Cultivation and Transmission of Etiological Agent of Kidney Disease in Salmonid Fishes. (22392)

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During recent years there have been increasing numbers of outbreaks of a condition called "kidney disease" in stocks of young salmon in hatcheries in the Pacific Northwest (1). In many cases catastrophic mortalities have resulted(1). The disease has been found in adult salmon on the spawning migration (2). Kidney disease has also been found in stocks of trout in hatcheries in the Pacific Northwest, and serious outbreaks of the disease have been reported in trout in Eastern United States(3,4). The histopathology of kidney disease in trout and salmon has been compared and found to be almost identical (5). Outbreaks of disease apparently similar to or identical with kidney disease have been reported in Atlantic salmon in Scotland(6) and in rainbow trout in Germany(7).

Tremendous numbers of tiny Gram-positive organisms often in pairs are characteristically found both intracellularly and extracellularly in the lesions of infected fish. These have been commonly referred to as diplobacilli. Except in its earliest phases, kidney disease in salmon and trout can be readily diagnosed by its pathology supplemented by examination of sections or smears from the diseased tissues stained by Gram's method. Nevertheless, studies on the disease and the nature of the etiological agent have been handicapped by a lack of satisfactory media for direct isolation and cultivation of the etiological agent. Apparently this difficulty has occurred in all laboratories where kidney disease has been studied. A number of investigators have failed in their attempts to cultivate the etiological agent(3,4,6,7). It has even been suggested that the organism is related to the etiological agent of salmon disease of canines(1,3), which is now considered to be one of the Rickettsia.

Methods of cultivation. After a number of unsuccessful attempts, cultivation of the etiological agent of kidney disease was achieved by several different methods. Cultivation of the organism was first achieved in suspensions of minced chick embryo tissues incubated at 15°C, and it was possible to make serial subcultures on this medium(1). However, this system did not prove convenient, and subsequently has been replaced by other methods. In one experiment a culture of the etiological agent of kidney disease, which had been grown on chick embryo tissues, was subcultured on a number of different types of culture media. Among these was Dorset's medium, as used for the cultivation of *Mycobacterium tuberculosis*. After approximately two months of incubation at 15°C, well developed colonies were found on this medium. These colonies contained organisms which were morphologically identical with those occurring in lesions of diseased fish. The bacteria were transferred on Dorset's medium, and after 5 serial subcultures were tested for pathogenicity to young salmon. Typical kidney disease was produced and the etiological agent reisolated from a number of the fish taken either in a moribund condition or shortly after death. In every case huge numbers of characteristic Gram-positive diplobacilli were found in smears taken from the kidneys of the experimental fish. Standard Dorset's medium did not prove satisfactory for the isolation of the etiological agent of kidney disease in natural outbreaks of the disease. A modified Dorset's medium fortified with cysteine, tryptone and yeast autolysate was prepared and subjected to greater heat treatment than is usual in the preparation of this medium. On this modified medium, direct inoculation from lesions of infected fish regularly resulted in good growth of the etiological agent. Serial subcultures were carried out without difficulty, and good growth was obtained in a period of 3 to 4 weeks at 17°C. This modified

Dorset's medium has been found useful in bacteriological diagnosis of kidney disease. Pure cultures were usually obtained except when the fish were dead or secondary bacterial infection had set in. It was found that only a small portion of the bacteria present in the inoculum developed on the modified Dorset's medium. This difficulty has been overcome by the use of a modified blood agar. It was noted that occasionally when kidney tissues from infected fish were streaked on plates of blood agar containing a tryptose base, growth of bacteria occurred in and around bits of kidney tissue. However, colonies did not develop on restreaking these bacteria on the same medium. It was also found that some stock cultures which had been maintained on modified Dorset's medium for a dozen or more transfers would develop when transferred to tryptose base blood agar. In a further search for a suitable plating medium, a variety of nutrient supplements were incorporated into tryptose base blood agar. The essential requirement proved to be cysteine. The addition of 0.05 to 0.1% cysteine to the tryptose base blood agar produced a medium on which growth occurred within 7 to 10 days when streaked directly from infected fish and incubated at 17°C. Somewhat better development occurred when the amount of blood (human) was increased from 10 to 20% by volume, and 0.05% yeast extract was added. The medium now in use is as follows:

	%
Tryptose	1.0
Beef extr.	0.3
NaCl	0.5
Yeast extr.	0.05
Cysteine-HCl	0.1
Human blood (by vol.)	20
Agar	1.5

Characteristic colonies are shown in Fig. 1. There is often a considerable variation in colony size on cysteine blood agar, although the colonies of all sizes contain bacteria of the same morphological characteristics. These are shown in Fig. 2. Cysteine blood agar has also proven satisfactory for the cultivation and isolation of the diplobacilli from fish in early stages of the disease before any characteristic pathology has developed. In this case the incubation period is often prolonged, presumably because the number of bacteria in the inoculum is small. In one experiment designed to test the requirement for cysteine, 10 fish were selected from a population of blueback salmon in which natural kidney disease

FIG. 1. Colonies on cysteine blood agar. Streaked directly from infected tissue. × 5.

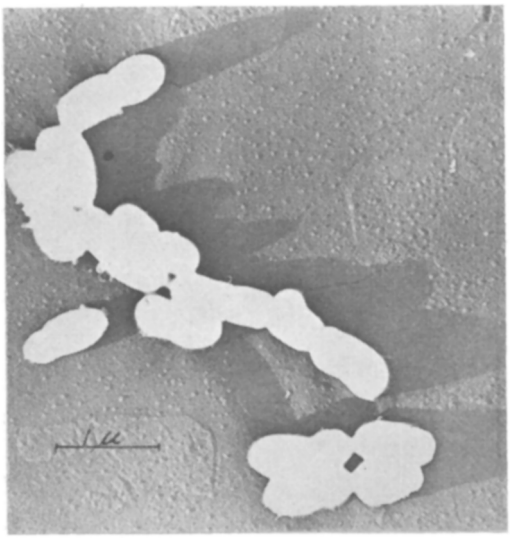

FIG. 2. Electron micrograph of diplobacilli from a cysteine blood agar plate. The cells were lightly shadowed with palladium at a 1:5 angle.

was present. None of the fish had been inoculated deliberately. At autopsy 5 of the fish showed gross lesions of kidney disease and 5 appeared normal. Material from the posterior portion of the kidney of each fish was inoculated on 2 blood agar plates lacking cysteine and on 2 blood agar plates in which cysteine was present. All plates were incubated at 17°C and observed at intervals up to 6 weeks. The plates from one fish remained sterile during the entire period of observation. The blood agar plates containing cysteine inoculated from all other fish developed colonies, many of them appearing at 9 days, others as late as 17 days after inoculation. No growth occurred on any of the blood agar plates deficient in cysteine until the 5th week when 1 to 6 colonies were found on each of 4 plates. The remaining 16 plates remained without growth. Whereas subinoculation from the four plates showing colonies to other tryptose blood agar plates deficient in cysteine yielded no growth, good growth occurred on subinoculation into blood medium containing cysteine.

Transmission experiments. The requirements of Koch's postulates as usually formulated have been met with the bacterium cultivated from fish with kidney disease. The bacterium is regularly present in lesions of the disease. Typical kidney disease has been produced by inoculation of pure cultures of the bacterium. Finally, the bacterium has been reisolated from fish with the experimentally produced disease.

In one experiment, cultures of the diplobacilli were isolated from the liver of an adult spring chinook salmon (*Oncorhynchus tshawytscha*). After replating 3 times on cysteine blood agar, 0.05 ml of a dilute suspension of the diplobacilli in saline was injected intraperitoneally into each of 20 yearling blueback salmon (*Oncorhynchus nerka*) held at 15°C. The first deaths occurred on the 12th day and all fish were lost by the 23rd day. The experiment was repeated after 2 further transfers of the culture. In this experiment all except one fish were lost between the 12th and 21st day. This fish survived until the 24th day. Characteristic diplobacilli were found in huge numbers in smears taken from each test fish, and the organisms were reisolated from a number of the fish taken shortly after death.

An additional experiment was carried out in order to compare the virulence of isolated cultures of the diplobacillus with that of organisms maintained in frozen fish tissues. A suspension was prepared from a portion of the liver of the adult spring chinook salmon and injected into a number of yearling blueback salmon. The liver had been kept at −20°C for about 4 months. After mortalities had set in, 3 of the fish were sacrificed. A suspension was made from the organs of these fish and injected intraperitoneally into each of 20 yearling blueback salmon in amounts of 0.05 ml. The fish were held at 15°C. The first death occurred on the 13th day, and all fish were lost by the 20th day. It is apparent from this experiment that the pure cultures grown *in vitro* were comparable in virulence with the organisms preserved in the frozen tissues of infected fish.

It has been our experience that kidney disease in trout is ordinarily less severe than in salmon, even though heavy mortalities have occurred in some trout hatcheries. Diplobacilli isolated from trout in the Pacific

Northwest are indistinguishable from those isolated from salmon, and are comparable in virulence toward salmon. In one experiment a dilute suspension of diplobacilli isolated on cysteine-blood agar from a trout during the course of an outbreak of kidney disease in a trout hatchery, was inoculated into a stock of blueback salmon under conditions similar to those given above. A heavy mortality set in on the 14th day and all fish were lost by the 19th day.

Etiological agent. In view of its morphology and its development on culture media, the etiological agent of kidney disease is clearly one of the bacteria. The organism is a Gram-positive, short rod, and occurs frequently in pairs. The dimensions are variable but most cells range from 0.3 to 0.5 μ by 0.5 to 1.0 μ. Chains are sometimes observed in old laboratory cultures. A considerable degree of pleomorphism has sometimes been noted in intracellular forms in infected tissues and in some laboratory cultures. No endospores or resistant forms have been found. The organism is proteolytic and an active catalase is present. On the basis of present information, the organism may be considered to be a species of *Corynebacterium*. However, further studies on the properties of the organism are being carried out, and the exact taxonomic status is still under study.

Summary. Media have been devised for the cultivation and isolation of the etiological agent of kidney disease in salmon and trout. It is noteworthy that growth occurs readily on addition of cysteine to complex blood media, which otherwise do not support growth. Koch's postulates have been fulfilled establishing that the organism isolated is the etiological agent of the disease.

1. Earp, B. J., Ellis, C. H., and Ordal, E. J., *Wash. Dept. Fish Spec. Rep. Ser.* No. 1, 1953, 74 pp.

2. Wood, J. W., and Wallis, J., unpublished manuscript.

3. Belding, D. L., and Merrill, B., *Trans. Am. Fish. Soc.,* 1935, v65, 76.

4. Snieszko, S. F., and Griffin, P. J., *Prog. Fish-Culturist,* 1955, v17, 3.

5. Wood, E. M., personal communication.

6. Blake, Isobel, Final Report of the Furunculosis Committee, HMS Stationary Office, Edinburgh, 1935.

7. Schäperclaus, W., *Fishkrankheiten. Akedemie-Verlag.,* Berlin, 1954, 3rd ed., 537 pp.

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Role of Thioctic Acid in Chick Nutrition. (22393)

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Thioctic acid (6, 8-dithiooctanoic acid) is known to be essential for the nutrition of several microorganisms and to be a component of keto acid oxidases. Evidence regarding its role in nutrition has been conflicting. Stokstad *et al.*(1) were unable to obtain any evidence of a thioctic acid deficiency in either chicks or rats on purified type diets containing alcohol extracted casein. DeBusk and Williams(2), however, reported that thioctic acid increased growth of chicks on a purified type diet and that 1 mg per kg per day was required to give maximum response. This communication deals with additional experiments on the dietary requirements of chicks for thioctic acid and on the synthesis of thioctic acid by the developing chick embryo.

Methods. Day-old Barred Rock x New Hampshire cross bred chicks were kept in thermostatically-controlled heated battery brooders and given feed and water *ad libitum.* Diets are shown in Table I. Casein was extracted continuously with hot ethanol for 20 hours. Thioctic acid was assayed by the pad plate procedure using *Corynebacterium bovis* (3). Samples were prepared for thioctic acid assay by grinding 5 g fresh tissue with 10 ml of 9 N H_2SO_4 in a tissue homogenizer, autoclaving for 3 hours at 120°C, neutralizing and diluting to 50 ml. Insoluble material was