high dosages. None of the treatments effected a complete cure.

Wolf and Dunbar (1959b) reported the results of a renewed search for a more effective and lasting treatment for kidney disease. Using the disk method of in vitro drug sensitivity screening, they tested the effect of 34 chemotherapeutic agents on 16 strains of the kidney disease bacterium that had been isolated during outbreaks of the disease.

They found that all strains were sensitive in varying degrees to penicillin G, as reported by Earp et al. (1953). The sulfonamides tested (sulfadiazine, sulfamerazine, sulfamethylthiadiazole, sulfadimetine, sulfathiazole, sulfisoxazole, and triple sulfa) greatly reduced the amount of growth, although some growth was observed next to the disks. All strains were strongly sensitive to the following antibiotics: bacitracin, candicidin, carbomycin, chloramphenicol, chlortetrachycline, erythromycin, novobicin, oxytetracycline, streptomycin, tetracycline, and thiocymetin. Both the nitrofurans (nitrofurantoin and nitrofurazone) and phenoxyethanol strongly suppressed growth. INH and PAS (used in the therapy of human tuberculosis) were without effect. Cycloserine, eulicin, filipin, fungichrome, neomycin, polymyxin B and viomycin also had little or no effect on the organism.

From this group of 34 drugs, 10 were selected for in vivo testing. Several of the drugs were not suitable for experimental feeding due to the normal route of administration; two others were discontinued by their manufacturers. Eastern brook trout weighing an average of 14.3 g were infected with kidney disease by intraperitoneal injection. Administration of the drugs was begun 11 days post-injection. The fish were fed twice a day and maintained at 12.5 C. All drugs were fed for 21 days and the fish observed for an additional 33 days. In this trial, sulfisoxazole clearly had the best therapeutic effect, with 1.5% mortalities 33 days after treatment was stopped, as compared to 64% to 85% mortalities with the other drugs.

In a second trial with fish weighing 33.4 g (other parameters were the same as the first trial), erythromycin-fed fish showed low mortalities and approximately half of the fish were pronounced cured after examination of Gram stains and inoculation of modified Mueller-Hinton agar with kidney material gave negative results. Sulfamethoxy-pyridazine-fed fish also showed low mortalities, but as with most other drugs in trials one and two, mortalities began to climb rapidly after therapy was discontinued.

Wolf and Dunbar concluded that erythromycin fed at the rate of 4.5 g per 100 lb of fish for 21 days was the preferred treatment. They stated that

the demonstrated capacity of most strains of the bacterium to grow to a limited extent in vitro in the presence of the sulfonamides used may well reflect an inherent ability to tolerate this group of drugs.

Since Wolf and Dunbar's experiments, the efficacy of erythromycin therapy for control of kidney disease has been confirmed in several field trials. In one test performed by the Washington Department of Fisheries, 40,000 fish received 100 mg of erythromycin per kg of fish per day for 21 days and an equal number of untreated fish served as controls. The incidence of disease was approximately the same in both groups before treatment. One month after the treatment was discontinued, the cumulative percent loss in the untreated group was 2.4% and in the treated group 0.5% (DeCew, 1969).

In trials with experimentally infected juvenile coho salmon, Banowetz (1974) found that erythromycin stearate fed at the rate of 100 mg per kg of fish for two 14-day treatment periods controlled the disease, while Ampicillin<sup>®</sup> (ampicillin trihydrate, Pfizer) and Pen V-K<sup>®</sup> (potassium phenoxymethyl penicillin, Pfizer) fed at the rate of 75 or 100 mg per kg per day for two 14-day treatment periods were ineffective.

Piper (1961) reported violent convulsive and spastic symptoms indicative of erythromycin thiocyanate toxicity during treatment of rainbow trout infected with kidney disease. The antibiotic was incorporated into the diet

and fed at the rate of 4.5 g per 100 lb of fish for 21 days.

Warren (1963b) attempted to reproduce these toxicity effects experimentally. He found that a dosage of 500 mg of erythromycin thiocyanate per kg of fish per day (five times the usual therapeutic level) was required to produce overt symptoms of toxicity.

The results of force-feeding trials at the Eastern Fish Disease Laboratory indicate that doses of over 2 g of sulfisoxazole per kg of fish weight are non-toxic to rainbow trout. However, young fish treated with sulfonamides often become more susceptible to fungus infections, especially of the branchial area.

Amos and Klontz (1975) studied the efficacy of erythromycin phosphate injected subcutaneously into spawning adult chinook salmon. They found that 17% (1974) and 9% (1975) of the mortalities among fish injected with erythromycin were due to kidney disease as compared to 45% (1974) and 37% (1975) of the mortalities among control (non-injected) fish. Twenty-one percent of the control fry (1974-1975) mortalities were observed among the fry from injected females.

DeCew (1972) tested penicillin G procaine, oxytetracycline-HCl, dihydrostreptomycin sulfate, benzathine penicillin G, erythromycin and neomycin sulfate alone and in several combinations for toxicity, efficacy, and

teratogenicity in adult spring chinook salmon infected with bacterial kidney disease and furunculosis. He found that none of the antibiotic formulations produced evidence of acute toxicity. The complex of Penicillin G procaine, dihydrostreptomycin sulfate and Oxytetracycline-HCl(PSO) effectively controlled the mixed infection and produced a three-fold increase in adult survival and production of viable eggs. "Swollen, broken, and reduced numbers" of kidney disease bacilli were observed in tissues from fish treated with the PSO mixture. Mandible and fin anomalies were observed in the progeny of adults treated with PSO close to spawning. However, these anomalies were reduced to a minimum when a 32 day period elapsed between the time of injection and spawning. DeCew recommended a series of three injections with a dosage of 15,000 to 30,000 units of penicillin G procaine, 19 to 38 mg dihydrostreptomycin sulfate and 4 to 8 mg oxytetracycline-HCl per kg of body weight.

Hoskins et al. (1976) have recommended the destruction of infected stock and the complete disinfection of holding facilities since control is difficult once the disease is established within a stock of fish. Although a drastic measure, this is probably the best method of control in cases where the number of infected fish is small and the chance of reinfection remote.

Ross and Smith (1972) found that kidney disease bacilli did not survive a 5 min exposure to either Beta-dine or Wescodyne at a concentration of 25 ppm active ingredient. These results indicate that iodophors may be effective egg disinfectants for the control of bacterial kidney disease.

Bullock et al. (1975) have summarized current treatment procedures in a pamphlet published by the United States Department of the Interior, Fish and Wildlife Service. They state (pages 4-5):

Kidney disease is the most difficult of the bacterial diseases to treat, possibly because the bacterium can occur intracellularly and is then beyond the reach of some antimicrobials. Under laboratory conditions, erythromycin USP given orally at the rate of 9-10 gm per 100 kg per day for 3 weeks gave the best, but not complete, control. Field applications have given similar results; cures were effected in some lots, but among others the disease recurred. All published accounts of treatment with sulfonamides report that mortality from the infection recurred after treatment ceased...None of the above mentioned antimicrobials have been approved by the Food and Drug Administration for use on fish meant for human consumption.

#### Immunology

Evelyn (1971) reported that sockeye salmon produced agglutinins specific for bacterial kidney disease in response to the intraperitoneal injection of heat-killed cells suspended in adjuvant. Antibodies were detectable for 16 months following a single injection; a second

injection, given 13 months after the first, elicited a clear-cut anamnestic response. The maximum agglutinating titers 90 days after the primary and secondary vaccinations were 1:2560 and 1:10,240, respectively. It is not known if the immune response observed confers protection against the disease.

Electrophoresis of sera from vaccinated fish revealed two fractions of low mobility occurring in the gamma and beta regions. These fractions were specific for the kidney disease bacillus since they disappeared when sera were absorbed with the bacteria.

Banowetz (1974) detected two antigens by immunodiffusion and immunoelectrophoresis in whole cell extracts of Corynebacterium. Both antigens appear to contain carbohydrate and protein.

Using agglutinin and precipitin reactions, Bullock et al. (1974) found that ten strains of the kidney disease bacterium from different parts of North America were antigenically homologous.

### Phagocytosis

Many workers have reported the intracellular occurrence of the kidney disease bacterium in vivo, but the role of phagocytosis in the disease process remains undefined. The relationship between intra- and extracellular growth is significant because it may hold the key to the



epidemiology and chemotherapy of BKD. In addition, it bears on the host-parasite relationship between the infected fish and the bacterium.

The ability of kidney disease bacilli to survive and multiply inside phagocytic cells may contribute to the chronic nature of BKD by sheltering the bacteria from circulating antibody and chemotherapeutic agents. Wolf and Dunbar (1959b) have suggested that intracellular survival may account for the refractility toward treatment.

#### Methods for Assessment of Phagocytosis

One of the greatest difficulties in phagocytosis studies is assessing the engulfment of bacteria and other particles. Light microscopy of stained and unstained preparations of phagocytes and particles has been used as a method for quantitating ingestion. However, it is often difficult, if not impossible, to distinguish between particles which are truly intracellular and particles which are merely superimposed on or attached to the phagocyte's surface. Numerous investigators have attempted to improve the quantitation of particle uptake through the use of radioisotopically labelled bacteria or particles (Brzuchowska, 1966; Carpenter, 1966; Carpenter and Barsales, 1967; Michell et al. 1969), paraffin oil particles containing oil red O (Stossel et al., 1972; Stossel et al., 1973). polystyrene or polyvinyl toluene particles (Weisman and

Korn, 1967), starch particles (Michell et al., 1969) and other methods. The major difficulty in these types of assays is efficient separation of cells containing particles from uningested particles. Elaborate control experiments are necessary to indicate that the separation process is functioning at an acceptable level.

Metabolic activities such as the rates of glucose oxidation to CO<sub>2</sub> and of nitroblue tetrazolium reduction have been used as indirect assays of ingestion (Sbarra and Karnovsky, 1959; Stossel et al., 1972). Unfortunately, the metabolic activities of resting cells contribute background activity. In addition, some chemical and biological agents can stimulate cellular metabolism without particle uptake (Cohn and Morse, 1960; Graham et al., 1967).

Another indirect technique, the bactericidal assay, measures a decrease in the number of viable (colony-forming) bacteria as an index of ingestion and killing by phagocytes. The limitations of this approach include its laboriousness, complexity, and design which precludes quantitation of initial and maximal rates of phagocyte-bacterial interaction. This particular method could not be used with kidney disease bacilli since they grow slowly and do not form discrete colonies.

The fluorescent antibody technique described in this paper was developed to study the intracellular growth kinetics of the kidney disease bacilli in salmonid cell cultures.

### Factors Affecting in vitro Phagocytosis

Many factors are involved in determining whether phagocytosis will occur in a given situation. These include the phagocyte-to-microbe ratio, the presence of serum proteins and opsonins, divalent cation levels, pH, a source of suitable carbohydrate, presence or absence of surfaces promoting phagocytosis, and the age of the phagocyte.

In phagocytosis assays, the ratio of particles to phagocytes affects the percent of phagocytes containing ingested particles as well as the number of ingested particles per phagocyte. There is some evidence to indicate that a high particle per phagocyte ratio leads to decreased ingestion (Rabinovitch, 1967). The reason for this is not clear, but may be due to changes in the configuration of the cell membrane or overburdening of the glycolytic pathways involved in providing energy for phagocytosis.

In a study of parameters affecting attachment and ingestion, Rabinovitch (1967) found that attachment of glutaraldehyde-treated red blood cells to mouse peritoneal macrophages did not require the presence of serum factors or divalent cations, whereas engulfment required both serum factors and divalent cations. Wood (1951, 1960) has emphasized the important role of surface phagocytosis in the ability of leucocytes to ingest encapsulated bacteria or bacteria which produce antiphagocytic substances. Coon (1965) found that cell density and the growth phase of cells can influence the

expression of the differentiated phenotype in embryonic chick cartilage cells.

### Phagocytosis in Teleosts

Post (1963) found that phagocytosis did not occur when whole blood or leucocyte preparations from immunized and non-immunized rainbow trout were incubated with Aeromonas hydrophila. Nor could he demonstrate phagocytosis in the blood or peritoneum of immunized and non-immunized trout that had been injected intracardially and intraperitoneally, respectively, with suspensions of Aeromonas hydrophila. However, phagocytosis in the peritoneum could be induced by injecting glycogen intraperitoneally 24 hr before the bacterial cells were injected.

Bell (1976) introduced Lactobacillus brevis into the blood stream of non-immunized, cultured sockeye salmon via cannulation of the dorsal aorta or intracardiac injection to study the ability of circulating leucocytes to phagocytize bacteria. No phagocytosis occurred in the peripheral blood when bacteria were introduced via the dorsal cannula, but bacteria were rapidly taken up by the kidney. Some phagocytosis by polymorphonuclear leucocytes did occur in the bloodstream when bacteria were introduced via intracardiac injection; however, these phagocytes may have drained from the renal portal system following caudal blood sampling.

Ellis and Munroe (1976) investigated the phagocytic properties of lympho-reticular tissues in the plaice (Pleuronectes platessa) after intraperitoneal injection of colloidal carbon. Peritoneal macrophages constituted a large population of phagocytic cells, but apparently, most of the carbon gained access to the circulation as free particles. Phagocytosis occurred chiefly in the ellipsoids of the spleen, the reticulo-endothelial cells in the hematopoietic tissue of the kidney and the reticulo-endothelial cells in the intermuscular spaces in the atrium of the heart. The carbon-containing macrophages in the kidney and spleen formed aggregates in the lymphoid areas, whereas the cardiac macrophages emigrated rapidly from the organ. Carbon-containing mononuclear phagocytes were observed in appreciable numbers in smears of blood and lymph only at 48 hr post-injection. These cells did not appear to be very active phagocytically, since there were never more than a few carbon particles present in the cytoplasm.

Hatai (1972a) has studied the clearance of Aeromonas hydrophila (liquefaciens) from the blood stream of eels (Anguilla japonica). He found that all viable bacteria were cleared from the blood within 52 hr of intravenous injection. Bacteria were removed from the blood stream predominantly by the kidney, rather than the liver, as in mammals (Rogers, 1960). Large numbers of bacteria were sequestered in the kidney, but they were eliminated with

difficulty. In experiments where a non-lethal dose was injected, the number of live cells in each organ decreased rapidly within 24 hr, but this decrease slowed down and the count was still  $10^3$  or  $10^4$  viable organisms after 1 week.

In a similar experiment, Bell (1977) injected sockeye salmon intravenously with Aeromonas hydrophila (liquefaciens). He found that at 15 min and 1 hr, and at 4 hr and 18 hr, the kidney held an average of 95% and 75% respectively, of all of the bacteria removed. However, the overall recovery, calculated on the basis of organ weights and blood volumes was approximately 28% at 15 min and 13% afterwards. Assuming that losses of bacteria at the injection site were low, this suggests that a high percentage of the inoculum was killed by the host and that bacteria were sequestered in other tissues. One to two times the number of bacteria were removed in the posterior half of the kidney as in the anterior half. Bacteria were most numerous in the kidney, then the spleen, followed by the blood and the liver.

### III. MATERIALS AND METHODS

#### Buffered Salines

Phosphate buffered saline (PBS), calcium magnesium free phosphate buffered saline (CMF-PBS), modified Dulbecco and Vogt's phosphate buffered saline (DV-PBS) (Wolf et al., 1960), and Hanks' balanced salt solution (HBSS) (Hanks, 1948) were used throughout this study. The PBS and CMF-PBS were prepared according to the following formulations:

#### PHOSPHATE BUFFERED SALINE, pH 7.2 (PBS)

	<u>g/l</u>
NaCl	8.33
$\text{KH}_2\text{PO}_4$	0.245
$\text{NaH}_2\text{PO}_4$	0.809
double distilled $\text{H}_2\text{O}$	to volume

#### CALCIUM-MAGNESIUM FREE PHOSPHATE BUFFERED SALINE, 10X, pH 7.2 (CMF-PBS)

	<u>g/l</u>
NaCl	80.00
KCl	3.00
$\text{Na}_2\text{HPO}_4$	0.73
$\text{KH}_2\text{PO}_4$	0.20
glucose	20.00
double distilled $\text{H}_2\text{O}$	to volume

### Culture of the Kidney Disease Corynebacterium

The strain of Corynebacterium used throughout these studies was supplied by J. E. Sanders, Associate Fish Pathologist, Oregon Department of Fish and Wildlife, who isolated it during a disease outbreak at the Leaburg Trout Hatchery, Leaburg, Oregon. Cysteine serum agar (CSA), a modification of Ordal and Earp's agar (1953) in which 10% fetal calf serum (FCS) is substituted for the human blood, was used for the maintenance of all cultures unless otherwise indicated. Mueller-Hinton agar supplemented with 0.1% cysteine (Wolf and Dunbar, 1959b) was also used for culture of the Corynebacterium.

### Tissue Culture Methods

Cell cultures were maintained in modified Eagle's minimum essential medium (MEM) with HBSS (Eagle, 1959), Eagle's minimal medium - Spinner modification (DIFCO, 1969), or Medium 199 (Morgan et al., 1950). The media were supplemented with 10% or 20% FCS (GIBCO), penicillin (100 IU/ml), and streptomycin sulfate (100 mg/ml), and buffered with sodium bicarbonate to pH 7.2. Double distilled water was used in all media preparations.

### Maintenance of Established Cell Lines

In 1964, Fryer established the STE 137 cell line, a heteroploid line of fibroblastic morphology derived from



steelhead embryos (Pilcher et al., 1968). The STE 137 line was used from passages 150 to 156 in this study. Stock cultures were grown at 18 C in 32 oz prescription bottles (Brockway Glass Co.) sealed with white latex stoppers (West and Co.). The cell line was subcultured at 20 to 30 day intervals (Fryer, 1964; McCain, 1967).

#### Preparation of Primary Monolayer Cell Cultures from Kidney and Spleen

Primary monolayer cell cultures were prepared from the kidneys and spleens of 12.5 to 17.5 cm, healthy, hatchery-raised brook trout according to the method of Wolf and Dunbar (1957). Tissues were dispersed by a modification (Wolf et al., 1960) of Bodian's (1956) cold trypsinization method. Final dilutions of 1:400 or 1:600 (approximately  $1 \text{ to } 3 \times 10^5$  cells per ml) in Eagle's MEM gave the best results. Cell cultures were incubated at 18 C and fed at 3 day intervals. Confluent monolayers developed in 5 to 6 days.

#### Preparation of Leucocyte Cultures

Leucocytes were harvested from healthy, hatchery-raised, 3 yr old male coho salmon by heparin/sedimentation or silicon/centrifugation. Culture medium consisted of 100 ml Eagle's minimal medium - Spinner modification, 10 ml FCS, 2 ml penicillin-streptomycin mixture and 0.2 ml of L glutamine (200 mM per l). This medium was saturated with pure

oxygen before use (Heckman et al., 1971); cultures were incubated at 18 C.

### Preparation of Mouse Macrophage Monolayers

Mouse macrophage monolayers were prepared by the method of Falk and Zabriskie (1971). Monolayers were fed with 0.75 ml of Medium 199 containing 10% or 20% serum and incubated overnight at 37 C before use.

### Phagocytosis Studies

#### Established Cell Lines

Leighton tubes and Sykes-Moore chambers were seeded with STE 137 cells and incubated at 18 C. After 24 hr, the medium was removed and a suspension of  $10^6$  bacteria per ml of fresh medium added to the cells. After an incubation period of 1 hr at 18 C, the coverslips were removed from the Leighton tubes, rinsed thoroughly in several changes of HBSS to remove excess bacteria and prepared for immunofluorescent microscopy. Sykes-Moore chambers were examined for phagocytic interactions by phase contrast microscopy.

This procedure was repeated with STE 137 cells at 2, 3, 4, and 5 days after passage. In another experiment, bacteria were added to the cell slurry at the time of passage to give a final concentration of  $10^6$  bacteria per ml of medium and incubated for 1 hr at 18 C. Then, the coverslips were removed from the Leighton tubes, rinsed and

prepared for immunofluorescent microscopy.

The above procedures were also performed with medium containing 10% fetal calf serum rather than the usual 20% serum.

#### Primary Monolayer Cell Cultures from Kidney and Spleen; Leucocyte Cultures

The methods described under "Phagocytosis: Established Cell Lines" were repeated using kidney and spleen primary cultures, and leucocyte cultures. DV-PBS was substituted for HBSS. Trypan blue was used to determine cell viability (Phillips, 1973).

#### Uncultured Leucocytes

Two methods were used to study the interactions between uncultured leucocytes and kidney disease organisms. Cover-slips and slides were acid cleaned in a 50% solution of nitric acid, then rinsed repeatedly in double distilled water and air dried. The bacterial suspension used in these experiments had a turbidity equivalent to that of the number three tube of the McFarland nephelometer, or about  $10^9$  organisms per ml. In both methods, control slides were stained with trypan blue to determine leucocyte viability (Phillips, 1973) and differential counts were performed on blood films stained with May-Grunwald Giemsa stain. No anticoagulants were used in the collection of blood samples.

In the first method, two to three drops of blood from a single fish (healthy, hatchery-raised, 12.5 to 17.5 cm brook trout) were placed on an acid-cleaned coverglass and incubated in a covered, humidified chamber for 30 to 45 min at 18 C to allow leucocyte attachment to the glass surface. The clot was then washed off with chilled DV-PBS and 0.5 ml of the bacterial suspension (about  $5 \times 10^8$  bacteria) added to the leucocyte monolayer. The mixture was immediately inverted onto a clean glass slide and the sides rimmed with paraffin. Interactions between the leucocytes and kidney disease organisms were observed under phase contrast.

In the second method, 0.1 ml of the bacterial suspension and 0.1 ml of freshly drawn blood were applied to an acid-cleaned coverglass and mixed thoroughly. The coverglass was incubated in a covered, humidified chamber for 30 min at 18 C and then rinsed repeatedly in DV-PBS to remove the clot and unphagocytized bacteria. The coverglass was mounted wet on a clean slide and examined by phase contrast microscopy.

In each method, 50 leucocytes were counted. The total number of intracellular bacteria divided by the total number of leucocytes counted gave the phagocytic index.

#### Mouse Macrophages

Macrophage monolayers were prepared as described and incubated overnight before use. To test for phagocytosis,

a suspension containing approximately  $5 \times 10^6$  bacteria per ml of HBSS and 10% or 20% serum was prepared. The old macrophage medium was discarded and replaced with 1 ml of the bacteria-HBSS-serum solution. This was incubated at 37 C, 2 to 5% carbon dioxide, for 1 hr. After incubation, the medium was discarded, the chamber and its housing removed, and the slides rinsed vigorously in HBSS three times. Slides were air dried, fixed in methanol for 30 sec, stained with Giemsa stain for 30 min and examined under oil immersion.

#### Preparation of Antigens and Sera

##### Soluble Corynebacterium Antigen

Soluble Corynebacterium antigen was prepared by washing the kidney disease bacilli from a culture bottle and centrifuging at 13,200 x G, 5 C, for 20 min to remove the cells. Extraction was not necessary, since sufficient antigen was present in the culture fluids.

##### Preparation of Anti-Corynebacterium Serum

Rabbit antiserum was prepared in a young, female New Zealand white rabbit according to the following method. Kidney disease bacilli were harvested in CMF-PBS from MHA cultures and centrifuged at 1500 x G, 22 C, for 15 min. The cells were resuspended in CMF-PBS to a concentration of 1.75 ml wet packed cells per 10 ml of CMF-PBS. The cell

suspension was placed in a 62 C water bath for 45 min to kill the bacteria and then cooled. Prior to injection, the suspension was emulsified with an equal volume of Freund's complete adjuvant. The rabbit was immunized according to the following schedule:

Day 1 - rabbit trial bled and serum titered  
2 ml of intramuscular in each flank

Day 29 - rabbit trial bled and serum titered  
2 ml intramuscular in each flank

Day 46 - rabbit trial bled and serum titered  
2 ml intramuscular in each flank

Day 53 - rabbit trial bled and serum titered  
2 ml intramuscular in each flank

Day 148 - rabbit trial bled and serum titered  
2 ml intramuscular in each flank

Blood was collected in sterile, vaseline-coated centrifuge tubes and incubated at 23 C for 1 hr. The clot was retracted at 4 C for 12 hr, then centrifuged at 455 x G, 22 C, for 15 min. The serum was harvested and merthiolate added to give a final concentration of 1:5000. The antiserum had an agglutination titer of 1:2048 and was used in diagnostic work.

## Immunological and Immunofluorescent Techniques

### Precipitin Analysis by Double Diffusion in Gels

Ouchterlony double diffusion in gels was used to detect soluble Corynebacterium antigens from salmonid tissues for rapid confirmation of a preliminary diagnosis of BKD. The gels consisted of a solution of 0.5% Agarose (VWR Scientific) in PBS and 1:5000 merthiolate. Fifteen ml aliquots of the gel were poured into disposable 100 x 15 mm Petri dishes and allowed to harden.

Then, a pattern consisting of one central antiserum well and six surrounding antigen wells was punched in the agar. All wells were 5 mm in diameter and the centers of adjacent wells 13 mm apart. The wells were filled with test solutions and antiserum, and the plates incubated at 23 C. Results were read in 18 to 36 hr.

### Titration of Anti-antigen and Anti-antibody for Immunofluorescent Techniques

A "checkerboard" titration was performed to determine the proper dilutions of anti-antigen and anti-antibody. Smears of bacteria were prepared on glass coverslips, air-dried, and fixed in cold acetone (-20 C) (Kaplan et al., 1975) for 10 min. A series of doubling dilutions of both the unlabeled antibody and the labeled antiglobulin (BBL caprine anti-rabbit globulin labeled with fluorescein isothiocyanate) were tested against each other (Beutner et al.,

1968; Hardy and Nell, 1971). The highest dilution which gave good staining with a minimum of non-specific fluorescence was selected for use. For diagnostic work, kidney smears were prepared, stained, and examined for fluorescence.

Indirect Fluorescent Antibody Technique for  
Differentiation of Intra- and Extracellular  
Bacteria in Phagocytosis Studies

STE 137 cells and primary cultures from kidney and spleen were prepared on glass coverslips and incubated with bacteria, as described previously. All procedures after this step were performed at 4 C. After incubation, the coverslips were rinsed in several changes of iced DV-PBS to stop phagocytosis and remove excess bacteria. Then, they were flooded with the appropriate dilution of anti-Corynebacterium serum and incubated in a covered, humidified chamber for 15 min. The serum was drained off and each coverslip washed repeatedly in two changes of DV-PBS. Diluted conjugate was added and the coverslips incubated for 15 min. Then, the coverslips were rinsed in two changes of DV-PBS followed by a final rinse in double distilled water, fixed in cold acetone (-20 C) for 10 min, and mounted smear side down on a very small drop of glycerol. The number of FA-staining bacteria associated with 50 leucocytes was counted using the oil immersion lens of a Leitz Ortholux fluorescence microscope.



After the FA count was completed, the overlying glass coverslip was carefully removed and the leucocyte monolayer counter-stained with a drop of 0.15% methylene blue in saline. The number of methylene blue staining bacteria associated with 50 leucocytes was recorded.

The percentage of cell-associated bacteria extracellularly attached to the leucocyte surface was calculated from:

$$\left( \frac{\text{FA count (extracellular bacteria)}}{\text{methylene blue count (intra- and extracellular bacteria)}} \right) \times 100$$

The percentage of cell-associated bacteria that were ingested by leucocytes was calculated from:

$$\left[ 1 - \left( \frac{\text{FA count}}{\text{methylene blue count}} \right) \right] \times 100$$

Controls consisted of routinely-stained monolayers which had not been exposed to bacteria and monolayers which had been exposed to bacteria but stained with normal rabbit serum substituted for the anti-Corynebacterium serum.

### Histopathology

Fish tissues for the histopathology and electron microscopy work were taken from a population of yearling eastern brook trout experiencing an outbreak of corynebacterial kidney disease. Control tissues came from disease-

free populations of the same age raised under identical conditions.

Diseased and control tissues were fixed in buffered neutral formalin for 2 weeks, then processed, dehydrated in a graded series of isopropyl alcohols, and infiltrated with Paraplast (Sherwood Medical Industries) in a Fisher tissuematon (Model 60). Bony material was decalcified for 3 hr in 10% citric acid in 10% buffered neutral formalin, then processed as indicated above.

Tissue sections were cut at 5  $\mu$  with an American Optical Spencer microtome. Serial sections were stained with Mayer's hematoxylin and eosin (Luna, 1968) or the Brown-Brenn Gram stain (Luna, 1968) and mounted in Permount (all dyes from Hartman-Leddon Co.).

### Electron Microscopy

#### Bacteria

Bacteria were harvested from 2 to 3 month old cultures in 1 to 2 ml of PBS and removed by centrifugation (13,200 x G, 0 C, 10 min). The supernatant was discarded and the cells were suspended in 4% glutaraldehyde for 2 hr. After fixation, the cells were centrifuged out and the supernatant removed. The bacteria were washed in Sorensen's buffer (17.2 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 17.8 g  $\text{Na}_2\text{HPO}_4$  per 1 double distilled water, pH 7.3, 0.125 M) overnight and then stained with 1% osmium tetroxide in Sorensen's buffer for 2 hr. After

staining, the bacteria were embedded in 2% ionager and dehydrated according to the following schedule:

<u>Percent</u>	<u>Time</u>
30 acetone	10 min
50 acetone	10 min
70 acetone + uranyl acetate	3 hr
100 acetone	10 min
100 acetone	10 min
100 acetone	10 min

The bacteria were placed in a 2:1 plastic:acetone mixture and left for 20 hr. Then, they were embedded in Bojax (Pangborn and Addison, U. Calif. Davis, unpublished), cured at room temperature for 2 hr and at 70 C for 18 hr.

Sections (silver to pale gold) were cut on a Porter-Blum MT-2 with a diamond knife and mounted on bare copper grids and copper grids coated with a thin film of Formvar. Specimens were examined at 60 kV with a Phillips EM-300 and at 75 kV with a Hitachi 125-E.

### Fish Tissues

All procedures through the ethanol dehydrations were performed at refrigerator temperatures. Fish were sacrificed, dissected immediately, and organ and tissue samples placed in individual vials of 0.1 M cacodylate buffer, pH 7.3 (Sabatini et al., 1962). The organ and tissue samples were removed from the cacodylate buffer and immediately

minced in a small amount of 2.5% glutaraldehyde, pH 7.3. Tissues were fixed in 2.5% glutaraldehyde for 75 min, then washed three times in 0.1 M cacodylate buffer and stained in 1% osmium tetroxide for 75 min. The specimens were left in cacodylate buffer overnight (18 hr, 5 C) and then dehydrated according to the following schedule:

<u>Percent</u>	<u>Time</u>
70 ethanol	5 min
80 ethanol	5 min
90 ethanol	5 min
95 ethanol	72 hr
100 ethanol	10 min
100 ethanol	10 min

Tissues were dehydrated in two changes of propylene oxide, 10 min per change, and infiltrated according to the following schedule:

3 parts propylene oxide to 1 part A/B epon mixture - 15 min on shaker
1 part propylene oxide to 1 part A/B epon mixture - 30 min on shaker
1 part propylene oxide to 3 parts A/B epon mixture - 2 hr on shaker
pure A/B epon mixture with 1.5% DMP 2 hr on shaker

The epon mixture was a modification of Luft (1961) and consisted of equal amounts of mixtures A and B:

<u>Mixture A</u>	<u>ml</u>
Epon 812	71.5
DDSA	100
<u>Mixture B</u>	
Epon 812	100
NMA	77

Tissues were embedded in pure A/B epon mixture with 1.5% DMP and cured at 60 C for 55 hr.

Sections 1  $\mu$ m thick were cut, stained with alkaline toluidine blue, and examined with the light microscope. Sections for electron microscopy were cut to gold thickness on a Porter-Blum MT-2 microtome, mounted on formvar coated copper grids and post-stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) for 15 min each. Sections were examined in a Hitachi 125-E at 75 kV.

#### IV. RESULTS AND DISCUSSION

##### Phagocytosis Studies

##### Indirect Fluorescent Antibody Technique

The indirect fluorescent antibody (IFA) technique described in this paper is a modification of methods used in gonococci research. It was developed because a more accurate method was needed for the differentiation of intra- and extracellular bacteria in phagocytosis studies with salmonid cells and kidney disease bacilli.

The IFA technique is based on the principle that living cells are impermeable to the fluorescein labeled antibody molecules. After the cells (STE 137 or primary cultures from kidney and spleen) interacted with bacteria at 18 C, phagocytosis was stopped with iced DV-PBS. Rabbit anti-Corynebacterium serum was applied to the living cell monolayer and incubated. Then, the living cell monolayer was washed free of excess serum and stained with caprine anti-rabbit globulin labeled with fluorescein isothiocyanate. All procedures were performed at 4 C to prevent further phagocytosis or internalization of antibody or conjugate.

Since the cells excluded conjugate, the IFA stained only extracellular bacteria. Once the cells were fixed in acetone, they became permeable to methylene blue. Thus, the methylene blue count reflects both intra- and extracellular bacteria.

## STE 137 Cells

Many factors can affect in vitro phagocytosis (see Review of Literature: Phagocytosis) and these were carefully considered in selecting experimental parameters. A ratio of ten bacteria to one phagocyte was chosen to ensure adequate contact without overloading the system. Serum and cation levels were well within what is considered the optimal range for phagocytosis (Rabinovitch, 1967; Kozel, 1977). Carbohydrate for glycolysis was provided by the dextrose in Eagle's MEM, and pH levels were carefully controlled throughout the experiments. Glass surfaces were pretreated to enhance phagocytosis.

The STE 137 line was used from passages 150 to 156 in these experiments. Contrary to previous observations made at four to five passages (Fryer, 1974) the STE 137 cells did not phagocytize kidney disease bacilli as determined by phase contrast and IFA microscopy. Phagocytosis was not induced by varying the serum concentration from 10% to 20% or by varying the age of the STE 137 cells (0, 1, 2, 3, 4, 5 days). Attachment of bacteria to STE 137 cells was observed in some cases.

Mouse macrophages avidly ingested kidney disease bacilli under similar experimental conditions, demonstrating that experimental parameters were conducive to phagocytosis and that kidney disease bacilli are phagocytizable particles.

How can the loss of phagocytic properties in this cell line be explained? There are two possibilities: 1) the

cell line consisted of a mixed population of cells at the time the observations of phagocytosis were made and the phagocytic cells have been selected against or died out; or 2) the STE 137 cells have dedifferentiated, losing the property of phagocytosis. Confirmation of either of these hypotheses would require characterization of the STE 137 line from primary to established cell line status. Such studies might include karyology; analysis of the cell cycle for generation time, duration of the DNA synthetic period, and duration of mitosis; and, possibly, the use of isoelectric focusing to give an isoenzyme profile of the cultured cell. Such studies are beyond the scope of this paper.

However, the plausibility of these two hypotheses is supported by information available in the literature. Dedifferentiation (the loss of differentiated phenotypic characteristics) has been reported from the early days of tissue culture, when Champy (1920) described the loss of secretory activity in monolayers of epithelium from prostrate tubules. Some of the phenotypic traits and the tissues involved include: the loss of contractile properties in explants of young rabbit aorta (Mauger, 1974), the lack of  $I^{131}$  uptake in cultivated thyroid tissue (Oppenheimer et al., 1956; Seaman and Stahl, 1956), the loss of



several enzymes by cultured liver cells (Perske et al., 1957), and the cessation of beating among cilia on epithelioid cells growing out of a human nasal explant (Jordan, 1956).

In two cases, there is some evidence to indicate that these changes in phenotype may be temporary and dependent upon the cell's microenvironment. Fisher (1938) reported loss of the prepigment of iris epithelium by the 25th serial passage. In more recent studies, Cahn and Cahn (1965) found that retinal pigment cells lost pigmentation only when they were grown as mass monolayer cultures ( $10^6$  cells/60 mm Petri dish) or when they were grown in a high-molecular weight fraction of embryo extract; however, these cells repigmented when grown as clones. Holtzer et al. (1958, 1960) noted that chondrocytes cultured in propagating monolayers irreversibly lost the ability to synthesize chondroitin sulfate and other cartilage matrix materials after a few days, but if the same cells were organ cultured as a nodular cell pellet, chondrogenesis and  $S^{35}O_4$  incorporation into chondroitin sulfate continued. Coon (1965) concluded that the

expression of the differentiated phenotype of embryonic chick cartilage cells is influenced by the medium (embryo extract supplement), the cell density and the growth phase of the cells...even after many cell generations most of the cells in a de-differentiated population are capable of re-expressing their differentiated phenotype.

Thus dedifferentiation appears to be a reasonable explanation for the loss of phagocytic capabilities by the STE 137 cell line; whether these capabilities could be recovered in the proper microenvironment is open to debate.

#### Primary Monolayer Cell Cultures from Kidney and Spleen

The viability of primary monolayer cell cultures from kidney and spleen was 90% as determined by trypan blue dye exclusion. Both the kidney and spleen cultures grew readily, producing epithelial-like monolayers in 2 to 4 days. However, the cells did not phagocytize the kidney disease bacillus, regardless of the serum concentration or the age of the cells.

These results are not fully understood. The absence of detectable phagocytosis may be a reflection of the methods used for harvest and culture of the kidney and spleen cells, rather than the phagocytic capabilities of these tissues. In trout, the kidney is the principal site of hematopoiesis. Because the hematopoietic tissue is somewhat loosely aggregated, it is conceivable that the bulk of the leucocytes and leucocyte blast population was released during the first hour's digestion and thus discarded. This would leave a heterogeneous population of tubule cells, melanocytes, fibroblasts and other cells which probably would not exhibit phagocytosis.

### Leucocyte Cultures

The viability of the leucocyte cultures was 85% as determined by trypan blue dye exclusion. Both the heparin/sedimentation and the silicon/centrifugation methods of leucocyte harvest gave good yields. The leucocytes grew readily; maximum mitotic activity occurred after 108 hours of incubation at 18 C. The leucocytes did not phagocytize the kidney disease bacilli, regardless of the serum concentration or the age of the cells.

### Mouse Macrophages

Mouse macrophages readily ingested the kidney disease bacilli (Figure 1) as determined by phase contrast microscopy; it was not uncommon for a single macrophage to contain 50 to 75 bacilli. These results show that the experimental parameters used in the STE 137 and kidney and spleen primary cell culture experiments are conducive to phagocytosis and that the kidney disease bacilli are phagocytizable particles. Mouse macrophages were chosen because many of the difficulties inherent in in vitro phagocytosis studies have been resolved in this system.

### Uncultured Leucocytes

Viability of uncultured leucocytes was approximately 95% as determined by trypan blue dye exclusion. Attachment without ingestion was observed in some cases; however,

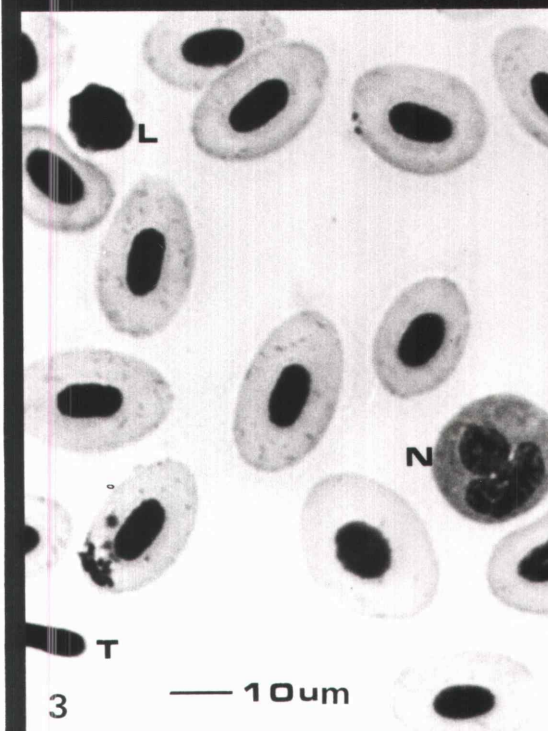
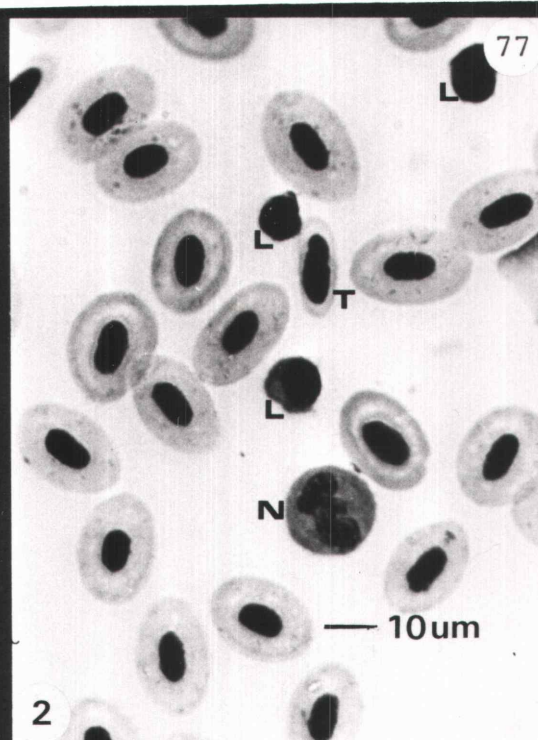
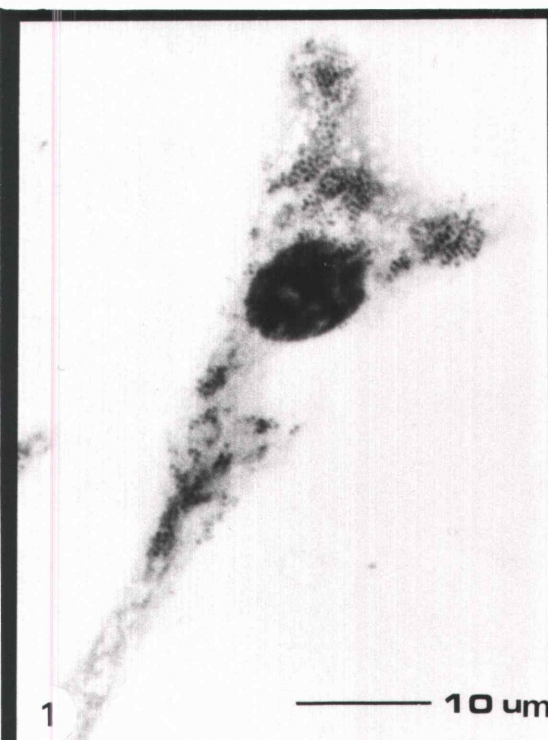


Figure 1. Mouse macrophage containing numerous phagocytized kidney disease bacilli. Giemsa stain. 2235 X.

Figures 2 & 3. Blood films from normal, hatchery-raised, 12.5 to 17.5 cm brook trout. Lymphocyte (L), neutrophil (N), thrombocyte (T). May-Grunwald Giemsa stain. Figure 2, 1260 X. Figure 3, 1375 X.

phagocytosis could not be demonstrated by phase contrast microscopy. These results are not surprising when the differential blood counts of the fish are taken into consideration.

The differential counts of blood films from normal, hatchery-raised, 12.5 to 17.5 cm brook trout ( $N = 60$ ) are given in Table 1. These counts are from the same fish used in the phagocytosis work described above. The criteria used in cellular classification were those of Lehmann and Stürenberg (1975), Finn and Nielsen (1971), and Jakowska (1956). Examples of each cell type are shown in Figures 2 and 3. Due to the many controversies in fish hematology over nomenclature, no attempt was made to distinguish between cells of the blast series.

The values given in Table 1 are in agreement with differential counts reported in the literature, some of which are summarized in Table 2. Other studies on sockeye salmon (Watson et al., 1956), rainbow trout (Weinreb, 1958), whitefish (McKnight, 1966), pike (Mulcahy, 1970), perch (Yokoyama, 1947), cyprinodontiform fishes (Gardner and Yevich, 1969), and platyfish and swordtails (Glucksman and Gordon, 1953) lend support to the observation that lymphocytes are the predominant white blood cells in fish and that in healthy fish, granulocytes are present as a small percentage of the white cell population.

Table 1. Differential blood counts from normal brook trout (Salvelinus fontinalis), May-Grunwald Giemsa stain.<sup>a</sup>

	Lymphocytes	Thrombocytes	Neutrophils	Macrophages <sup>b</sup>	Blast forms
Range	70-98	0-27	0-7	0-1	0-9
Mean	82.4	12.1	2.4		2.6
Standard deviation	7.1	7.0	1.7		2.1
Standard error	0.9	0.9	0.2		0.3
95 percentile range	80.6-84.3	10.3-13.9	1.9-2.8		2.1-3.2

<sup>a</sup>All information is for healthy, hatchery-raised, 12.5 to 17.5 cm fish; complete data given in Appendix Table 1.

<sup>b</sup>Macrophages were present in the blood films from 3 fish; most authors consider the presence of macrophages in the circulating blood abnormal.

n = 60

Table 2. A summary of differential blood counts of normal salmonid fishes reported in the literature.

Investigator/species of fish	Percent lymphocytes		Percent granulocytes		Percent thrombocytes	
	mean±SE	range	mean±SE	range	mean±SE	range
McCarthy <u>et al.</u> (1973): Rainbow trout, Kamloops variety ( <u>Salmo gairdneri</u> Richardson)	93.5	85- 99	4.8	1- 9	1.8	1-6
McCarthy <u>et al.</u> (1975): Rainbow trout, Shasta variety ( <u>Salmo gairdneri</u> Richardson)						
immature		79- 98		2-18		
mature males		91-100		0- 9		
mature females		89- 99		1-11		
Conroy (1972): Atlantic salmon ( <u>Salmo salar</u> L.)						
fingerlings	84	76- 92	16	8-24		
parr	88.25	80-100	11.75	0-20		
smolts	95	92-100	5	0- 8		
Blaxhall & Daisley (1973): Brown trout ( <u>Salmo trutta</u> )	90±8.9		<sup>a</sup> 6.6±6.5	0-25		
			<sup>b</sup> 1.4±2.3	0.11		
			<sup>c</sup> 1.6±1.9	0- 8		
			<sup>d</sup> 0.3±0.7	0- 4		
Lieb <u>et al.</u> (1953): Alaskan lake trout ( <u>Cristovomer namaycush</u> Walbaum)	91.2		<sup>e</sup> 8.2			
			<sup>f</sup> 0.6			
American grayling ( <u>Thymallus signifer</u> Richardson)	97.5		<sup>e</sup> 1			
			<sup>f</sup> 1.5			

<sup>a</sup> neutrophils; <sup>b</sup> metamyelocytes; <sup>c</sup> myelocytes; <sup>d</sup> blast forms; <sup>e</sup> monocytes; <sup>f</sup> neutrophils

Infection with the kidney disease diplobacilli may trigger granulocytopoiesis. Yasutake (1969) has reported that hematopoietic hyperactivity as evidenced by lymphocytopenia and an increased number of blast cells in the kidney as well as vascular leucocytosis, occurs during the first week of experimental infection with kidney disease. Further studies of hematological changes during the course of infection would add greatly to our knowledge of the pathology of this disease.

Taken together, the work of Post (1963), Bell (1976), and the results presented in this paper suggest that phagocytosis in the peripheral blood of healthy salmonids is not a major factor in the removal of invading bacteria due to the small number of cells with phagocytic capabilities.

Where, then, does phagocytosis take place? The experiments of Ellis and Munroe (1976), Hatai (1972a) and Bell (1977) suggest that the kidney is an important site of phagocytosis in the teleost. The renal portal system may provide an extensive filtration bed for returning venous blood, thereby allowing entrapment of bacteria and other foreign bodies. The liver, heart, peritoneum, spleen and gills also play an important role in phagocytosis. These conclusions are further supported by a close examination of the histopathology of this disease.



## Histopathology

The histopathology of bacterial kidney disease is described by organ system in the following sequence: kidney, liver, pancreas, gut, heart, gills, brain. A brief description of the normal histology as observed in normal trout is given, followed by the gross and microscopic pathology.

### Kidney

In trout, the kidneys occupy a dorsal retroperitoneal position along the entire length of the body cavity and are bounded by the vertebrae, the ribs and the gas bladder. Ogawa (1961) has classified the teleostean kidney into five configurational groups. In this classification, the Salmonidae belong to type I in which the two sides of the kidney are completely fused and there is no clear distinction macroscopically between the anterior and posterior kidney.

In normal trout, the anterior kidney is composed of hematopoietic tissue interspersed with melanin granules. This tissue serves as the primary blood-forming organ in teleosts although some hematopoiesis occurs in the circulatory system. Interrenal tissue, homologous to the adrenal cortex in mammals, is found scattered along the larger blood sinuses (branches of the post-cardinal veins). Renal tubules are sparse in the anterior kidney but become more numerous toward the caudal region. The corpuscles of

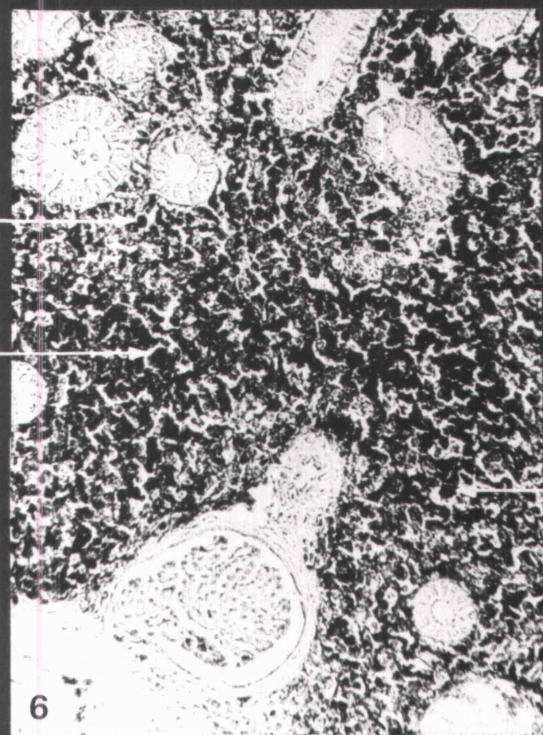
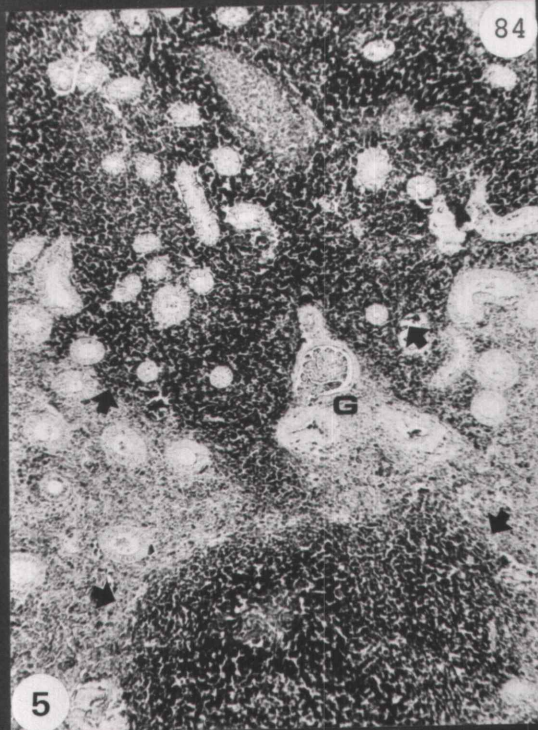
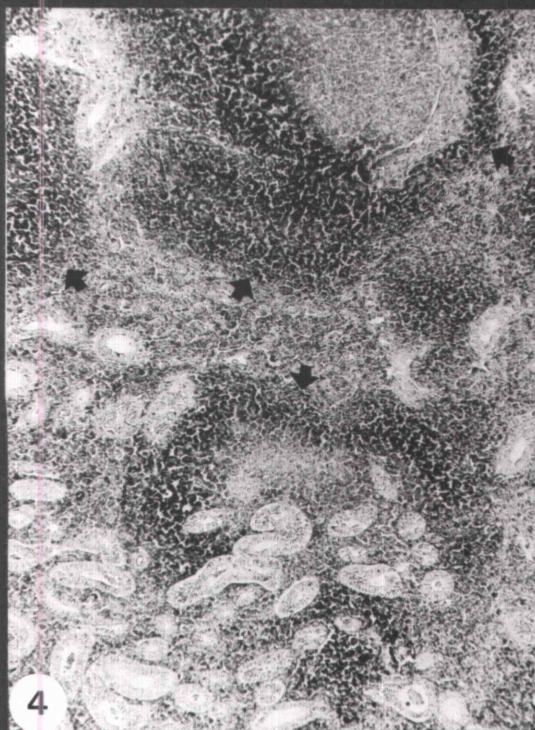
Stannius are located on the dorsal side of the middle part of the kidney.

The posterior kidney consists of numerous nephrons and ducts surrounded by hematopoietic tissue and melanin granules. The typical nephron is composed of the renal corpuscle (glomerulus and Bowman's capsule), neck segment, first and second segments of the proximal convoluted tubule, distal segment and the collecting tubules, which empty into the mesonephric duct. Chromaffin tissue, homologous to mammalian adrenal medullary tissue, is found in the posterior kidney within the walls of the renal blood vessels.

In fish with bacterial kidney disease, the kidney appeared swollen and grayish-white upon gross examination. Distinct grayish-white lesions 1 to 5 mm in diameter were observed beneath the capsule of the kidney on the ventral side. Upon dissection, these lesions were found throughout the kidney although they were especially prominent in the posterior kidney. In some specimens the disease had progressed to the point where tissue destruction was so widespread that all integrity was lost, and the kidney appeared as a soft, pulpy mass.

Histologically, the lesions occurred in the interstitium as distinct colonies of bacterial cells surrounded by inflammatory cells (Figures 4-6). At first, the interstitial infiltration appeared to consist predominantly of

Figures 4-16. All sections are from brook trout (Salvelinus fontinalis) naturally infected with coryne-  
bacterial kidney disease. Brown-Brenn stain,  
unless indicated otherwise.



Figures 4-6. Kidney.  
Extensive destruction  
(arrows) of interstitium  
and tubules.

Figure 4. 75 X.

Figure 5. An intact glomer-  
ulus (G). 90 X.

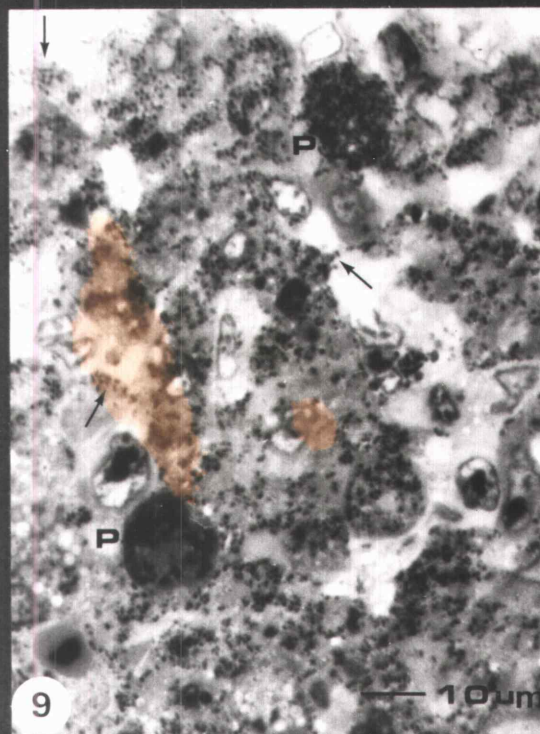
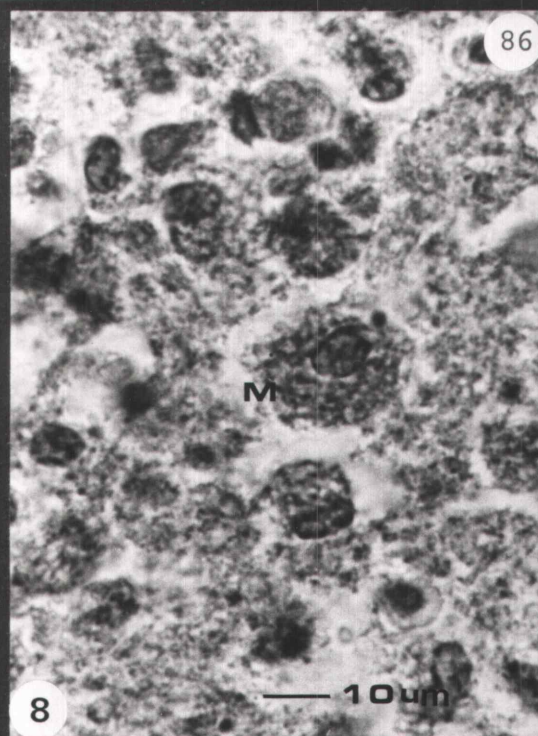
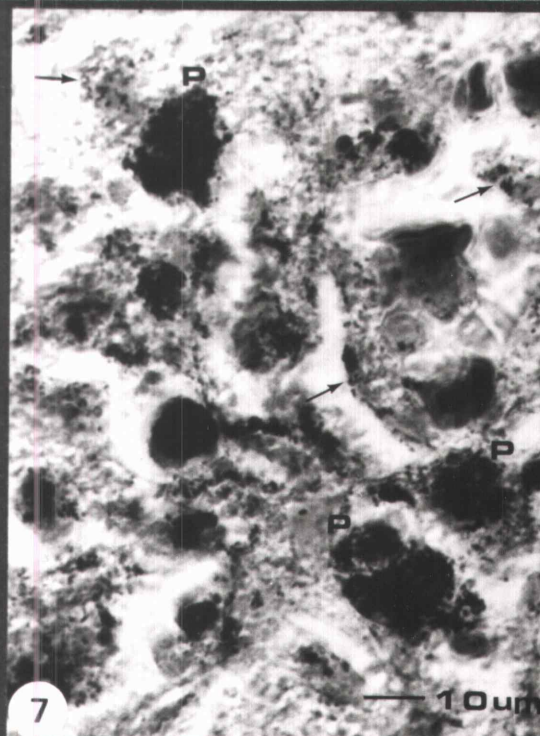
Figure 6. Macrophages  
(arrows) filled with  
bacteria. 450 X.

lymphocytes, but closer examination revealed an underlying network of macrophages obscured by the presence of other cells (Figure 7). The cytoplasm of most macrophages was amphophilic, granular and vacuolated. Several degenerating macrophages with karyorrhectic nuclei were noted; some were filled with bacteria, while others appeared to have burst, releasing bacteria into the interstitium (Figures 7-9). Very few polymorphonuclear leucocytes (PMNs) were observed. This was especially surprising in an infection of such magnitude; one would expect to see massive numbers of PMNs with some monocytic infiltration. The large (20  $\mu$ m), polygonal, inflammatory cells described by Wolke (1975) were probably epithelioid cells. No epithelioid cells were observed in the fish examined.

Some nephrons remained intact even when the interstitium surrounding them was completely destroyed (Figures 4-6). In most instances, though, the tubules in the center of a lesion were completely obliterated and the necrotic focus appeared as amorphous, granular debris seemingly composed of fragmented, coagulated cells. It is remarkable that fish survived to the point of such extensive tissue destruction.

Extensive bleeding was noted in the center of larger lesions. This, combined with the vast destruction of hematopoietic tissue, was undoubtedly responsible for the lowered hematocrits reported in fish suffering from bacterial kidney disease (Hunn, 1964). Large accumulations of





#### Figures 7-9. Kidney.

Figure 7. Typical lesion: amorphous, granular debris, intra- and extracellular bacteria (arrows). Note the phagocytic cells (P) packed with bacteria. 1305 X.

Figure 8. Hematoxylin and eosin stain. Macrophage (M). 1375 X.

Figure 9. Alkaline toluidine blue stain, embedded in modified Epon mixture. Two phagocytes (P), numerous extracellular bacteria. 1205 X.

fat were observed in some sections stained with toluidine blue. However, this was probably a non-specific response to stress, rather than part of the specific pathology of this disease. No giant cell formation, fibrosis or walling off of lesions was observed.

### Liver

In normal trout, the liver ranges in color from light to dark reddish-brown. It is surrounded by a capsule of connective tissue which extends inward as trabeculae along the ducts and major blood vessels. The lobules are generally less defined than in higher vertebrates. The muralia are two cell layers thick and are often irregularly arranged around the central veins. Frequent rosettes of six to eight hepatic cells suggest some cord-like arrangement. The hepatocytes (parenchyma) are polyhedral with a spherical nucleus and are often filled with glycogen, giving a vacuolated appearance. Sinusoids are lined by reticular and von-Kupffer-like cells.

The gross pathology of the liver was highly variable, ranging from no noticeable changes to extremely enlarged, pale livers studded with lesions 5 mm in diameter. In most cases, the liver was noticeably enlarged and had a discolored or mottled appearance.

The microscopic pathology was basically the same as in the kidney, with localized growth of bacteria forming

discrete lesions surrounded by a mixed, non-specific inflammatory response consisting of lymphocytes, some degenerating neutrophils, and macrophages (Figures 10, 11). As in the kidney, the center of the lesions was a mass of amorphous, granular debris apparently composed of fragmented, coagulated cells.

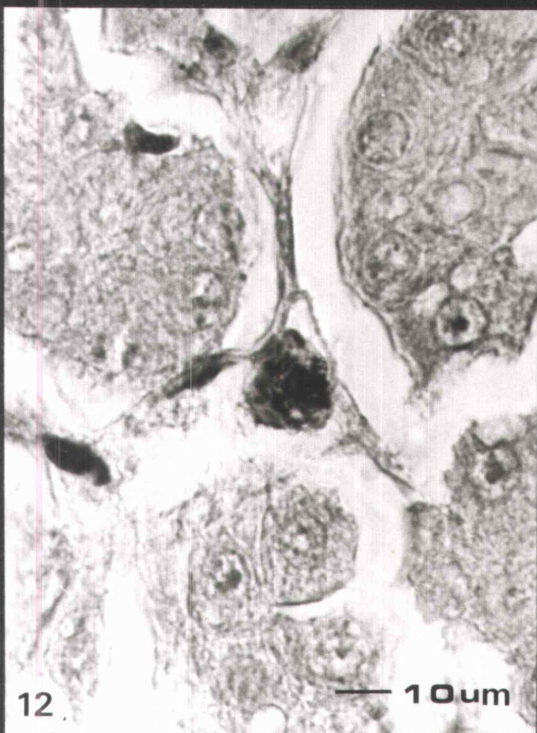
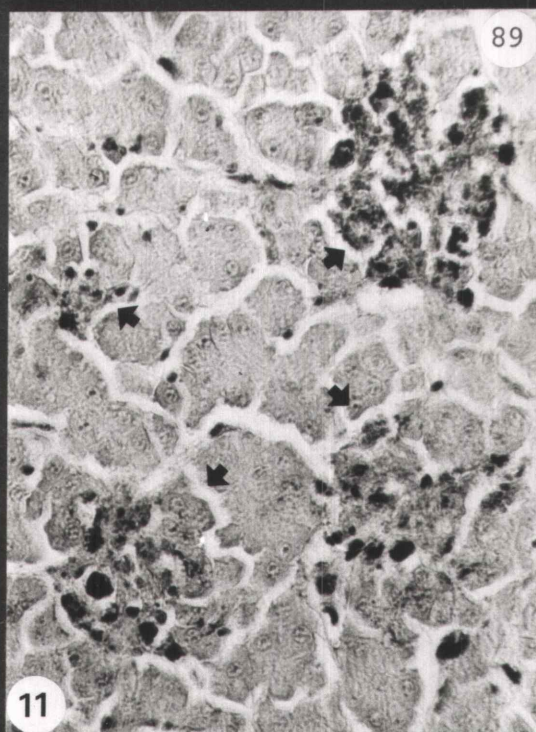
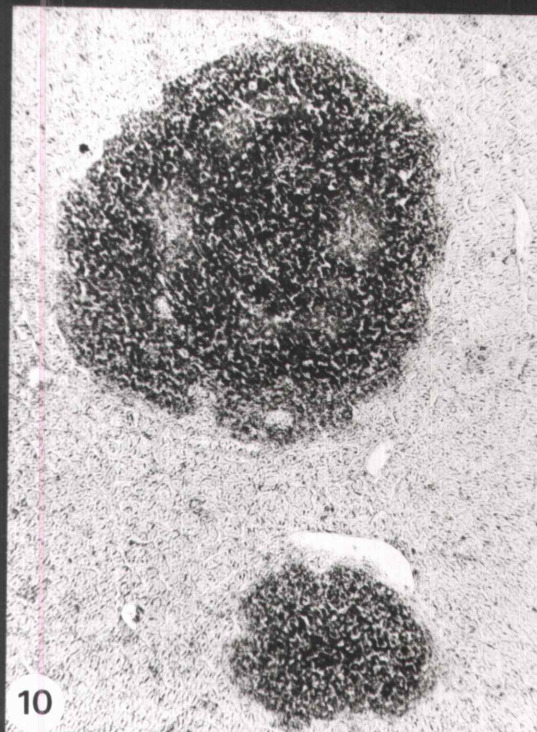
The inflammatory response was less dramatic in the liver; this was probably due to the larger numbers of stem cells and leucocytes present in the kidney, as compared to the liver. Although macrophages were present, there was no extensive infiltration. No cytoplasmic inclusion bodies (Wood and Yasutake, 1956a) were observed in the parenchymal cells although intracellular bacteria were common.

Pearsall and Weiser (1970) state that Kupffer cells constitute approximately 30% of the nucleated cells of the liver. These cells were especially prominent at 400X with the Brown-Brenn stain in which scattered pockets of bacteria appeared bluish-purple in contrast with the yellowish-green cytoplasm (Figure 11). A single von-Kupffer-like cell gorged with bacteria is shown in Figure 12.

#### Pancreas

The pancreatic tissue of the trout is diffuse, occurring predominantly in the mesentery or fascia of the caeca. The pancreas is made up of two types of tissue: the endocrine islets of Langerhans surrounded by exocrine





Figures 10-12. Liver.

Figure 10. Two hepatic lesions. 30 X.

Figure 11. Scattered pockets of infection. 400 X.

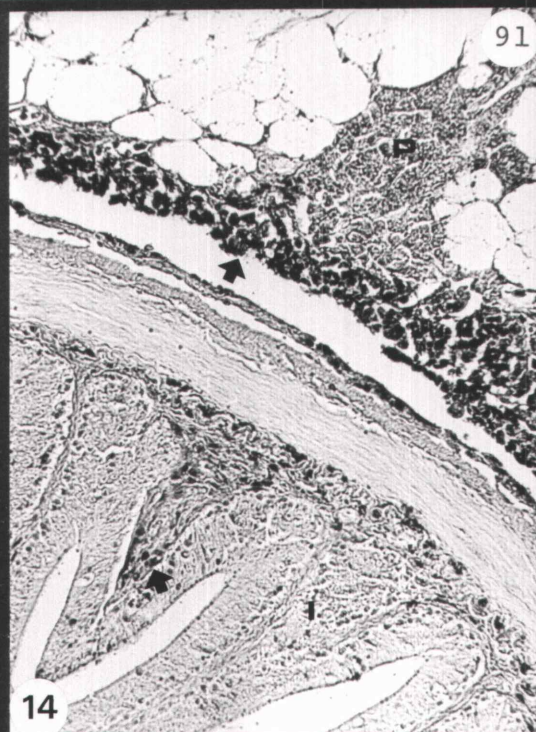
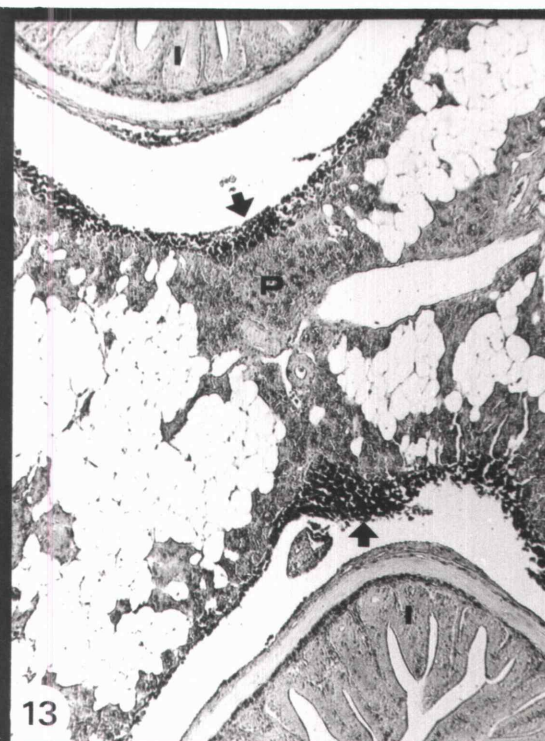
Figure 12. Von Kupffer-like cell with phagocytized bacteria. 1375 X.

glandular tissue which secretes digestive enzymes. Fat is often interspersed among the pancreatic acini. The islets of Langerhans occur as irregular, branching cords of cells of varying size which are scattered throughout the more compact acinar cells. The cells of each acinus are pyramidal, with a characteristic basal nucleus. The apex of the cell contains numerous zymogen granules.

Bacterial cells were observed primarily along the margins of the pancreas (Figures 13, 14). This location implies spread via a peritonitis. Destruction and necrosis of the acinar cells with minimal inflammation occurred where bacteria were present in sufficient numbers. Some intracellular bacteria were observed within acinar cells. The lack of involvement of the islets of Langerhans was probably due to their relative inaccessibility rather than any particular characteristic of the cells. No cytoplasmic inclusion bodies such as those described by Wood and Yasutake (1956a) were observed.

#### Gut

The stomach wall in trout, as in other vertebrates, is composed of four layers; the tunica serosa (outermost layer), the muscularis externa, the submucosa, and the mucosae (innermost layer). The serosa consists of a single layer of epithelial cells and supportive loose connective tissue. The muscularis externa is made up of two layers:



Figures 13, 14. Pancreas  
(P), intestine (I).

Figure 13. 75 X.

Figure 14. Lesions (arrows)  
occur predominantly along  
the margins of the pan-  
creas and in the tunica  
serosa of the intestine.  
400 X.



an outer longitudinal layer and an inner circular layer twice the thickness of the outer layer. The submucosa consists of irregular connective tissue containing muscle fibers and scattered blood vessels. The mucosa includes five distinct regions: the muscularis mucosa, the stratum granulosum, the stratum compactum, the lamina propria, and the epithelium. The muscularis mucosae is an irregular network of circular and longitudinal smooth muscle fibers adjacent to a layer of granular cells (stratum granulosum). The stratum compactum is a dense, longitudinally oriented layer of collagenous fibers apposed to the lamina propria, a layer of compact areolar tissue containing numerous capillaries and nerves.

The wall of the intestine is similar to the stomach wall except that it lacks the submucosa and muscularis mucosae.

Bacilli were present intra-and extracellularly in the serosa and the lamina propria (Figures 13, 14). Some bacteria were also observed in the stratum granulosum and, rarely, in the muscularis. Inflammatory cells were uncommon.

### Heart

The heart of the trout consists of a series of four enlargements: the sinus venosus, the atrium, the ventricle, and the bulbus arteriosus. Histologically, the

cardiac muscle resembles that of other vertebrates, but the atrial and ventricular walls are relatively thinner while the bulbus arteriosus is strongly elastic. The ventricular wall is made up of two layers: a superficial (cortical) layer in which the fibers form a branching network and an inner (spongy) layer where the fibers extend into the lumen as trabeculae. Intercalated discs and cross-striations can be observed at high magnifications.

Although reticulo-endothelial cells surrounding muscle groups in the heart showed intense phagocytic activity, there was no "massive myocarditis" as described by Wood and Yasutake (1956a). This may be due to the fact that the cases reported here were from fish suffering acute, rather than chronic infections. Other workers (Mackmull and Michels, 1932; Ellis and Munroe, 1976; Herman, 1975) have reported active phagocytosis by teleost cardiac tissue.

### Gills

Tapered and flattened gill filaments extend from the four branchial arches arising on either side of the pharynx. Each gill filament contains an afferent and efferent branch from the corresponding branchial artery. These vessels are separated by a rod of hyaline cartilage, and secondary lamellae extend from the dorsal and ventral surfaces of the filaments. Lamellae are covered by a layer (one or two cells thick) of interdigitating squamous epithelial cells

resting on a basement membrane and lined by a thin layer of connective tissue. The epithelial cells are separated from one another by pillar (pilaster) cells and blood capillaries in the form of a capillary plexus. Mucus-secreting goblet cells are scattered among the epithelial cells of the gill lamellae as well as in the basilar region. Chloride cells are located primarily in the epithelium separating successive lamellae and near the afferent vessel in the gill filament.

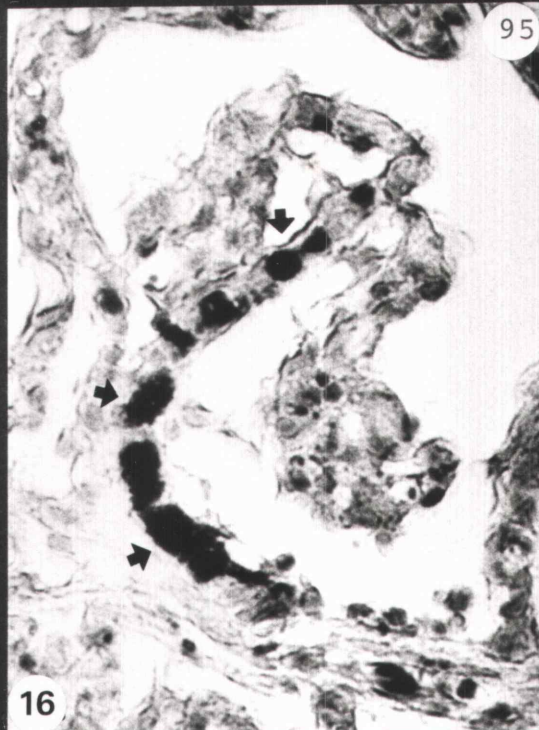
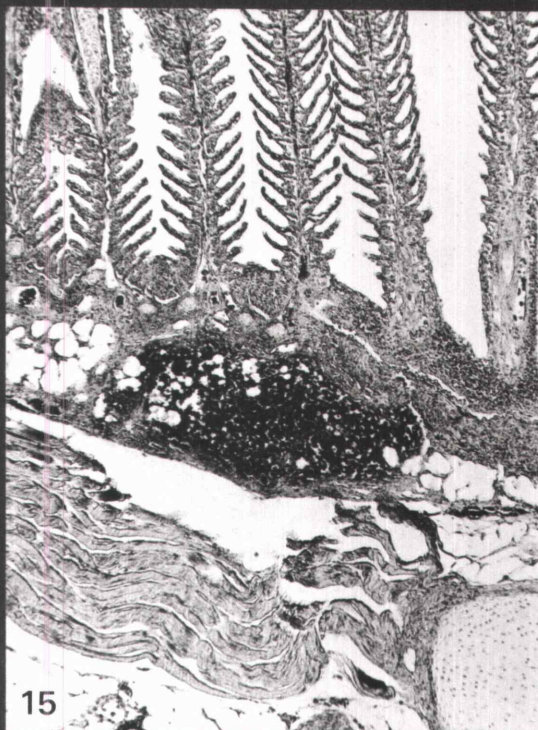
Typical lesions with a mild inflammatory response were noted at the base of the gills in several specimens (Figure 15). Free macrophages which had phagocytized large numbers of the bacteria were also observed in the gill filaments of fish with basilar lesions (Figure 16).

#### Brain

An excellent discussion of the normal histology of the trout brain and nervous system is presented in Anderson and Mitchum (1974) and Wales (in press). No lesions such as those described by Wood and Yasutake (1956a) were observed in the meninges or brain, although extracellular bacteria and macrophages which had phagocytized bacteria were noted.

Brunson and Gall (1971) define a granuloma as:

a nodular lesion characteristically containing a central mass of epithelioid and giant cells and a peripheral collection of small lymphocytes.



Figures 15, 16. Gill.

Figure 15. Large lesion at the base of the gills. 55 X.

Figure 16. Macrophages (arrows) containing phagocytized bacilli in the capillary of a gill filament. 350 X.

Robbins and Angell (1976) state:

a granuloma consists of a microscopic aggregation of plump fibroblasts or histiocytes (macrophages) that have been transformed into epithelial-like cells and are therefore designated epithelioid cells, surrounded by a collar of mononuclear leucocytes, principally lymphocytes and occasionally plasma cells...the identification of a granulomatous reaction actually rests with the recognition of the conversion of macrophages into epithelioid cells.

According to these two widely accepted definitions, the lesions of bacterial kidney disease in this population of fish could not be called true granulomas, since epithelioid cells, giant cells and fibrosis are absent. Furthermore, the lesions are distinctly different from the classic focal granulomas of piscine tuberculosis or flavobacteriosis (Parisot, 1958; Nigrelli and Vogel, 1963; Parisot and Wood, 1966; Wolke, 1975). Perhaps the disease is best described as a "bacteremia characterized by a systemic, diffuse...histocytic inflammation" (Wolke, 1975).

The magnitude of the inflammatory response was much less than would be expected with the massive numbers of bacteria present. This implies that the bacteria are of low virulence and show a high degree of host adaptation. Some of the extensive tissue destruction may have been caused by the accumulation of toxic bacterial metabolites. However, most of the pathological changes in this disease appear to be produced by the host's response rather than the bacterium.



The following sequence of events in the course of infection can be postulated. The kidney disease bacteria gain access through an unknown portal of entry and bacteremia ensues. Some phagocytosis occurs in the blood, but this is small in comparison to the uptake by other organs, especially the kidney. In the kidney, the renal portal system acts as an extensive filtration bed, causing mechanical sequestration of the bacteria which in turn enhances opportunities for engulfment by phagocytic cells. The bacteria are able to multiply within the host cells; this may be due to some intrinsic property of the bacteria (such as cell wall composition) which prevents lysis, or an inadequacy (or lack) of lysosomal enzymes in the phagocytic cells. Finn and Nielsen (1971) have reported the persistence of bacteria in trout phagocytes for up to 16 days, implying that lysosomal enzymes, if present, are only weakly active. Wolke (1975) states that on the basis of gross and histopathological examination, true pus is not a product of acute inflammations in fish. Further work in this area is needed to clarify our understanding of the piscine inflammatory response.

Eventually, the host cell is killed and bursts, releasing the bacteria; other cells phagocytize the free bacteria and the cycle continues, or the bacteria multiply extracellularly. The renal portal system, which originally sequestered bacteria, may also serve to disseminate them.

The systemic distribution of lesions implies a bacteremia early in the course of infection. Apparently, the kidney is the first organ affected, since lesions in this organ were always in a more advanced stage.

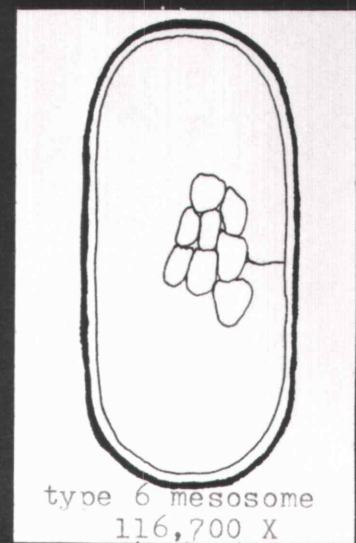
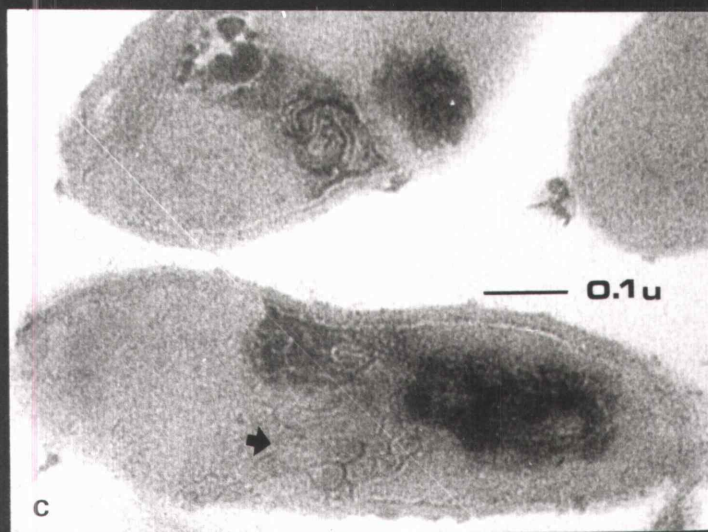
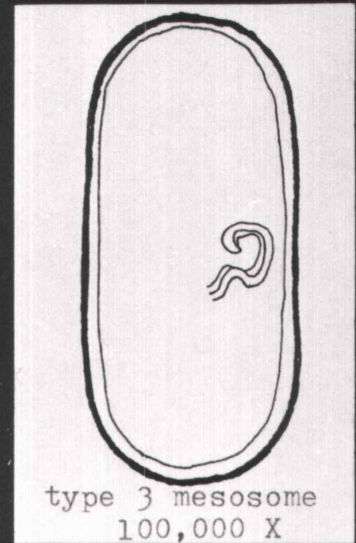
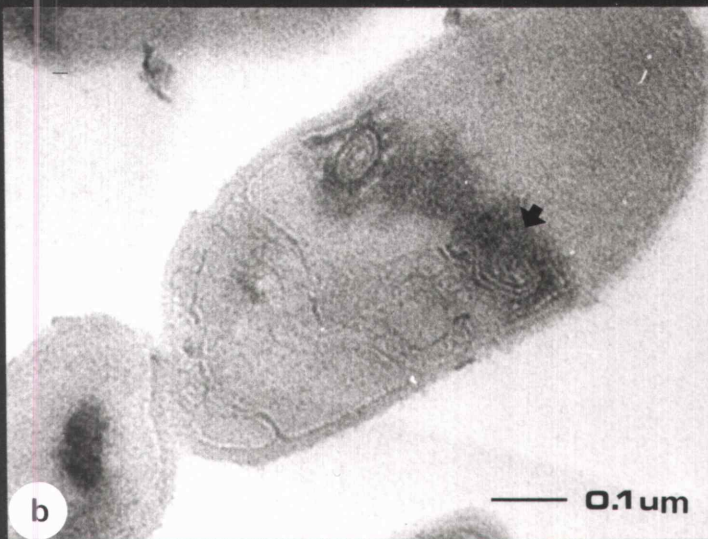
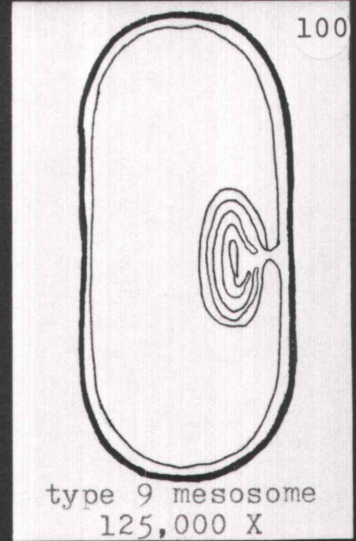
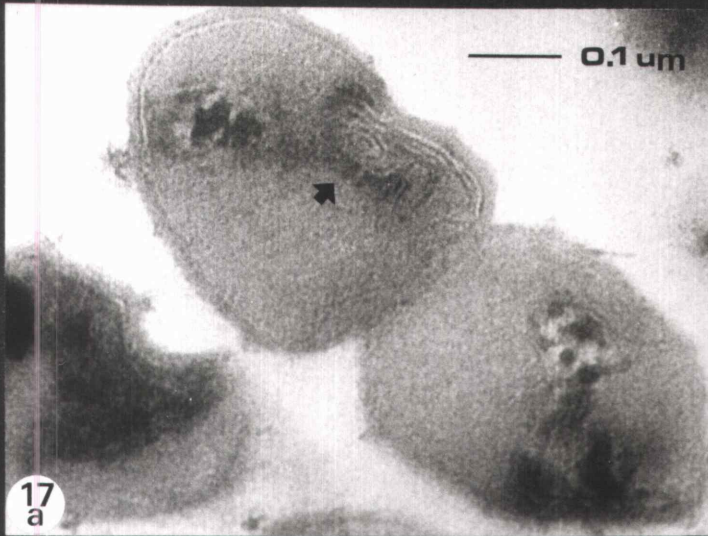
Intracellular sequestration as well as the inability of the phagocytic cells to kill the bacteria or degrade the dead organisms to diffusible products stimulates the inflammatory response. The pathological response of chronic inflammation is the result of this continued irritation. The macrophage is the basic architectural unit of chronic inflammation, while the presence of lymphocytes implies an immune (hypersensitivity) reaction.

### Electron Microscopy

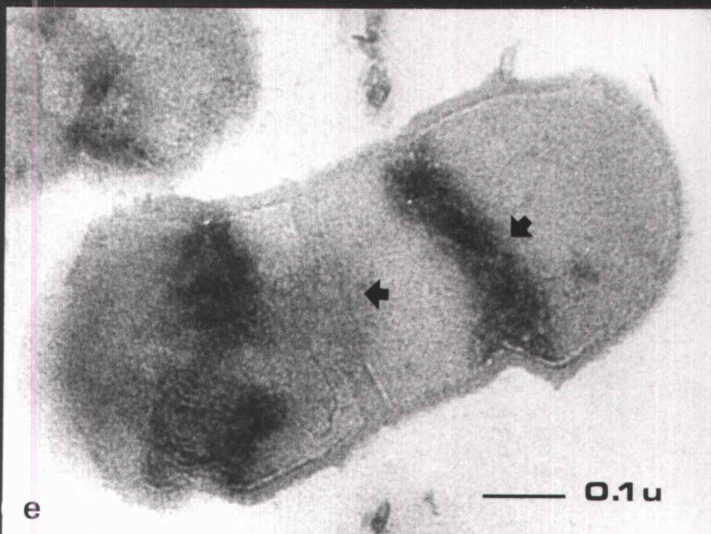
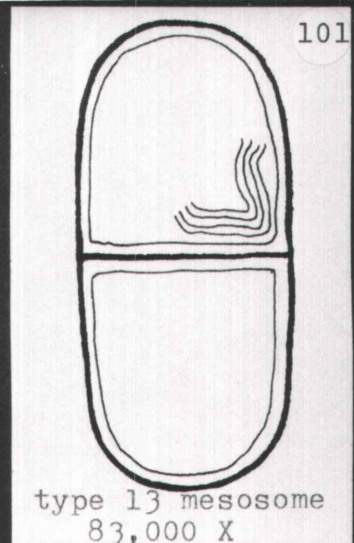
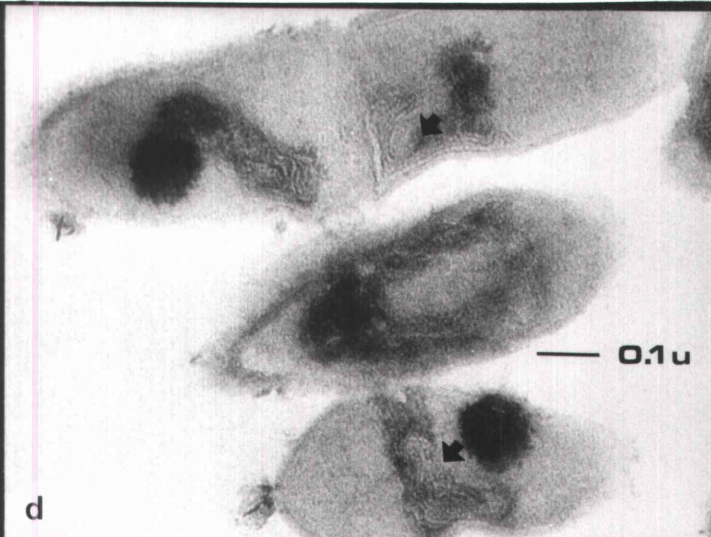
#### Cultured Kidney Disease Bacilli

The ultrastructure of the kidney disease bacterium is seen in Figure 17a-h. The cell wall thickness as seen in thin section varied from 8.0 to 18.6 nm. The wall appeared to consist of two layers: an outer layer of electron-dense fibrils of "fuzz," approximately 4.5 nm thick and an inner layer of homogeneous electron-density, approximately 14.0 nm thick. The outer layer was rarely intact, but vestiges of it could be seen in most bacteria. These dimensions are summarized in Table 3. It should be emphasized that these are relative, not absolute values, since growth conditions and differences in the method of fixation

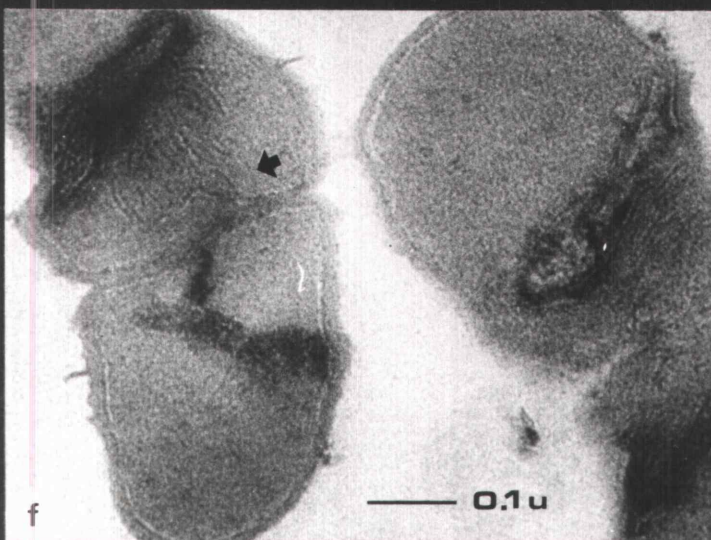
Figure 17, a-h. Morphology of the kidney disease bacillus cultured on cysteine serum agar. Types of mesosomes according to Ghosh (1974).

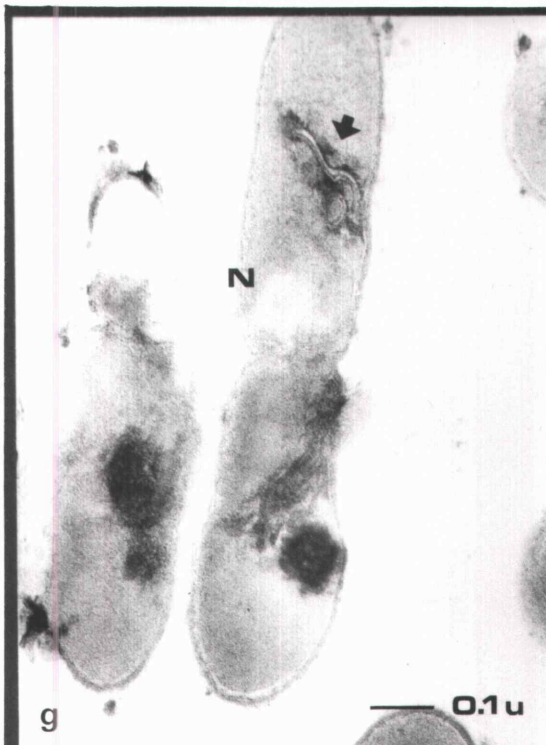




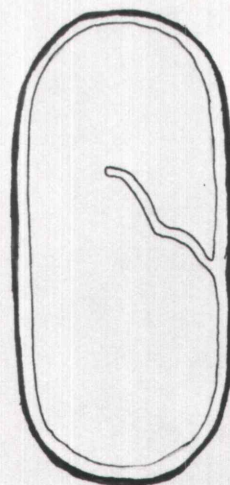


e, f. Note the involvement of mesosomes with septation. both photos 110,000 X.





type 5 mesosome  
79,500 X  
Nucleoid (N).



Not described by Ghosh;  
similar to type 9 meso-  
some. 74,000 X

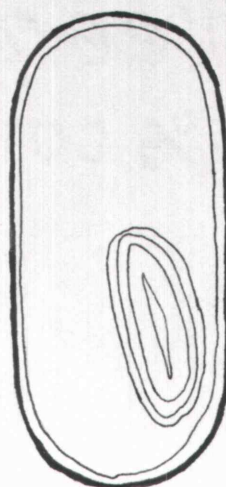


Table 3. Dimensions (in nm) of the cell wall and cytoplasmic membrane of kidney disease bacilli cultured on cysteine serum agar.<sup>a</sup>

	Cell wall	Cytoplasmic membrane			
		Total membrane	Outer layer	Middle layer	Inner layer
Mean	14.0	14.3	4.1	6.2	4.0
Range	8.0-18.6	8.0-21.3	2.7-5.3	4.0-10.6	2.7-5.3
Standard deviation	2.2	3.1	1.0	1.6	1.0
Standard error	0.3	0.6	0.2	0.3	0.2
95 percentile range	13.3-14.7	13.1-15.4	3.8-4.5	5.6-6.8	3.6-4.4

cell wall n = 43

cytoplasmic membrane n = 29

<sup>a</sup>Complete data given in Appendix Table 2.

can alter the dimensions of the cell wall and plasma membrane (Glauert and Thornley, 1969). The values reported are in agreement with those in the literature. Glauert (1962) found that the cell wall of Gram-positive bacteria varied in width from 15.0 to 35.0 nm; the microcapsule of Nocardia calcaria was 50 nm thick and the cell wall 15.0 nm. Holt and Leadbetter (1976) reported an outer layer of 4.0 to 5.0 nm and a cell wall of 7.0 to 16.0 nm in oral streptococci. The cell wall of Listeria monocytogenes varied from 18.0 to 30.0 nm (North, 1963; Edwards and Stevens, 1963; Ghosh and Murray, 1967).

The trilaminar-appearing cytoplasmic membrane lay immediately beneath the cell wall. The total membrane thickness varied from 8.0 to 21.3 nm with a mean of 14.3 nm, nearly twice the thickness of a unit membrane (Table 3). The membrane was composed of two electron-dense layers, each approximately 4.0 nm thick, separated by an electron-transparent layer 6.2 nm thick. In several places, the cytoplasmic membrane had "pulled away" from the cell wall, revealing the periplasmic space. This was probably the result of shrinkage during the dehydration process.

Mesosomes<sup>1</sup> were observed in 85 to 90% of the bacteria examined. Most bacteria contained only one mesosome, but

---

<sup>1</sup>Membranous cytoplasmic organelles, also referred to as peripheral bodies (Chapman and Hillier, 1953), chondrioids (Van Iterson, 1961), intracytoplasmic membranous elements (Koike and Takeya, 1961), and plasmalemmasomes (Edwards and Stevens, 1963).



approximately 30% of the bacteria examined appeared to have two or more. These may have actually been one continuous mesosome which appeared as two because of the plane of section.

Internal mesosome structure has been described by different investigators as tubular (Ryter, 1968), vesicular (Ryter, 1968), lamellar (Highton, 1969) or a combination of these. The mesosomes in this study consisted of concentric, lamellar whorls, although in a few instances the membranes appeared lamellar-vesicular or vesicular. Ghosh (1974) has classified mesosomes into 13 different types on the basis of appearance. Types 1, 3, 4, 6 and 9 were observed in this study (Figure 17a-h).

The intracytoplasmic membranes resembled the trilaminar limiting cytoplasmic membrane. They consisted of two electron-dense layers separated by an electron-transparent layer. Dimensions were similar to those described for the cytoplasmic membranes.

The location of mesosomes within the cytoplasm varied. They were frequently associated with what appeared to be nuclear material. There also appeared to be extensive mesosomal involvement with septa formation and division (Figure 17a-h).

The nuclear region of the bacterium was characterized by an area containing fine fibrils. The fibrils were numerous and compact and appeared as aggregates. The

cytoplasm of the bacterium was composed of fine granules and was similar to the cytoplasm of other bacteria examined by thin section.

In spite of a substantial body of literature, the origin and function of bacterial mesosomes remain obscure (Reusch and Burger, 1973; Ghosh, 1974; Greenawalt and Whiteside, 1975; Salton and Owen, 1976). Almost all of the major functions of the bacterial cell have been ascribed to the mesosome at one time or another. The proximity of the mesosome to the septal region of the cell has led some investigators to propose that mesosomes may be the site of some wall-membrane biosynthetic processes. However, comparisons of wall and membrane polymer and lipid synthesizing enzymes in mesosomal and plasma membrane fractions clearly show that only the muralytic enzyme is preferentially located in the mesosome (Owen and Freer, 1972; Forsberg and Ward, 1972; Ellar and Postgate, 1972). This has been confirmed in three independent studies using two different organisms. Two other enzymes, DD-carboxypeptidase and LD-transpeptidase appear to be equally distributed between plasma and mesosomal membranes or slightly greater in the plasma membrane (Forsberg and Ward, 1972; Joseph and Stockman, 1975). Workers agree that isolated mesosomal vesicles either lack or have substantially reduced complements of other plasma membrane enzymes and electron transport components such as ATPase, dehydrogenases and

cytochromes (Greenawalt and Whiteside, 1975; Owen and Freer, 1972; Reusch and Burger, 1973).

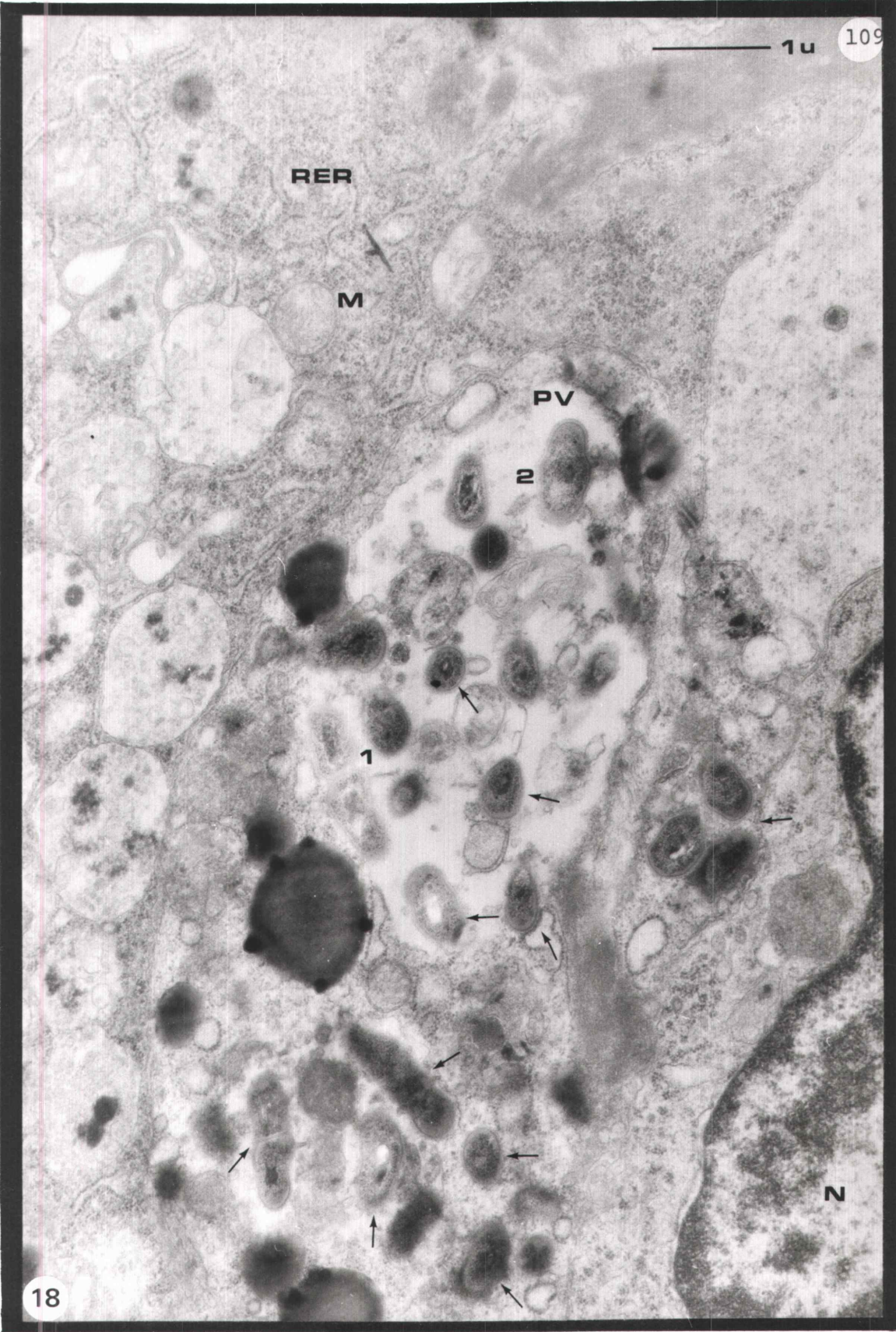
A few investigators feel that mesosomes are an artifact of fixation (Silva et al., 1976; Fooke-Achterrath, 1974; Higgins et al., 1975). There is some evidence to indicate that the size, number and distribution of mesosomes are affected by the method of fixation, but "irrespective of the technique of preparation, peripherally located and tightly organized small mesosomes could always be demonstrated" (Ghosh, 1974). Ghosh (1974) has suggested that mesosomes represent a stage in membrane evolution.

#### Kidney Disease Bacilli in Trout Tissue

Micrographs of the kidney disease bacilli in naturally infected kidney tissue from eastern brook trout are seen in Figures 18-20. The most striking difference between the cultured bacteria and the bacteria from infected tissues is the uniform size of the latter. This is not surprising since the host offers optimal conditions for growth. The mean length of bacilli from infected tissues was 0.65  $\mu\text{m}$  and the mean width was 0.39  $\mu\text{m}$  (Table 4). It was not possible to compare these dimensions with similar measurements from cultured bacteria since there was so much variation in the size and shape of the latter.

The mean cell wall thickness was 16.1 nm as compared to 14.0 nm in the cultured bacteria. The mean thickness

Figure 18. Morphology of the kidney disease bacillus in kidney tissue from naturally infected brook trout (Salvelinus fontinalis). Bacteria (arrows) occur predominantly within the membrane-bound phagocytic vacuole (PV). Mitochondrion (M), nucleus (N), rough endoplasmic reticulum (RER). Enlargements of selected bacilli (1, 2), page 110. 21,800X.





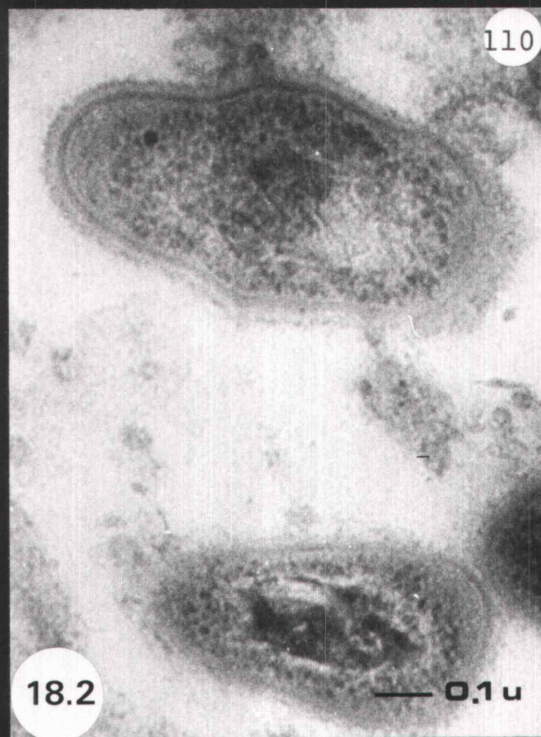
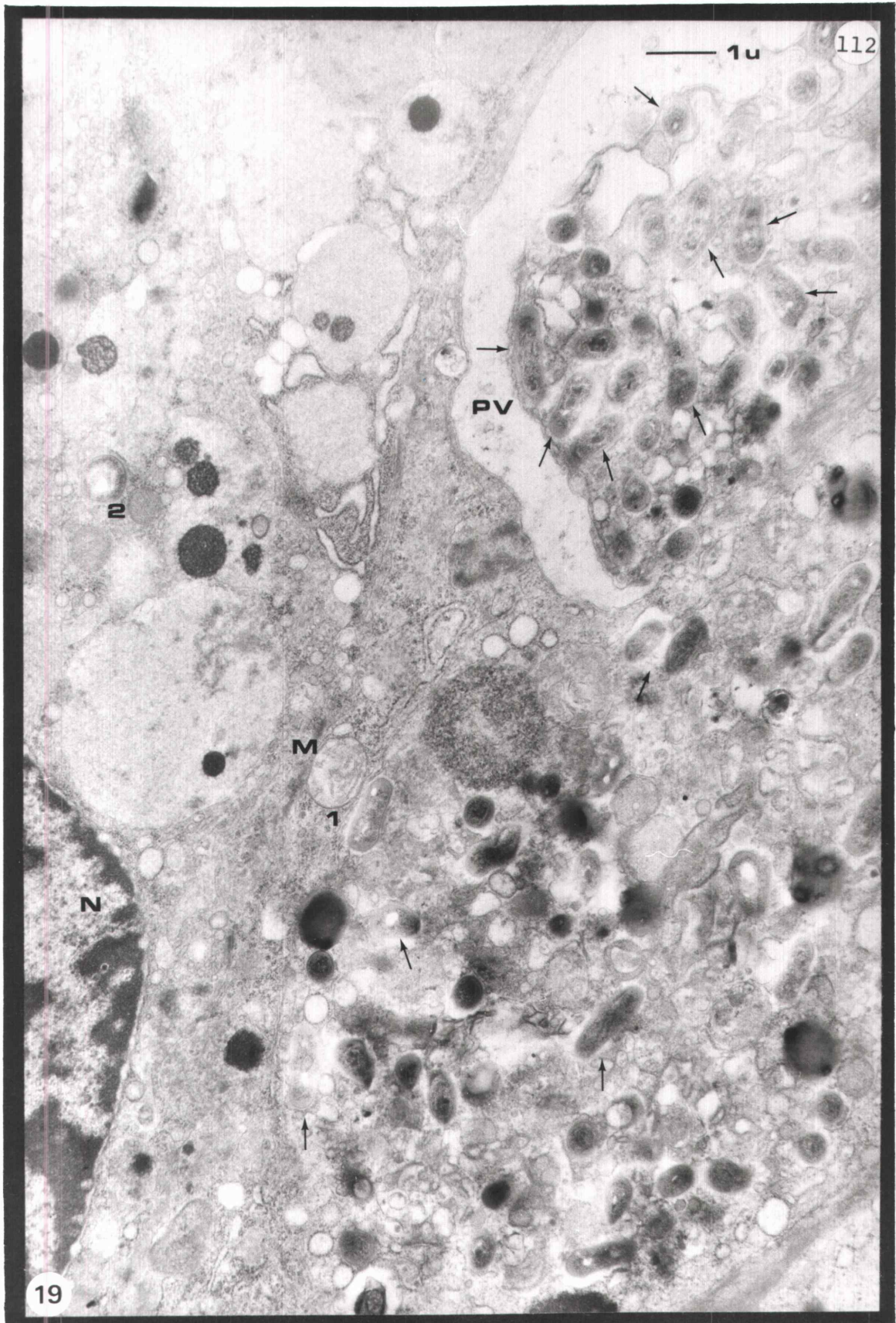


Figure 18.1. Enlargement  
of selected bacilli.  
70,000 X.

Figure 18.2. Same.  
77,000 X.

Figure 19. Morphology of the kidney disease bacillus in kidney tissue from naturally infected brook trout (Salvelinus fontinalis). Bacteria (arrows), mitochondrion (M), nucleus (N), phagocytic vacuole (PV). Enlargements of selected bacilli (1, 2), page 113. 13,400X.





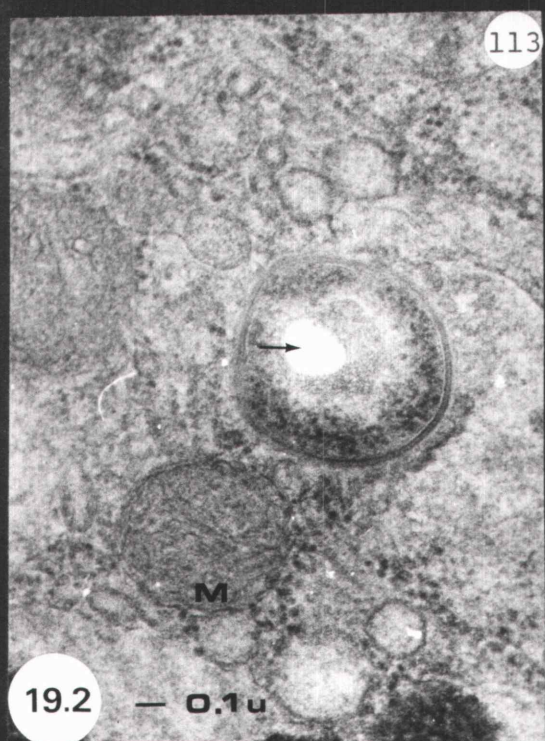
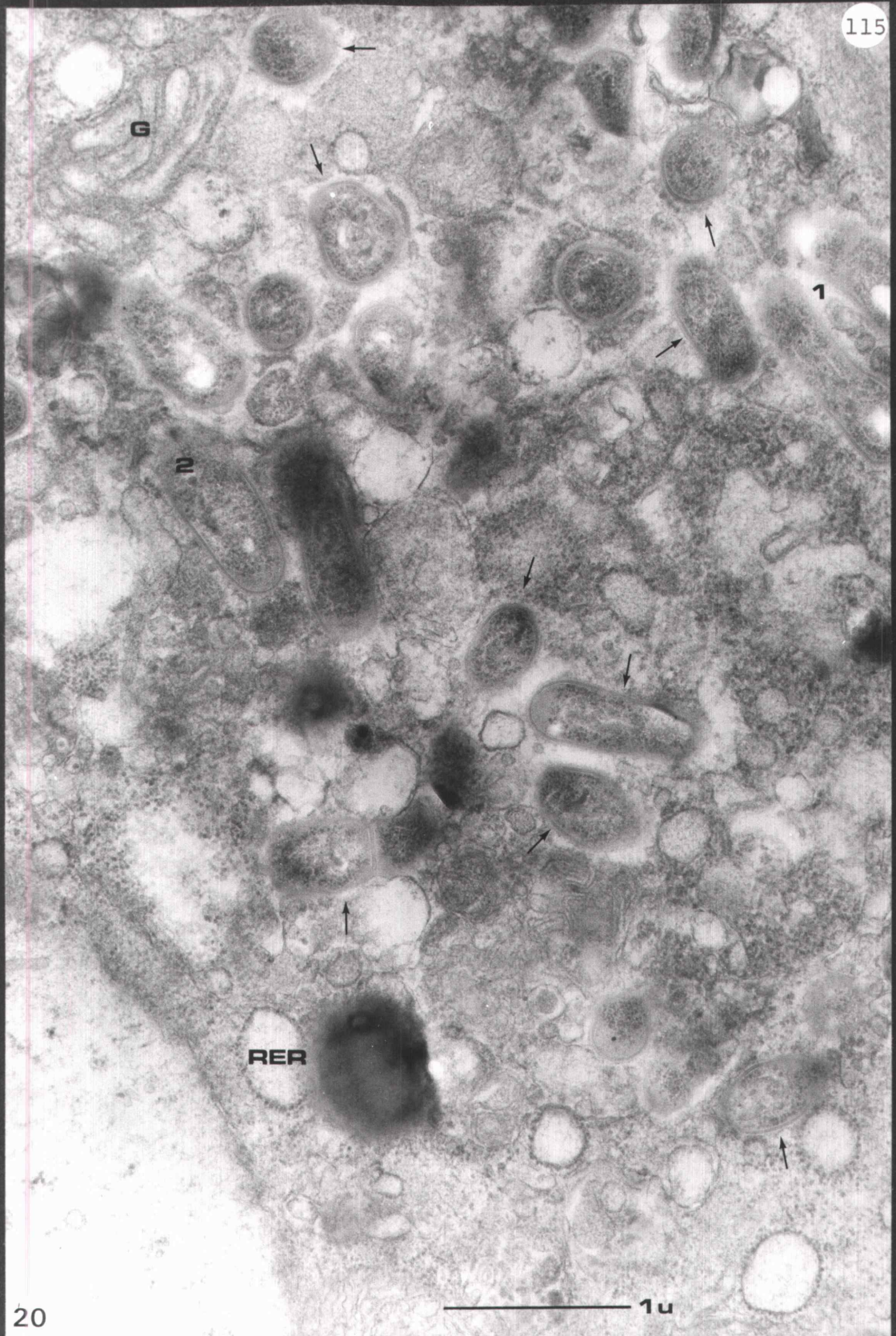


Figure 19.1. Enlargement  
of selected bacilli.  
56,400 X.

Figure 19.2. The electron-  
transparent areas may be  
lipid deposits similar to  
those observed in Coryne-  
bacteria and Mycobacteria  
43,900 X.

Figure 20. Morphology of the kidney disease bacillus in kidney tissue from naturally infected brook trout (Salvelinus fontinalis). Bacteria (arrows), Golgi apparatus (G), rough endoplasmic reticulum (RER). Enlargements of selected bacilli (1, 2), page 116 . 32,900X.





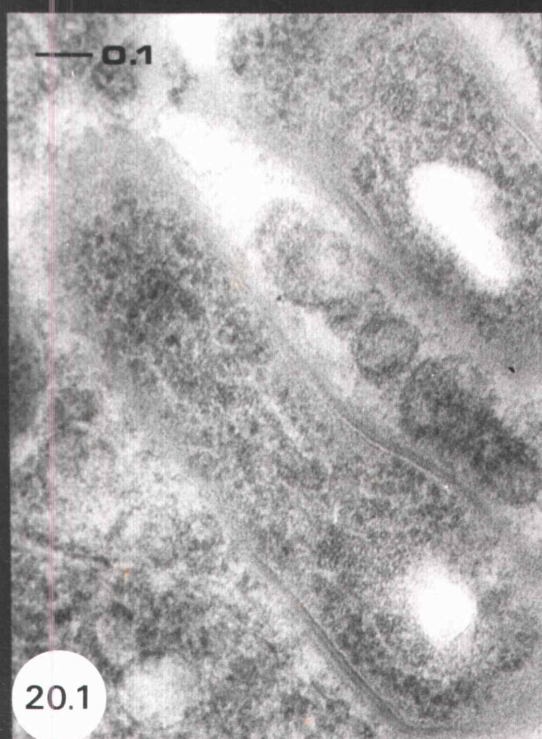


Figure 20.1. Enlargement  
of selected bacilli.  
70,500 X.

Figure 20.2. Same.  
86,900 X.

Table 4. Dimensions of the kidney disease bacillus, its cell wall and cytoplasmic membrane in kidney tissues from diseased brook trout (Salvelinus fontinalis).<sup>a</sup>

	Length ( $\mu\text{m}$ )	Width ( $\mu\text{m}$ )	Cell wall thickness (nm)	Cytoplasmic membrane (nm)
Mean	0.65	0.39	16.1	8.8
Range	0.37-0.96	0.33-0.44	7.2-28.8	7.2-13.2
Standard deviation	0.19	0.03	4.7	2.4
Standard error	0.05	0.01	0.9	1.0
95 percentile range	0.56-0.74	0.37-0.40	14.2-17.9	6.9-10.7

length,width n = 18

cell wall n = 23

cytoplasmic membrane n = 6

<sup>a</sup>Complete data given in Appendix Table 3.



of the cytoplasmic membrane was 8.8 nm in the bacilli in infected tissues and 14.3 nm in the cultured bacteria.

Mesosomes were observed in some bacteria but were not as abundant as in the cultured bacilli. This may have been due to the difference in the method of fixation. Electron-transparent areas were observed within some bacteria; these may have been lipid deposits similar to those seen in species of Corynebacteria and Mycobacteria (Barksdale, 1970). Cytoplasmic granularity was pronounced.

Both intra- and extracellular bacteria were observed in host tissues. There was no evidence of lysis or degradation of bacteria. Myelin figures and fat deposits, both characteristic of non-specific pathological stress, were common in the host tissues.

## V. SUMMARY AND CONCLUSIONS

1. An indirect fluorescent antibody technique was developed which allowed differentiation of intra- and extracellular bacteria in phagocytosis studies.
2. Contrary to previous observations made at four to five passages, STE 137 cells (used from 150 to 156 passages) did not exhibit phagocytic capabilities as determined by phase contrast and immunofluorescent microscopy. Attachment without ingestion was observed in some cases. Mouse macrophages avidly phagocytized kidney disease bacilli under similar experimental conditions. Potential explanations for the loss of phagocytosis in the STE 137 cells were explored. It was concluded that either (1) the cell line consisted of a mixed population of cells at the time the observations of phagocytosis were made and the phagocytic cells have been selected against or died out; or (2) the STE 137 cells have dedifferentiated, losing the property of phagocytosis. Some studies to confirm either of these hypotheses were recommended.
3. Primary monolayer cultures from kidney and spleen, and leucocyte cultures were established and grew vigorously (viability 90%, 85% respectively), but failed to phagocytize the kidney disease bacillus. The reasons for this are not fully understood, but may be related to the

cell types recovered by the methods used for cell harvest and culture.

4. Kidney disease bacilli are phagocytizable particles and the experimental parameters in this study are conducive to phagocytosis as evidenced by work with mouse macrophages.
5. Uncultured leucocytes failed to phagocytize the kidney disease bacillus; this is not surprising, since differential counts revealed a paucity of leucocytes with phagocytic capabilities. Differential counts of blood films from normal, hatchery-raised, 12.5 to 17.5 cm brook trout showed:  $82.4 \pm 0.9\%$  lymphocytes,  $12.1 \pm 0.9\%$  thrombocytes,  $2.4 \pm 0.2\%$  neutrophils, and  $2.6 \pm 0.3\%$  blast forms. Studies of hematological changes during the course of infection were recommended.
6. The histopathology of bacterial kidney disease was described by organ system (kidney, liver, pancreas, gut, heart, gills, brain). The typical lesion was a distinct, localized growth of bacteria and central mass of amorphous, granular debris surrounded by a mixed, non-specific inflammatory response consisting of lymphocytes, some degenerating neutrophils, and macrophages. Very few polymorphonuclear leucocytes were observed. The lesions are distinguished from the classic focal granulomas of piscine tuberculosis or flavobacteriosis. The disease is described as a "bacteremia



characterized by a systemic, diffuse...histiocytic inflammation" (Wolke, 1975).

7. The ultrastructure of the kidney disease bacterium and its relationship to host tissues was studied via electron microscopy. The mean length was 0.65  $\mu\text{m}$  and the mean width 0.39  $\mu\text{m}$ . The mean cell wall thickness was 16.1 nm and the mean thickness of the cytoplasmic membrane 8.8 nm. These dimensions are within the range of values reported for other bacteria.

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## APPENDIX

Appendix Table 1. Differential blood counts from normal brook trout (Salvelinus fontinalis), May-Grunwald Giemsa stain.<sup>a</sup>

Slide number	Lymphocytes	Thrombocytes	Neutrophils	Macrophages	Blast forms
1	82	15	1	0	3
	76	16	1	0	6
	79	17	2	0	2
	82	13	4	0	2
2	73	23	0	0	4
	81	13	1	0	5
	79	13	3	0	5
	85	11	1	0	3
3	72	14	6	1	7
	88	7	4	0	1
	89	3	3	0	5
	90	2	1	1	6
4	70	27	2	0	1
	83	14	1	0	2
	80	15	5	0	0
	82	15	2	0	1
5	77	19	1	0	3
	72	21	1	0	6
	71	24	2	0	3
	70	24	3	0	3
6	98	0	2	0	0
	85	3	7	0	5
	93	2	2	0	3
	97	0	3	0	0

Appendix Table 1 (continued)

Slide number	Lymphocytes	Thrombocytes	Neutrophils	Macrophages	Blast forms
7	88	7	5	0	0
	82	9	4	0	5
	89	7	3	0	1
	85	12	1	0	2
8	98	0	0	1	1
	85	3	3	0	9
	88	7	0	0	5
	95	1	2	0	2
9	80	13	2	0	5
	79	20	0	0	1
	79	13	4	0	4
	75	18	5	0	2
10	91	6	2	0	1
	86	10	2	0	2
	90	3	3	0	4
	91	8	0	0	1
11	71	21	4	0	4
	81	17	2	0	0
	81	16	2	0	1
	78	20	2	0	0
12	86	12	2	0	0
	88	4	5	0	3
	88	6	4	0	2
	90	7	2	0	1

Appendix Table 1 (continued)

Slide number	Lymphocytes	Thrombocytes	Neutrophils	Macrophages	Blast forms
13	83	14	2	0	1
	73	22	4	0	1
	78	14	3	0	5
	85	14	0	0	1
14	81	13	3	0	3
	88	8	1	0	3
	76	20	0	0	4
	84	10	1	1	4
15	89	5	6	0	0
	85	14	0	0	1
	75	21	3	0	1
	80	18	2	0	2

<sup>a</sup>All information is for healthy, hatchery-raised, 12.5 to 17.5 cm fish.

Appendix Table 2. Dimensions (in nm) of the cell wall and cytoplasmic membrane of kidney disease bacilli cultured on cysteine serum agar.

Wall	Cytoplasmic membrane			
	Total membrane	Outer layer	Middle layer	Inner layer
16.0	17.3	4.0	8.0	5.3
14.6	14.6	5.3	5.3	4.0
16.0	14.6	5.3	5.3	4.0
18.6	16.0	5.3	5.3	5.3
16.0	10.6	2.7	5.3	2.7
13.3	13.3	4.0	5.3	4.0
10.6	16.0	5.3	5.3	5.3
16.0	14.6	4.0	6.7	4.0
16.0	14.6	4.0	6.7	4.0
12.0	14.6	4.0	5.3	5.3
14.6	10.6	2.7	5.3	2.7
13.3	10.6	2.7	5.3	2.7
16.0	21.3	5.3	10.6	5.3
13.3	13.3	4.0	5.3	4.0
13.3	14.6	4.0	6.7	4.0
14.6	21.3	5.3	10.6	5.3
10.6	17.3	4.0	9.3	4.0
13.3	13.3	4.0	5.3	4.0
14.6	14.6	4.0	6.7	4.0
14.6	18.6	5.3	8.0	5.3
10.6	12.0	4.0	5.3	2.7
16.0	10.6	2.7	5.3	2.7
10.6	13.3	5.3	5.3	2.7
8.0	10.6	2.7	5.3	2.7
16.0	8.0	2.7	5.3	2.7
16.0	14.6	4.0	6.7	4.0
18.6	16.0	4.0	6.7	5.3
13.3	12.0	4.0	4.0	4.0
13.3	14.6	5.3	5.3	4.0
14.6				

Appendix Table 2 (continued)

Wall	Total membrane	Cytoplasmic membrane		
		Outer layer	Middle layer	Inner layer
16.0				
10.6				
13.3				
13.3				
14.6				
16.0				
13.3				
13.3				
16.0				
10.6				
13.3				
14.6				
12.0				

Appendix Table 3. Dimensions of the kidney disease bacillus, its cell wall and cytoplasmic membrane in kidney tissues from diseased brook trout (Salvelinus fontinalis).

Length ( $\mu$ )	Width ( $\mu$ )	Cell wall thickness (nm)	Cytoplasmic membrane (nm)
0.49	0.42	14.6	7.2
0.52	0.38	14.6	13.2
0.67	0.40	18.3	8.4
0.95	0.37	18.3	7.2
0.53	0.39	14.6	7.2
0.49	0.41	21.9	9.6
0.42	0.41	18.3	
0.57	0.43	16.4	
0.93	0.36	14.6	
0.68	0.41	12.8	
0.96	0.36	14.6	
0.51	0.36	16.4	
0.66	0.34	21.9	
0.85	0.38	16.4	
0.91	0.44	14.4	
0.67	0.34	14.4	
0.37	0.40	10.8	
0.50	0.33	12.0	
		12.0	
		7.2	
		7.2	
		28.8	
		21.6	