A STUDY TO CONTROL THE DISEASES INFLUENCING THE SURVIVAL OF ADULT CHINOOK SALMON IN THE COLUMBIA RIVER BASIN

by

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INTRODUCTION

This study was initiated in April 1962 to develop methods for the detection, prevention, and control of infectious diseases in adult chinook salmon. Three major research categories were to be studied: (1) determine if an immune response could be induced in fish by their exposure to antigens; (2) test certain drugs to determine their usefulness in the control of fish diseases; and (3) screen a selected group of chemicals to observe their effects on external infections caused by microbial agents. Additionally, pathogens affecting adult salmon were to be identified, a stock culture collection of these pathogens was to be maintained, and insofar as possible, physiological studies of the organisms involved were to be carried out. Within these categories, investigations have been conducted as follows:

IMMUNE RESPONSE

INTRODUCTION

The lack of extensive information on the immunological character of poikilothermic animals has been emphasized by Hildemann (1962). With the exception of temperature (Cushing, 1942; Bisset, 1948; Elek, Rees, and Gowing, 1962), conditions effecting the formation of antibodies in cold-blooded vertebrates can be assumed to be the same as those in other animals (Hildemann, 1962), and the techniques employed in the study of immunological reactions of other vertebrates are usually found adaptable to similar investigations with poikilothermic animals (Ridgway, 1962).

Anti-bacterial agglutinins have been produced in rainbow and brown trout at 10° C. (Smith, 1940), in cutthroat trout at averages of 7 and 9.0° C. (Duff, 1942), and in carp at both 10° C. (Smith, 1940) and 20° C. (Pliszka, 1939). Duff was able to show the formation of significant titers through oral administration of Aeromonas salmonicida vaccine, however, as in most instances of the intraperitoneally-injected bacterial vaccines, high titers occurred among many of the control fish.

Though isoagglutinins could not be detected in fish held at from 3-5° C., they were produced in sockeye salmon at 9-14° C. and in rainbow trout held at 15° C. (Ridgway and Klontz, 1960; Ridgway, 1962). Ridgway (1962) also shows the production of hemagglutinating antibodies by sablefish at temperatures as low 5-7° C.

Duff (1942) was able to develop resistance to furunculosis disease in cutthroat trout by feeding an oral vaccine containing the causative agent A. salmonicida. It has also been recently shown that newly-hatched fry of Amazonian discus fishes apparently receive some form of passive immunization by feeding on the epidermal mucous secretion of the parental skin (Hildemann, 1962).

Temperatures favoring the most rapid production of antibodies, or indeed, the release of antibodies into the circulation (if the situation in fish is found to be analogous to that in frogs) (Elek, Rees and Gowing, 1962) are at the higher temperature tolerance limits of cold-water fishes. Since most trout and

salmon are reared in relatively cold water, it becomes important to determine what concentrations can be expected under these conditions, and the relationship of these antibodies to actual protection for the fish. A study was undertaken to investigate active and passive immunization of rainbow trout, spring chinook and coho salmon against A. salmonicida.

MATERIALS AND METHODS

Experimental Animals

Agglutinating antibodies were prepared in 2-, 3-, and 4-year-old adult rainbow brood trout. The fish were obtained from the Oregon Game Commission Roaring River Hatchery and were held in circular tanks having a total water volume of 1,070 liters per tank, fed from spring water which maintained an average temperature of 12° C. These fish were fed Clark's "New Age" brood-fish feed at 30 grams per fish per week.

Juvenile coho used for passive immunization were kept in stock ponds at 13° C. and fed Oregon pellets until needed for experimentation. Water temperature was slowly raised to 17° C. over 3 days after the fish had been transferred to 16-liter tanks in the laboratory. These fish and those employed in the oral immunization experiment were obtained from Oregon Fish Commission (OFC) Sandy Hatchery and were of the 1961 brood. Their average weight was 19.3 gm. The production feeding of oral vaccine involved 1962-brood juvenile coho at the OFC Siletz Hatchery.

Adult spring chinook used in the experiment reported here were obtained from Eagle Creek National Hatchery.

Serum Preparation

Blood was removed from the fish by severing the tail. Blood samples from each fish were kept separate and were immediately placed at 12° C. for clotting. After about one hour all samples were placed at 2° C. for complete clot retraction. Rainbow serum samples held for passive immunization on coho were frozen. Other serum samples were stored at 2° C.

Agglutination

Agglutinating antigens consisted of washed suspensions from 24-hour (at 18° C.), modified furunculosis medium (MF) broth culture (0.25 gm 1-tyrosine, 5 gm yeast extract, 2.5 gm NaCl, and 10 gm tryptone). The cells were centrifuged, washed twice, then resuspended in cold 0.85% NaCl and the pH adjusted to 7.0. Fresh antigens were prepared before each experiment.

Agglutination was carried out according to the method outlined by Kolmer, Spaulding and Robinson, (1951) for brucella antigens (4 hours incubation at 52° C. followed by overnight at 4° C.

A. salmonicida stock cultures used in antigen preparation included 5000H

(Snake River) 1/, 5006Z (Siletz Hatchery), 5007W (Willamette Hatchery), 5010B (Big Creek Hatchery), 5012T (Trask Hatchery), 5016K (Klaskanine Hatchery), and ATCC 14174.

Experimental Infection

Juvenile coho involved in passive immunization were experimentally infected using a combination of two methods which had experimentally proved to be effective and measurable in our laboratory. First, immediately after injection of the serum, and while the fish were still anesthetized, (1:17,500 MS-222 Tricaine methanesulfonate) an area of about 1 cm^2 (midway down the lateral line) was scraped clean of scales and slime, and a concentrated suspension of A. salmonicida 5000H in MF broth applied with a swab to the scraped area. The fish were then immediately placed back in the fresh running water. After 24 hours a 50 ml MF broth suspension of the same strain (incubated for 24 hours at 18° C.) was added to each tank. Immediately after introduction of the bacterial suspension the water to each tank was stopped, and the water-contact treatment carried out for one hour with aeration. The same procedure was repeated again at 48 hours. All fish dying during this experiment were autopsied to determine the specific cause of death.

Fish which had received the oral vaccine in the laboratory were experimentally infected by introducing a 50 ml MF broth culture (24 hours at 18° C.) of A. salmonicida 5000H into the water supply daily through the first 15 days of the experiment.

Juvenile coho at the Siletz Hatchery experienced a natural infection of furunculosis. Had the infection not occurred, fish would have been removed to the laboratory for challenge.

Vaccine Preparation

Organisms used in antigen production were inoculated onto bottle slant cultures and incubated for 72 hours at 18° C. After incubation the growth was scraped from the agar with a glass rod and suspended in aqueous 0.85% NaCl at pH 7.0. Formalin was then added to 0.2% and the suspension placed at 25° C. for one hour. The suspensions were then placed, and permanently stored at 2-4° C.

For injected vaccines, the final cell concentration was adjusted to 3×10^{10} cells per ml. The vaccine was mixed 2:1 with Freund's (complete) adjuvant (FA) prior to inoculation, and injected intra-abdominally. A. salmonicida 5000H was employed in all experiments which involved intra-abdominal inoculations.

Vaccine for use orally in the laboratory immunization experiments was prepared in the same manner as the injectable vaccine, and was incorporated into Oregon pellets by addition of the preparation to the thawed food. The mixture was then repelleted with a modified home food grinder, and refrozen. This oral vaccine preparation contained A. salmonicida 5000H.

1/ Parenthesis indicate location where culture was isolated. All are from OFC hatcheries except the ATCC strain, and the Snake River culture which was obtained from the Idaho Fish and Game Commission hatchery at Oxbow Dam.

Vaccine for incorporation into Oregon pellets used in the production experiment at the Siletz Hatchery was prepared the same as the oral vaccine used in the laboratory experiment, except that incubation of the formalin preparation at 25° C. was omitted. A. salmonicida 5006Z, 5012T, and 5016K were employed in the ratio of 3:1:1 as antigens for this experiment.

Active Immunization

Organisms for antigen production were inoculated onto slant cultures of 5000H incubated for 72 hours at 18° C. After incubation the growth was scraped from the agar with a glass rod and suspended in 0.85% NaCl at pH 7.0. Formalin was then added to 0.2% and the suspension placed at 25° C. for one hour. The suspensions were then placed, and permanently stored at 2-4° C. The final vaccine cell concentration was 3 x 10^{10} cells per milliliter. The vaccine was mixed 2:1 with FA prior to inoculation, and injected intraperitoneally. Two-year-old rainbow received three injections, the second injection occurring one week after the initial inoculation, the third after an additional five weeks. Control fish in this group received either aqueous 0.85% NaCl or 0.85% NaCl plus FA. An injection of 2 ml vaccine was made initially, thereafter, each fish receiving 1 ml each time. Blood was removed from these fish four weeks after the final injection.

The first five injections of the 3- and 4-year-old rainbows were carried out one week apart; the sixth after an additional two weeks; the seventh after an additional week; and the final injection after an interval of five more weeks. One ml was inoculated per fish each time. Control fish received 1 ml of 0.85% NaCl each time the other group received vaccine. Serum was removed from this group one month after the final injection.

Spring Chinook Immunization

Information relative to spring chinook immunization was obtained on only 3 fish, since most of the test fish died prior to what was considered a minimum stimulatory period. These survivors were in a group which received a single 9-ml injection of the same vaccine used for intraperitoneal inoculation of the adult rainbows. Controls received the same amount of a saline-FA preparation.

Blood was removed eight weeks after the single injection and assayed for agglutinating antibodies.

Passive Immunization

Antiserum and control serum used for injection of juvenile coho consisted of pooled 20-fish samples (filter sterilized) from the same rainbow that were tested for agglutinating antibodies.

Three groups of coho were involved in this experiment: (1) 37 negative controls which received no serum; (2) 36 controls which received 0.5 ml undiluted sterile serum from control rainbows; and (3) 40 fish which received 0.5 ml undiluted sterile anti-5000H serum from vacinated rainbow. Each group was divided approximately in half and placed in two tanks (16 liter water volume) on a controlled water-temperature table. The experimental water temperature was held at 17° C. Serum was injected intraperitoneally after anesthetization with 1:17,500 MS-222. Four hours after inoculation of the serum the fish received their initial exposure to A. salmonicida 5000H.

Oral Immunization

Four groups of juvenile coho were involved in the laboratory experiments: (1) the first group received a total of 406×10^9 cells per fish over a 98-day period (4.14×10^9 cells/fish/day); (2) the second received a total of 122×10^9 cells per fish over a 46-day feeding period (2.65×10^9 cells/fish/day); (3) a third received a total of 42.3×10^9 cells per fish over a period of 22 days (1.9×10^9 cells/fish/day); and (4) a fourth consisted of control fish. One group of controls had remained in the laboratory for 98 days and a second had remained in the laboratory for 46 days. All fish had been held in the laboratory for at least 46 days. Vaccination temperature was held at 13° C. throughout the experiments.

During field studies at Siletz Hatchery, 1962-brood juvenile coho received a total of 22×10^{13} cells over a period of 81 days. There were 72,000 vaccine fish and 72,000 control fish fed during this period giving a total of 3.82×10^7 cells/fish/day. The average high temperature during this period was 16.6° C. with a maximum high of 20.5° C. The average low temperature was 13.4° C. with a minimum of 11.1° C. The total overall average was 15° C. for the 24-hour day.

RESULTS

Test for Agglutinins in Actively Immunized Rainbow

Uniformly high levels of agglutinating antibodies were attained in the 2-, 3-, and 4-year-old adult rainbow trout (Tables 1 and 2). Both groups of vaccine-injected fish showed relatively high levels of agglutinin formation, while the controls all lacked similar antibody levels. The low levels found in some of the controls probably reflects the residual of previous non-artificial stimulation.

Table 1. Agglutination of *A. salmonicida* 5000H by Immune and Control Serum from 2-Year-Old (300 gm) Adult Rainbow Trout.

Serum Sample	No Agglutinins	Dilution						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
Vaccinated	0	0	0	0	0	0	2	1
Control (saline)	4	1	0	0	0	0	0	0
Control (Freund's adjuvant)	5	0	0	0	0	0	0	0

Table 2. Agglutination of *A. salmonicida* 5000H by Immune and Control Serum of 3- and 4-Year-Old (900-1000 gm) Adult Rainbow Trout.

Serum Sample	No Agglutinins	Dilution								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Vaccinated	0	0	0	0	0	2	5	8	6	1
Controls	20	7	1	0	0	0	0	0	0	0

Test for Agglutinins in Actively Immunized Adult Spring Chinook

As shown in Table 3, antibody formation was detected among the fish tested within eight weeks following the single vaccine injection. Loss of additional test animals through death hampered this experiment, and although the results obtained clearly show a difference between the control and vaccine fish, the relative differences one could expect among larger groups must be extrapolated conservatively.

Table 3. Agglutination of *A. salmonicida* 5000H by Immune and Control Serum from Adult Spring Chinook Salmon.

Serum Sample	No Agglutinins	Dilution				
		1:2	1:5	1:10	1:20	1:40
Vaccinated	0	0	0	1	1	0
Control (saline plus Freund's adjuvant)	0	1	0	0	0	0

Specificity of Anti-5000H Serum from Rainbows

An attempt to detect gross serological differences between strains of *A. salmonicida* was made on five additional isolates from OFC hatcheries and an ATCC strain. The homogeneity of the strains is apparent in Table 4.

Passive Immunization of Juvenile Coho

The protective value of immune rainbow serum is shown by the hourly mortality in Figure 1. Each line on the graph represents an average between two samples giving a total of 37 negative control fish, 36 positive control fish, and 40 antiserum-receiving fish. It can be clearly seen that the administration of antiserum prior to infection not only delays the onset of furunculosis disease, but also supresses the normal mortality dynamics. The cumulative mortality is shown in Figure 2. It can be seen that the antiserum-treated fish follow a similar, though delayed curve, from 36 to 48 hours behind those of the control fish. Total mortality is shown to reach 72.5% in the antiserum fish, and 88.9% and 91.9% in the positive and negative controls, respectively.

Table 4. Specificity of Anti-5000H Serum.

Antigen	Serum	Agglutination								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
5000H	Anti-5000H	4+	4+	4+	4+	4+	2+	2+	-	-
	Control	-	-	-	-	-	-	-	-	-
5006Z	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	4+	2+
	Control	-	-	-	-	-	-	-	-	-
5007W	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	3+	2+
	Control	-	-	-	-	-	-	-	-	-
5010B	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	3+	3+
	Control	-	-	-	-	-	-	-	-	-
5012T	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	3+	1+
	Control	-	-	-	-	-	-	-	-	-
5016K	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	4+	2+
	Control	-	-	-	-	-	-	-	-	-
ATCC 14174	Anti-5000H	4+	4+	4+	4+	4+	4+	4+	3+	2+
	Control	-	-	-	-	-	-	-	-	-

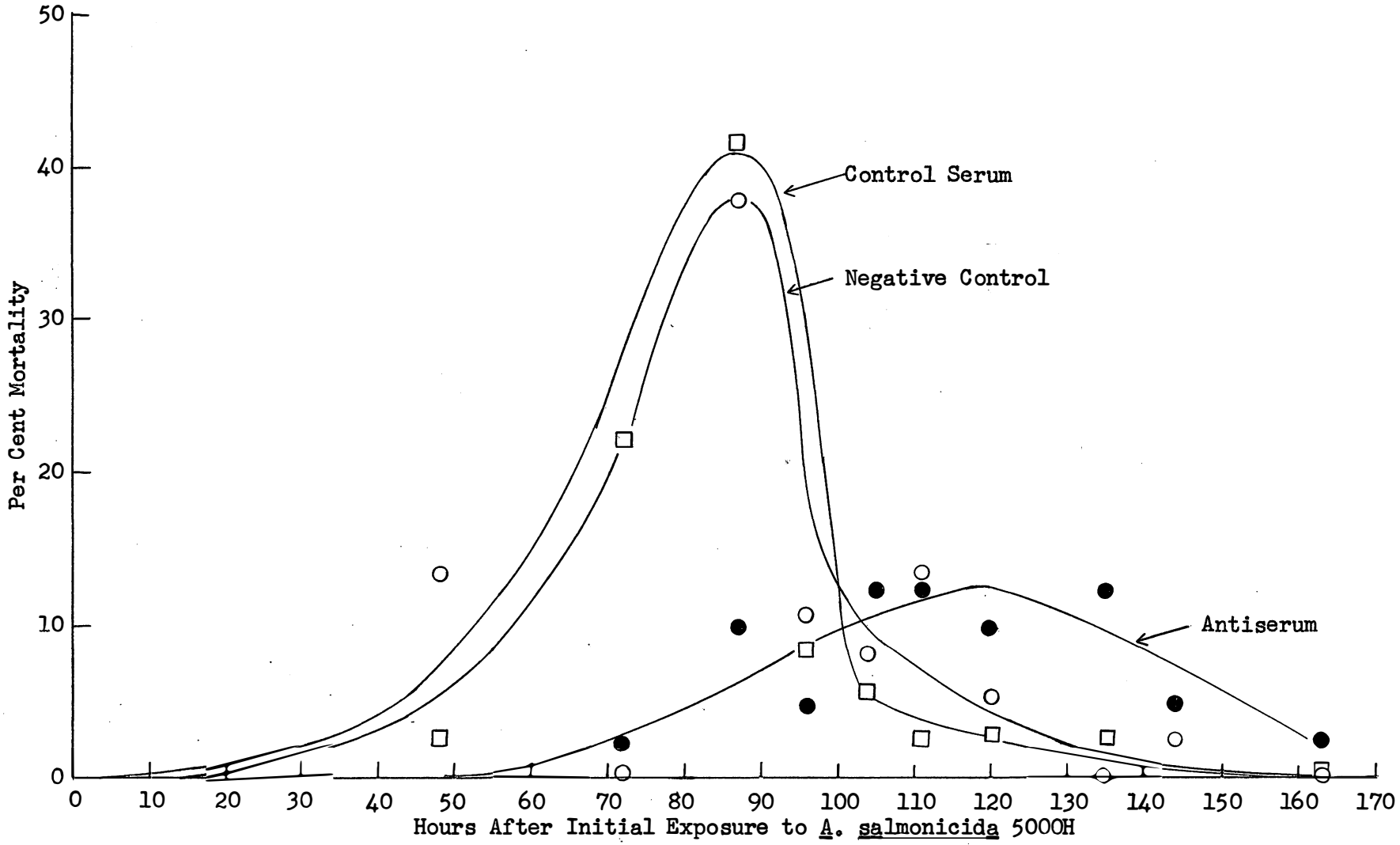

Figure 1. Hourly Per Cent Mortality of Negative Control, Serum Control, and Antiserum-Treated Fish.

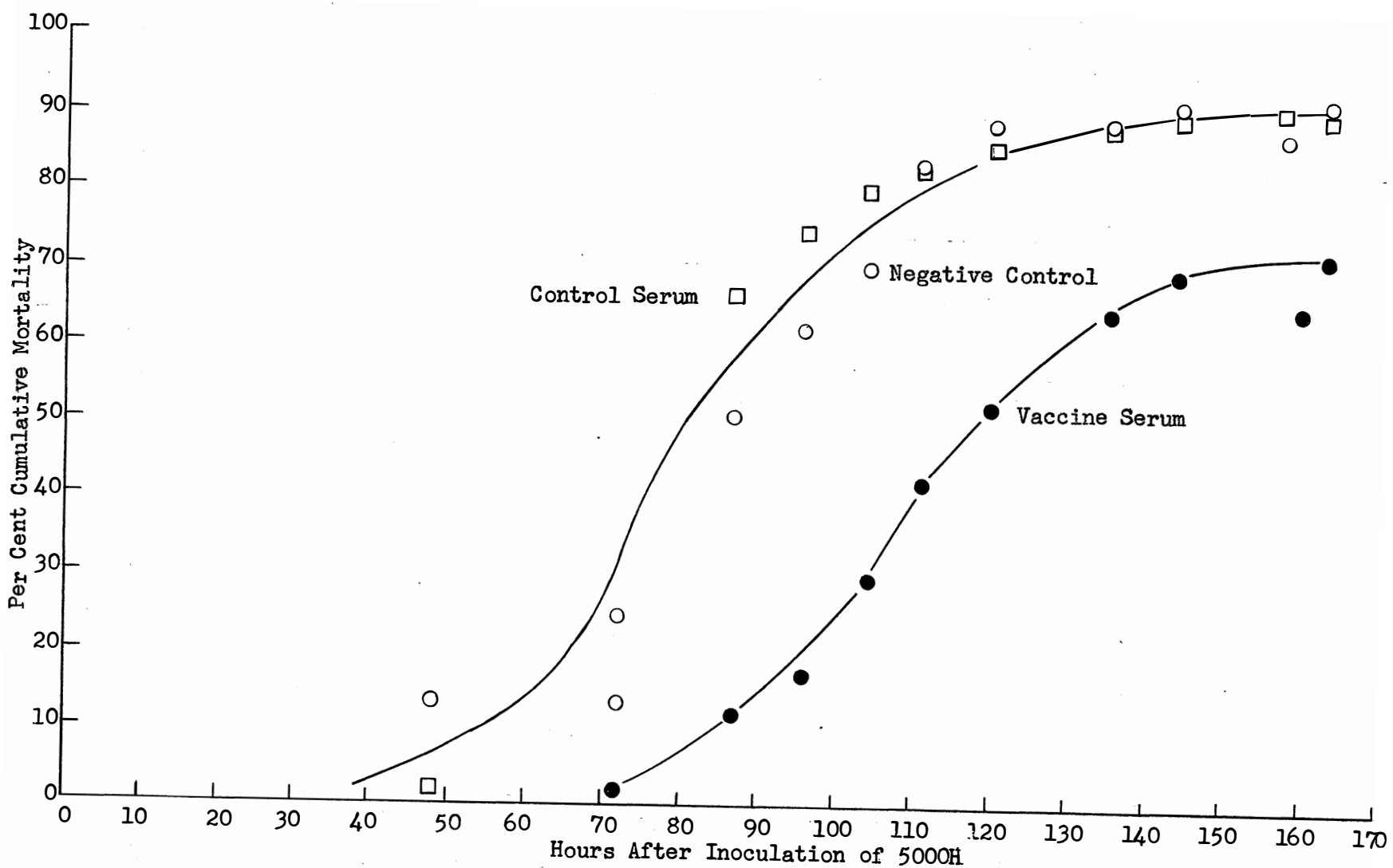

Figure 2. Cumulative Mortality of Juvenile Coho Infected with A. salmonicida 5000H After Receiving No Serum, Control Serum, and Antiserum From Adult Rainbows.

Oral Immunization Experiments

Although the mortality data on juvenile coho treated in the laboratory presented in Tables 5 and 6 would seem to suggest that some protection was conferred on the fish fed through 98 days, this was not true since when this group was analyzed statistically by X^2, the computed value (1.22 with one degree of freedom) was not significant at the 5% level.

No protection was observed among fish involved in the production experiment at the Siletz Hatchery. This was expected since these fish received much lower levels of vaccine than those held in the laboratory. It was hoped, however, that a smaller effect might be more easily detected in the large populations of this experiment.

These fish were also tested for agglutinating antibodies. None were detected.

DISCUSSION

Without exception, all of the vaccinated rainbow trout showed a relatively high level of antibody production. Though the history of the 3- and 4-year-old fish included their involvement in several furunculosis epidemics during both the time that they were being raised and during their life as brood stock in holding ponds, no control showed antibodies comparable to even the more limited production found in two of the vaccinated fish. To eliminate the possibilities that there might still be *A. salmonicida* agglutinating antibodies present, though undetectable through lack of homogeneity, between strains, a group of other isolates was tested with the serum. These not only included organisms from various OFC hatcheries, but also an ATCC strain, 14174. All showed high agglutination titers with the anti-5000H serum making it unlikely that agglutinins against another *A. salmonicida* was present, though undetected. The lower titer experienced with the vaccine culture, 5000H, when compared with the other *A. salmonicida* can be explained by the fact that it had been repeatedly transferred throughout the experiments, and over a period of two years, being used almost exclusively in these experiments. The other strains employed experienced no such vigorous culturing stress, most being isolated only recently.

It is well known that although agglutinins may be present at high levels, it does not necessarily indicate the existence of "protecting", of "immunizing" antibodies. The co-existence, and stimulation of both these factors remained to be shown by passive protection experimentation. It was seen in this experiment that immunizing substances did exist in the rainbow serum, whether related or unrelated to the existence of the agglutinins; their presence conferring a relatively high level of protection on the juvenile coho. The success of immunizing these juveniles passively could have been more absolute had the method of infection been less drastic, however it had been earlier determined experimentally that this method of infection gave rise to sensitive and reproducible measurement of the disease progression resulting in a uniform mortality curve. 1/

1/ See section "The Pathogenicity of a Phage-Sensitive Strain of *Aeromonas liquefaciens*", following.

Table 5. Oral Immunity of Juvenile Coho After 98 Days Treatment with *A. salmonicida* 5000H Vaccine.

Sample	No. Fish	Per Cent Cumulative Mortality Days after Initial Infection Attempt 0-6	7	8	9	10	11	12	13	14	15	16	17
Vaccine	(13)	0	15.4	15.4	30.8	30.8	30.8	30.8	30.8	30.8	30.8	30.8	30.8
Vaccine	(15)	0	0	6.7	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3
Control	(13)	0	7.7	7.7	15.4	23.1	30.8	30.8	46.2	46.2	46.2	46.2	53.8

Table 6. Oral Immunity of Juvenile Coho After 22 and 46 Days Treatment with *A. salmonicida* 5000H Vaccine.

Sample	Fish	Per Cent Cumulative Mortality Days after Initial Infection Attempt 0-6	7	8	9	10	11	12	13	14	15	16	17
Vaccine	(16) 1/	0	12.5	18.8	18.8	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3
Vaccine	(14) 1/	0	0	0	0	7.1	14.2	14.2	14.2	21.3	21.3	21.3	21.3
Vaccine	(12) 2/	0	0	8.3	8.3	16.7	16.7	16.7	16.7	16.7	25.0	25.0	25.0
Vaccine	(11) 2/	0	0	0	9.1	9.1	9.1	9.1	18.2	18.2	18.2	18.2	18.2
Control	(21)	0	0	9.5	14.3	14.3	19.0	19.0	19.0	23.8	23.8	23.8	23.8
Control	(24)	0	0	0	0	4.1	20.8	25.0	25.0	37.5	37.5	37.5	37.5

1/ Fish samples which received vaccine over a period of 22 days.
2/ Fish samples which received vaccine over a period of 46 days.

Passive protection in fish is not surprising, since it had been quite commonly employed for some time in combatting diseases of warm-blooded animals. Its effectiveness has been found to be of relatively short duration in the latter case, giving protection for up to 2-4 weeks. Its lack of practicality for treatment of large numbers of juvenile fish is obvious, since it would benefit the fish for so short a time. Its best application appears to be in the treatment of adult salmon after their return to the holding facilities. Since this is during the final phase of the salmon's life cycle, no consideration need be made for continuing protection, nor long-range effects as in the case of juveniles. In addition, the salmon are always immediately available during this period, and are usually present in numbers which could be treated practically. They are usually held, at most, from 3-4 months prior to spawning, encountering most disease problems either during the latter part of this period, or during high temperature phases of the holding pond water, or conditions of a similar predictable nature. With these factors in mind, a system of passive immunization or protection could be created, and as in the case of other animals, not just one, but several diseases could be combatted simultaneously with proper serum preparations. It is also logical to predict that other animals may be utilized as a source of immune serum as happens with other animals. Information from this study suggests that serum may possibly be prepared in the adult salmon themselves, perhaps in sufficient quantities to aid succeeding generations which return later to the holding ponds.

These results are limited, however, and do not show the high antibody titers found among fish which received a larger number of vaccine injections. Production of antibodies is apparently slow among all cold-water fishes, and this disadvantage is helped neither by the fact that these fish have a limited life expectancy, nor by the degenerative physiological state in which they exist. However, even though these fish may not be good suppliers of antiserum, they may be able to produce sufficient antibodies to protect themselves until the eggs or sperm can be harvested.

Although intra-abdominal inoculation of 2-, 3-, and 4-year-old adult rainbows, and spawning age salmon (to a lesser degree) proved to result in the production of significant amounts of A. salmonicida agglutinating antisera, the oral administration of similar vaccines proved ineffective when incorporated into juvenile coho diets.

Statistical analysis of mortality is supported in its non-significance revelation by the lack of agglutinins among these fish. No agglutination could be detected with serum removed from any of the fish involved in any of the oral vaccination experiments. Several circumstances could explain the lack of stimulatory effect by the oral vaccine, including insufficient time to allow antibody formation, lack of optimal temperatures, presence of vehicle inhibitors, suboptimal antigen concentrations, and fish strain and physiological factors. The question also arises as to whether the vaccine was of a nature conducive to antibody formation. Although it is shown that this vaccine causes the formation of "protective" substances in the blood after intra-abdominal inoculation, it would not necessarily follow that this same preparation should elicit as dramatic a result by the more circuitous, and chemically vulnerable oral route.

CONCLUSIONS

1. Intra-abdominal inoculation of a formalin-killed vaccine preparation of A. salmonicida causes the formation of agglutinins, and whether related or

unrelated, the formation of protective antibodies in adult rainbow trout.

2. This method of vaccine administration also results in the formation of agglutinating antibodies in adult spring chinook salmon after only one injection.

3. Passive immunization was found to give a relatively high degree of protection when adult immune rainbow serum was injected into juvenile coho salmon prior to contact with the disease.

4. Active oral immunization of juvenile coho were unsuccessful both in laboratory and field attempts, however, these failures should not preclude additional studies on this method.

DRUG TESTS

INTRODUCTION

The objectives of the following experiments were to: (1) determine the ability of the sulfonamides, Sulmet (sulfamethazine), Gantrisin (sulfasoxizole), Bactrovet (sulfadimethoxine), and S.E.Z. (sulfaethoxypyradiazine) to produce blood or tissue levels in fish greater than 4 mg per 100 ml blood (mg%); (2) develop a suitable method for administering the sulfas; and (3) conduct antibiotic sensitivity tests on two organisms, *A. salmonicida* and *A. liquefaciens.*

Previous *in vitro* sensitivity tests indicated that Sulmet, Gantrisin, Bactrovet and S.E.Z. were effective in controlling the growth of *A. salmonicida.* These preliminary experiments, mostly involved with development and standardization of methodology have been omitted from this report in the interest of continuity and brevity.

The most extensive sulfonamide experiments were conducted with Bactrovet as the previous experiments on juvenile salmon indicated that this sulfa provided sustained blood sulfa levels without toxic effects.

In vitro antibiotic sensitivity tests were conducted on two strains of fish pathogens.

MATERIALS AND METHODS

Adult Experiments

As the adult salmon is presumably non-feeding when returning to the parent stream, two basic sulfa application techniques were exploited: oral forced-feeding and intramuscular injections.

Bactrovet was administered first to spring chinook from the Eagle Creek National Hatchery, then to coho from the Sandy Hatchery followed by steelhead and spring chinook from the Marion Forks Hatchery. The oral forced-feeding was investigated first because this technique seemed the simplest as it required no sterile equipment. Two forms were fed; tablets, and two injectable solutions. One solution was the 10% commercial preparation and the other a 25% laboratory preparation. The other sulfas fed orally were injectable 25% commercial S.E.Z. solution,

Sulmet tablets, and Gantrisin tablets. These were fed only to spring chinook from Marion Forks. Intramuscular injections of Bactrovet were given to spring chinook, coho and steelhead.

Crystalin Bactrovet was dissolved in DMSO (Dimethyl sulfoxide) in the hopes of producing a 25% injectable solution.

Accessory Experiments on Juvenile Coho

As an adjunct to the adult salmon experiments, a test was conducted on juvenile coho to determine if sulfa levels could be analyzed from some part of the fish other than the blood with no sacrifice of accuracy. The fish in these experiments were fed Oregon pellets containing Sulmet. Blood was taken from the fish, the blood analyzed for sulfa content, and the remaining carcasses of each sample homogenized in a blender. The homogenized fish were then centrifuged and the supernatant body fluids analyzed. A similar experiment, utilizing a new veterinary quadrasulfa (sodiam sulfathiazole, sulfamerazine, sulfamethazine, and sulfaquinoxaline) was conducted on juvenile coho. The quadrasulfa was incorporated in Oregon pellets and fed at three concentrations: 2.5, 5, and 10 grams of sulfa per 100 pounds of fish.

In Vitro Antibiotic Experiments

In vitro sensitivity tests were conducted on Sandy River strains of *A. salmonicida* and *A. liquifaciens* to determine which antimicrobial agents might be best utilized for controlling furunculosis and possibly other bacterial and mycotic infections of adult salmon. *In vivo* tests are being formulated. Laison with several pharmaceutical supply firms is being maintained with the hope that as new drugs become available, those with special promise for fish disease control will be furnished for testing.

RESULTS

Adult Experiments

The forced-fed Bactrovet tablets produced only slight blood levels in the steelhead (Table 7 and Figure 3) but none in the chinook or coho. The injectable Bactrovet solutions administered orally to these fish produced only short-term, low blood levels (Tables 8 and 9 and Figure 3) in all species.

The most persistent blood sulfa levels from a single force-feeding were achieved with Sulmet and Gantrisin tablets fed to spring chinook (Table 9 and Figure 4). The water-soluble injectable S.E.Z. and Bactrovet solutions force fed to these fish produced blood sulfa levels, but of lower concentrations (Table 9 and Figure 4).

The commercial 10% intramuscular injections given to chinook, coho, and steelhead produced satisfactory blood sulfa levels (Tables 7 and 10 and Figure 5) but caused hemmorrhagic abscesses at the injection site.

The Bactrovet dissolved in DMSO precipitates out of solution of contact with body fluids thereby producing no sulfa levels in the fish.

Table 7. Blood Sulfa Levels in Mg-Per Cent[1] of Adult Steelhead Force Fed Bactrovet Tablets and Capsules and Given Intramuscular Injections of Bactrovet at a Water Temperature of 54° F.

Drug Form	Rate of Mg Sulfa Per Lb of Fish	Hours After Treatment			
		24	48	72	96
Control	0	0	0	0	0
Tablets	120	0.06	1.60	0.80	1.50
Capsules	120	0.03	1.30	0.30	0
26% Lab Prep I.M. Injection	9.3	14.50	11.20	10.70	7.30

1/ Mg sulfa per 100 ml blood.

Table 8. Blood Sulfa Levels in Mg-Per Cent of Adult Coho Orally Force Fed Injectable Forms of Bactrovet at a Water Temperature of 54° F.

Drug Form	Rate in Mg Sulfa Per Lb of Fish	Hours After Treatment			
		24	48	72	96
10% Commercial Solution	100	3.60	0	--	--
25% Lab Prep Solution	100	1.90	0	--	--

Table 9. Blood Sulfa Levels in Mg-Per Cent of Adult Spring Chinook Orally Force Fed Sulfonimides at a Water Temperature of 54° F.

Drug	Rate in Mg Sulfa Per Lb of Fish	Hours After Treatment				
		24	48	72	96	120
Sulmet Tablets	100	0.60	4.00	4.50	4.60	4.50
Gantrisin Tablets	100	2.60	5.40	6.40	6.00	4.50
Bactrovet 30% Lab Prep. Sol.	200	1.80	1.60	1.40	0.60	--
S.E.Z. 25% Commercial Sol.	100	0.80	0.15	0.14	0	--
S.E.Z. 25% Commercial Sol.	200	4.00	2.60	1.70	1.20	--
Control	0	0	0	0	0	0

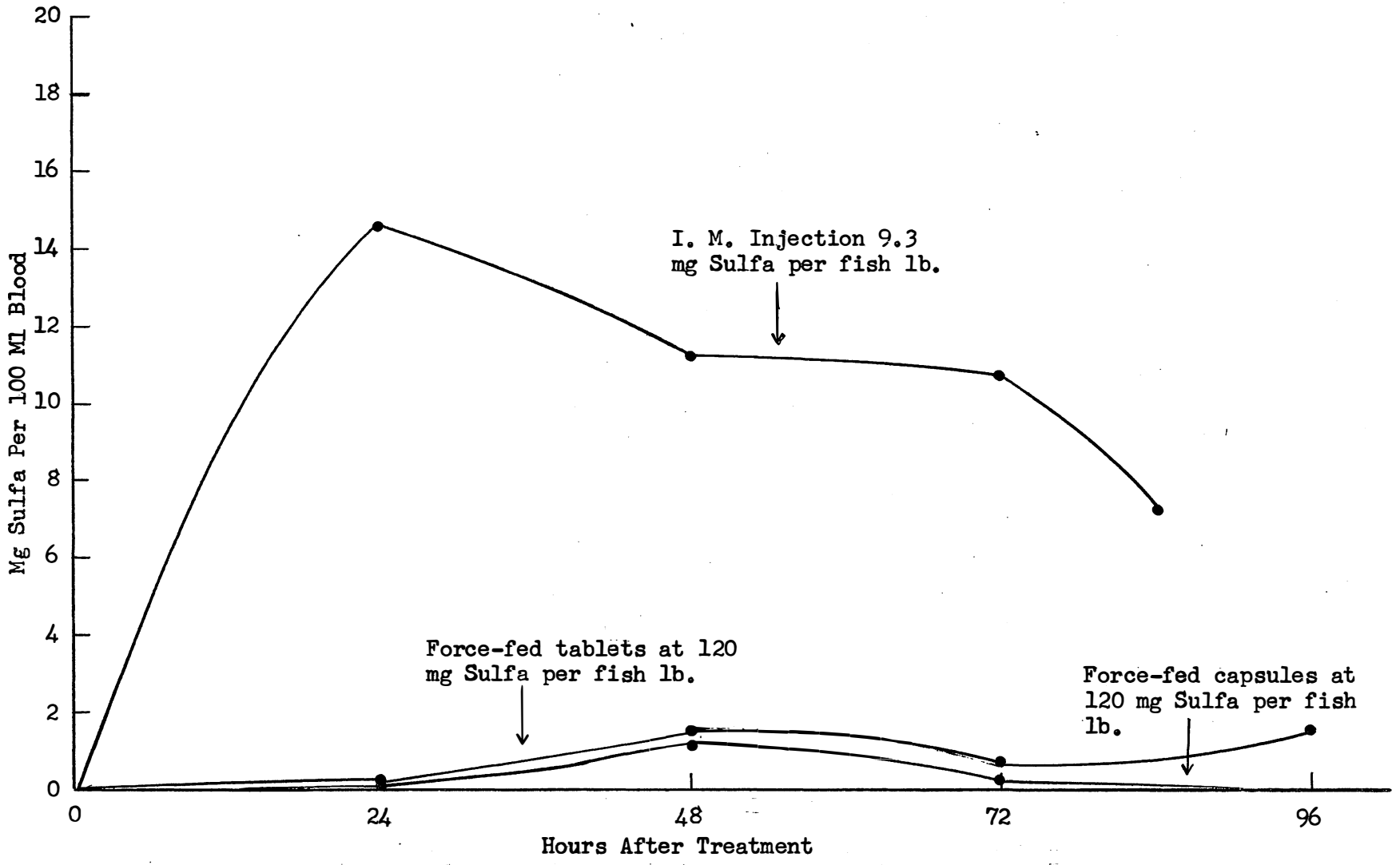

Figure 3. Blood Sulfa Levels of Adult Steelhead Given Intramuscular Injections and Force-fed Bactrovet Tablets and Capsules.

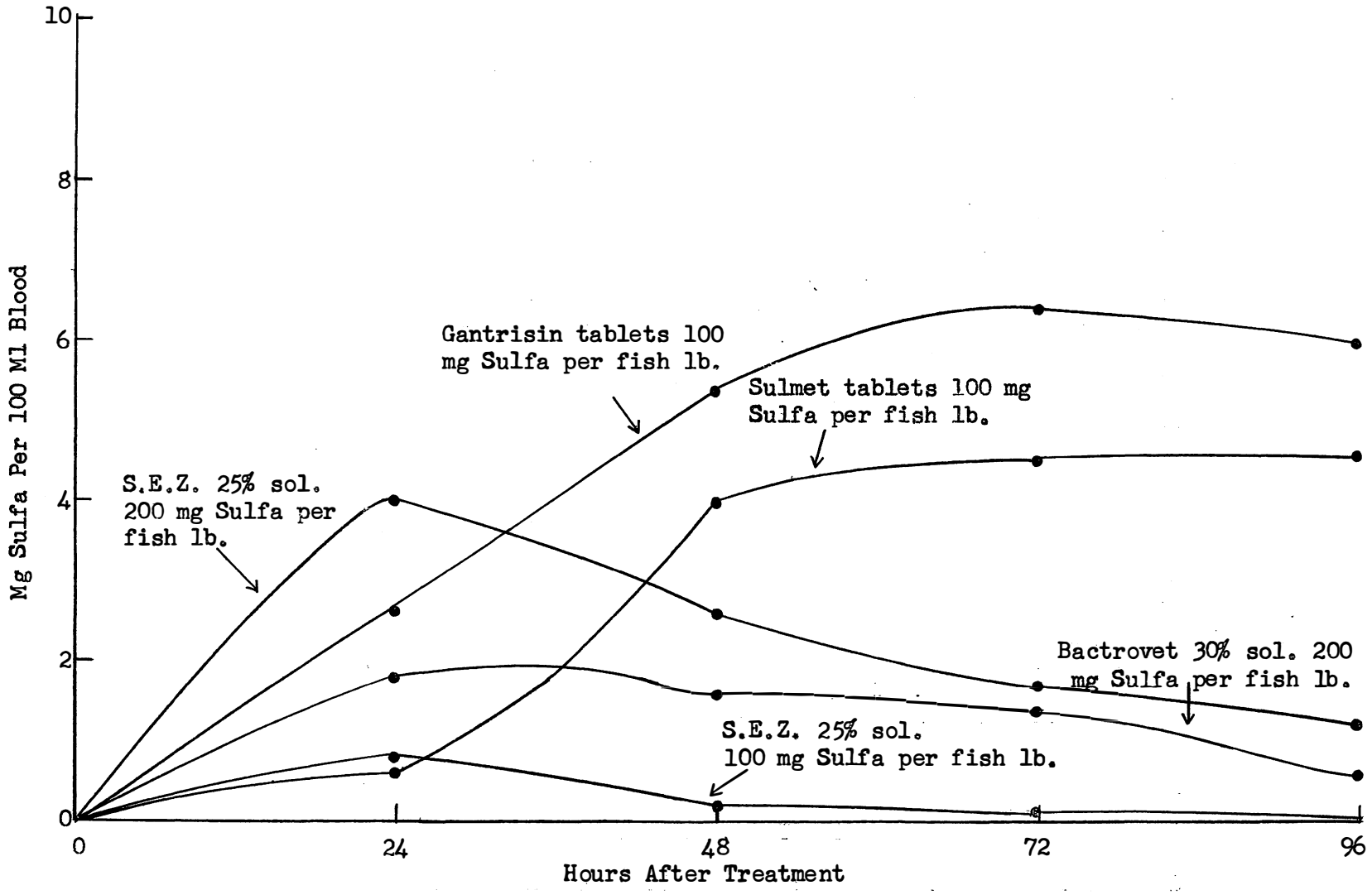

Figure 4. Blood Sulfa Levels of Adult Spring Chinook Force-Fed Sulfonamides.

Table 10. Blood Sulfa Levels in Mg-Per Cent of Adult Chinook and Coho Given Intramuscular Injections of Bactrovet at a Water Temperature of 54° F.

Drug	Rate in Mg Sulfa Per Lb of Fish	Hours After Treatment						
		12	24	48	72	96	120	144
Chinook								
10% Commercial Sol.	70	9.2	9.7	9.0	7.8 1/	12.8	12.0	9.3
10% Commercial Sol.	70	12.3	12.2	11.2	8.0 2/	15.1	16.3	13.5
Coho								
10% Commercial Sol.	100	--	12.8	8.0	6.2	--	3.5	--
25% Lab. Prep. Sol.	100	--	11.3	8.4	6.3	--	3.5	--
50% Lab. Prep. Sol.	100	--	18.8	15.8	13.0	--	4.5	--
Control	0	0	0	0	0	0	0	0

1/ Booster shot of 35 mg sulfa per lb of fish.
2/ Booster shot of 70 mg sulfa per lb of fish.

Accessory Experiments on Juvenile Coho

The resulting blood and tissue levels obtained from this experiment were comparable (Table 11), indicating that if a fish is not large enough to furnish adequate blood for analysis, that tissue analysis method might be employed.

The coho receiving the quadrasulfa produced blood levels proportional to the amount of sulfa received (Table 12). No toxic effects were observed in any lot.

In Vitro Antibiotic Experiments

The resulting sensitivity values of the antibiotic tests are listed in Table 13. Indications are that for this particular strain of *A. salmonicida*, only bacitracin would be non-effective. By contrast, only 9 of the antibiotics tested were effective in controlling the growth of *A. liquifaciens*.

CONCLUSIONS

Adult Experiments

Bactrovet force fed in any form to adults does not produce satisfactory blood sulfa levels. Consequently, this sulfa would have to be employed in the form of intramuscular injections. These injections produce high sulfa levels but the resulting hemorrhagic abscesses make this route undesirable. However, as these abscesses apparently do not hinder the fish's mobility or metabolism this method might be used in any emergency when no other course is effective. The sulfas, Sulmet and Gantrisin show the best possibilities as these seem to be absorbed by the fish when administered orally.

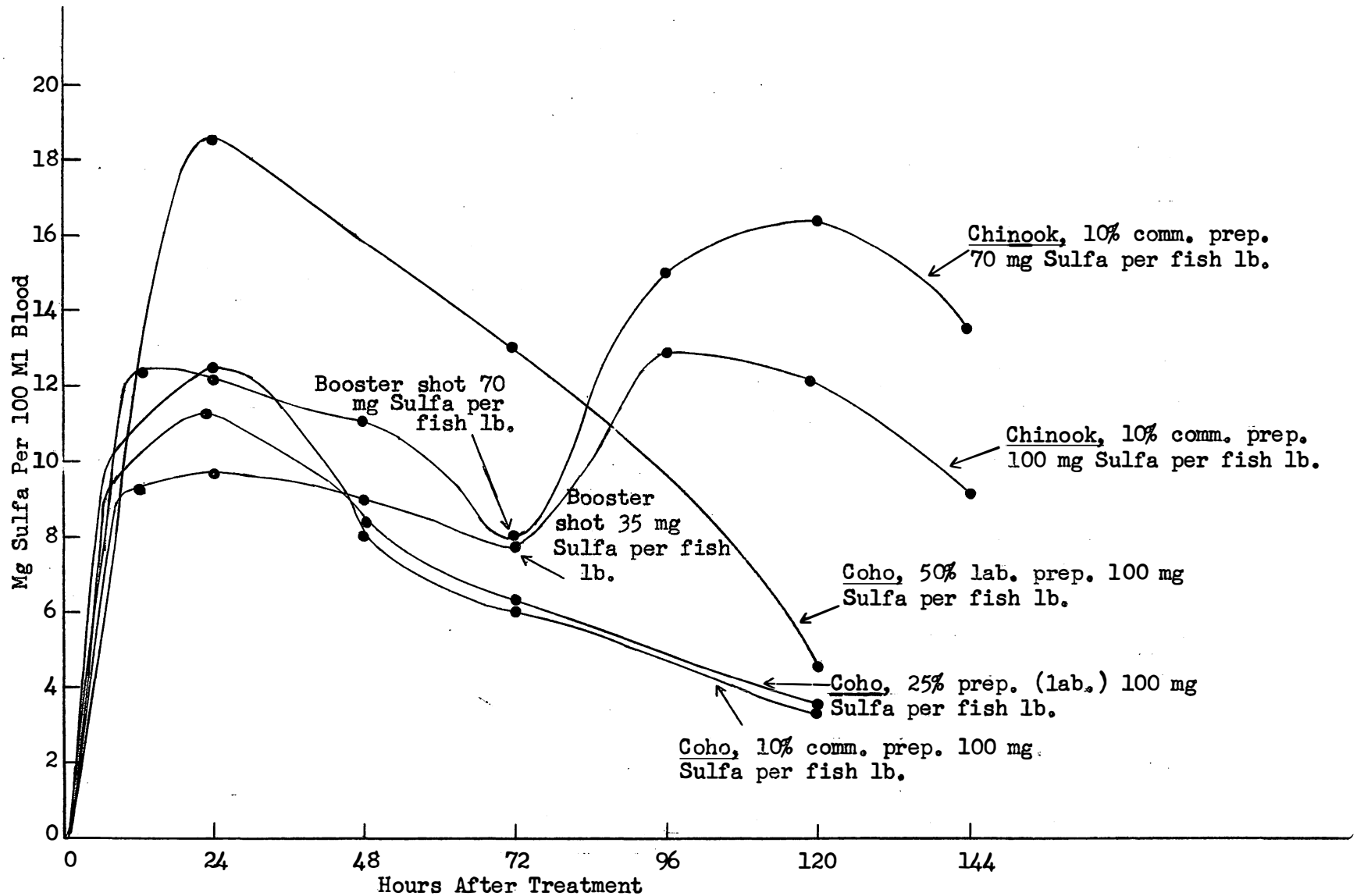

Figure 5. Blood Sulfa Levels of Adult Coho and Chinook Given Intramuscular Injections of Bactrovet.

Table 11. Comparison in Mg-Per Cent Between Blood Sulfa Levels and Tissue Sulfa Levels of Juvenile Coho Fed Oregon Pellets Containing 5 Gms and 2 Gms of Sulmet per 100 Pounds of Fish.

Fish Size	Replication	Treatment	Water Temp.	Hours After Start of Feeding 24	48	72	96
Yearling 1/	A	Blood only	54° F.	3.5	2.0	--	--
		Carcass only		5.2	2.4	--	--
		Whole fish		6.5	2.0	--	--
	B	Blood only		6.5	6.5	8.5	--
		Carcass only		7.2	6.8	9.3	--
	C	Blood only		1.5	6.5	4.8	--
		Carcass only		1.5	7.3	4.8	--
Yearling 2/	A	Blood only	44° F.	--	--	--	0.7
		Carcass only		--	--	--	0.7
		Whole fish		--	--	--	0.7
Fry 2/	A	Whole fish		--	--	--	0.8

1/ Feeding rate--5 gm Sulmet per 100 pounds fish.
2/ Feeding rate--2 gm Sulmet per 100 Pounds fish.

Table 12. Tissue Levels in Mg-Per Cent of Juvenile Coho Fed Quadrasulfa (S-4) at a Water Temperature of 54° F.

Lot	Feeding Rate in Mg Sulfa per Lb. Fish	Hours After Start of Feeding 48	96
1	2.5	1.8	1.9
2	5.0	4.4	4.4
3	10.0	8.1	--

Table 13. Sensitivity of a Sandy River Strain of *A. salmonicida* and *A. liquifaciens* to Fourteen Antimicrobial Agents.

Antimicrobial Agent	Organisms	
	A. Salmonicida	A. Liquifaciens
Furacin	S	R
Furadantin	S	R
Tetracycline	S	S
Colymycin	S	S
Carbomycin	MS	MS
Penicillin	MS	R
Polymyxim-B	S	S
Terramycin	S	S
Neomycin	S	MS
Chlorotetracycline	S	S
Erythomycin	S	MS
Aureomycin	S	S
Bacitracin	R	R
Dihydrostreptomycin	S	R

S - Sensitive
MS - Moderately sensitive
R - Resistant

Accessory Juvenile Coho Experiment

These tissue sulfa level experiments indicate that if a fish is too small to furnish adequate blood for analysis, the fish may be homogenized and the body fluids analyzed for sulfa levels with comparable accuracy. This technique might well be applied to fry or fish of similar size.

The quadrasulfa (S-4) produces satisfactory sulfa blood levels in the coho when fed at 5 and 10 gm of sulfa per 100 pounds of fish. No toxic effects were noted in the coho of 50 fish per pound, but might cause different results in fish of other species. Therefore, experimentation is recommended on each species of fish for which this drug might be used.

Antibiotic Experiments

The *in vitro* sensitivity tests indicate that on the strain of *A. salmonicida*, only Bacitracin was not effective in controlling growth, while in the case of *A. liquifaciens*, 5 antimicrobial agents were non-effective. These tests are not conclusive. *In vivo* tests may produce quite different results.

EXTERNAL INFECTIONS CONTROL

I. Topical Application of Malachite Green for Control of Common Fungus Infections in Adult Spring Chinook Salmon

INTRODUCTION

During adult chinook studies in 1962 at OFC holding ponds at Dexter Dam on the Middle Willamette River, the usual problem with external fungus infection was experienced. Treatment of the water supply with 1 ppm malachite green was not effective in curing this malady once it had become well established. Experience with 12 experimental fish suggested that topical application of a strong solution of malachite green to fungus-infected areas might prove effective in combatting the disease. Although these fish were concurrently involved in another experiment, and were being handled frequently, the original lesions were free of fungus and advanced healing was evident at the end of 6 weeks following one topical application of malachite green per week. In a few instances there was complete healing. Some benefit was noted at the OFC Sandy Hatchery after only one topical treatment of fungus-infected areas of fish upon their arrival at the holding ponds.

In 1963 an attempt was made to duplicate these earlier results in a more refined study. Fish were chosen which exhibited advanced fungus infections on the head, commonly called "sore heads". These were treated by various methods expected to accomplish results similar to those found earlier.

MATERIALS AND METHODS

Adult female chinook salmon were obtained from Eagle Creek National Hatchery. "Zinc-free" malachite green regularly used in OFC hatcheries was made up as a 10% aqueous solution (weight/volume) and applied directly to the lesions with a paint brush. Fish were anesthetized (1:17,500 MS 222) before treating and photographing.

RESULTS

Fish number 15 was treated for the first time on July 18, 1963 (Figure 6). Figure 7 shows the same fish one week after treatment, and while not as evident photographically as visually, the mycelial mat was reduced. Healing of the area had not yet begun. Figure 8 shows the same fish 3 weeks after the first treatment. The area above the eye clearly shows tissue repair. Close examination of the dark tissue projection at the back of the lesion also revealed regeneration around its tip. Figure 9 was taken 4 weeks after initiation of treatment. By this time the area above and just behind the eye was almost healed. Reexamination of the photographs reveals many other, more subtle spots of tissue repair.

Fish number 29 as shown in Figure 10 had an extensive fungus lesion on the right side of the head. Figure 11 shows the same fish 13 days after the first treatment. By comparison of the two photographs it can be seen that the mycelial mat was gone from the lesion, and the area just above the eye showed slight tissue repair at this time. The forward part of the lesion also exhibited healing. This fish did not survive past this treatment. The cause of death was diagnosed as furunculosis.

Fish number 17 (Figures 12 and 13), had an extensive fungus infested lesion covering the greater portion of the head. Figure 12 shows the fish before treat-

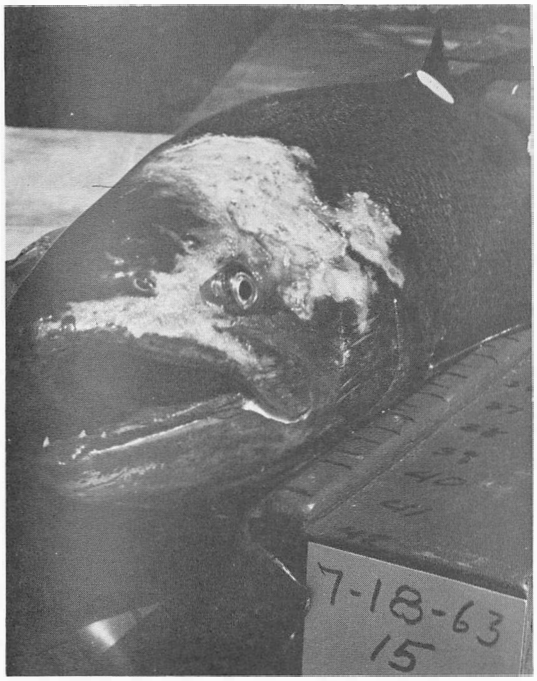

Figure 6.--Female Chinook, Before Topical Malachite Green Application.

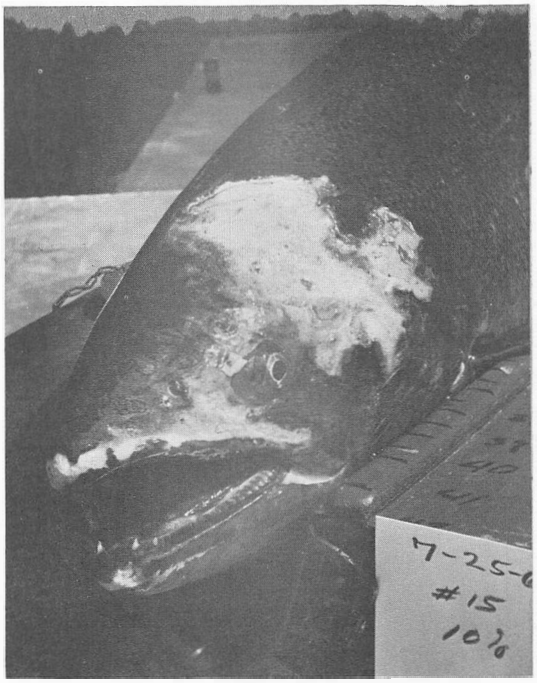

Figure 7.--Same as Figure 6, One Week After Topical Application of 10% Aqueous Malachite Green.

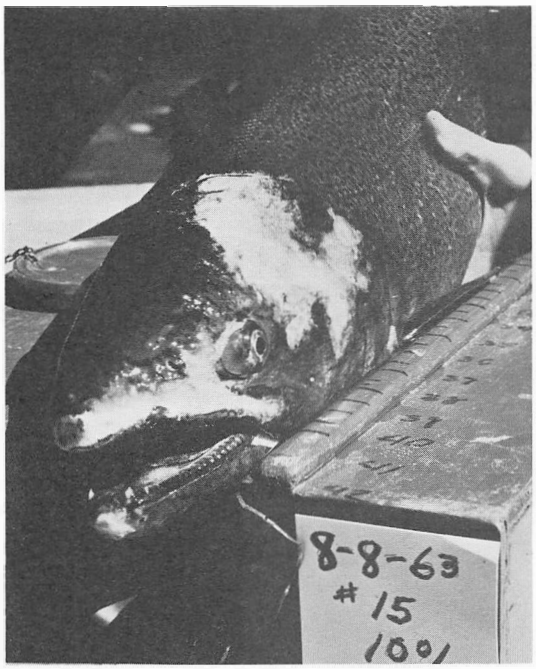

Figure 8.--Same as Figure 6, Three Weeks After Weekly Topical Application of 10% Aqueous Malachite Green.

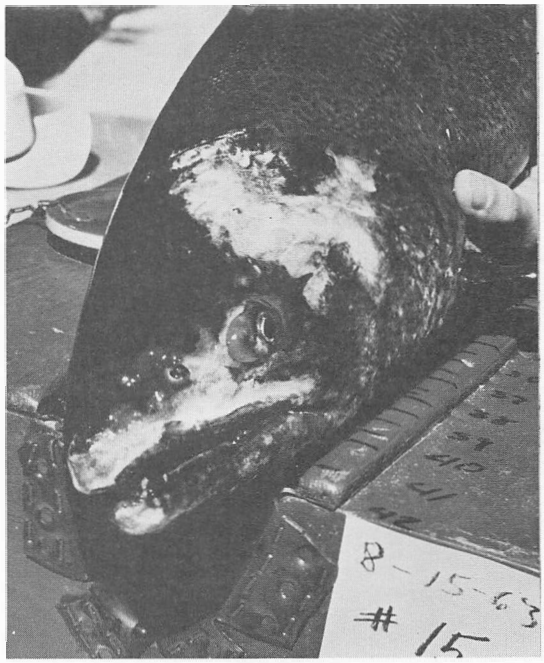

Figure 9.--Same as Figure 6, Four Weeks After Weekly Topical Application of 10% Aqueous Malachite Green.

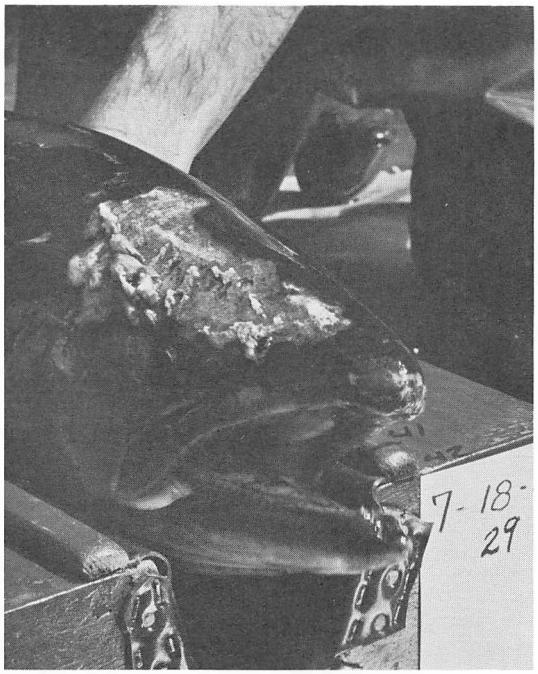

Figure 10.--Female Chinook, Before Treatment with Malachite Green.

Figure 11.--Same as Figure 10, Thirteen Days After Weekly Topical Application of 10% Aqueous Malachite Green.

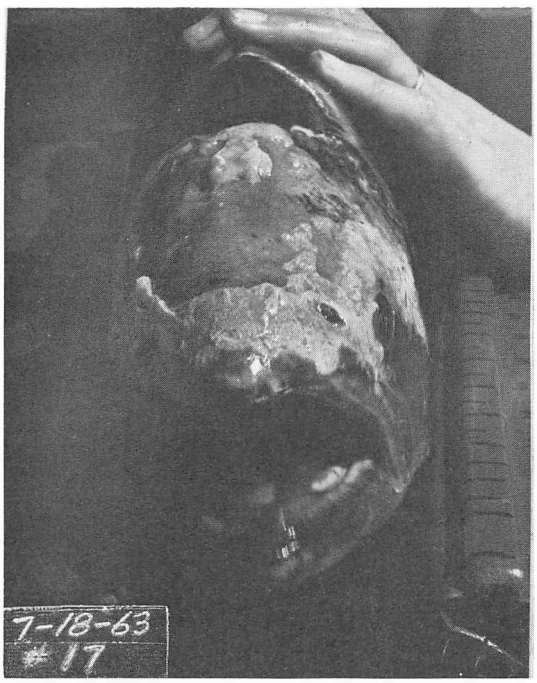

Figure 12.--Female Chinook, Before Treatment with Malachite Green.

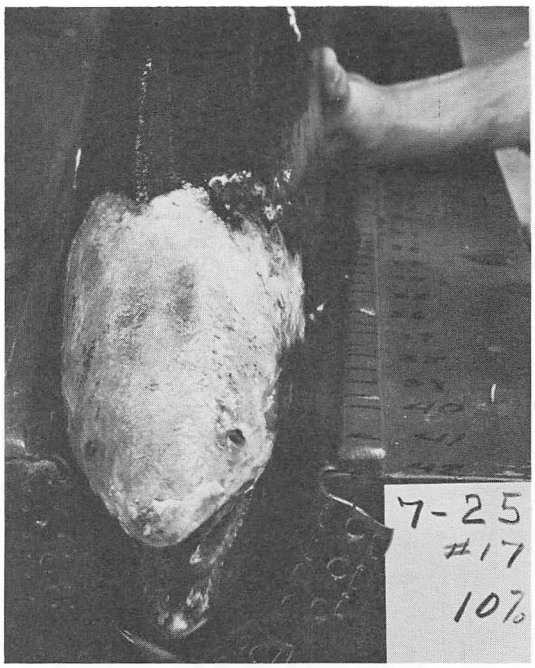

Figure 13.--Same as Figure 12, One Week After Topical Application of 10% Aqueous Malachite Green.

ment and Figure 13 shows the fish one week after treatment. By comparison most of the fungus mat had become less extensive during this short period, however, healing could not be recorded since the animal did not survive until the next examination. The cause of death was diagnosed as a secondary A. liquefaciens infection.

DISCUSSION

It is evident that an extensively "fungused" lesion on adult chinook salmon can be cleared of the infection by topical application of malachite green with subsequent regeneration of the damaged tissues. Topical application affords an opportunity to subject the fungused area, with safety to the fish, to a concentrated solution of fungicide, and to rid the area of the infectious agent. Once the lesion is free of the agent, healing will follow with relative rapidity.

Treatment of each fungused fish in this manner, each week, on an operational basis is in many cases impractical. However, if situations were to arise in which a small number of females were on hand, or in which the fish were being frequently handled (as experimental animals or ripeness tests during spawning periods) one could easily justify the topical application procedure.

CONCLUSIONS

Concentrated malachite green, when applied directly to a fungused lesion clears the lesion of fungus and weekly treatments promote healing of the area. No fungus tested resisted this treatment.

II. Water Treatment for Control of Common Fish Diseases: Chemical Toxicity Studies

INTRODUCTION

This investigation was undertaken to establish total immersion toxicity levels of malachite green, ethyl mercury phosphate (lignasan) and pyridyl-mercuric acetate (P.M.A.) for chinook and coho salmon. Each has been employed in water treatment procedures for the control of fish diseases. The primary objective of this investigation was to establish toxicity levels for adult salmon, but juvenile studies were conducted during the winter when adults were not available.

MATERIALS AND METHODS

All toxicity studies were conducted by subjecting the fish to the chemical for one hour. The fish were observed for 72 hours after the treatment period and the number of deaths noted at 24-hour intervals. All studies on adult chinook were conducted at Dexter holding ponds, in water temperatures of 13-14° C. The test on coho adults (jacks) were performed at Sandy Hatchery, in water temperatures of 6-10° C. All experiments on juveniles were conducted at Clackamas with water temperatures maintained at 15° C. Chinook yearlings were obtained from Willamette Hatchery, and the coho came from Sandy Hatchery. The juveniles of both species were subjected to identical experimental conditions, therefore the toxicity for each species is plotted on the same graph for comparison.

RESULTS

Adult Experiments

Figure 14 shows malachite green is toxic for adult chinook at concentrations greater than 5 ppm with an LD_{50} between 8-9 ppm; LD_{100} was experienced at 10 ppm. An LD_{50} is the dosage or concentration at which 50% death can be expected. Lignasan (ethyl-mercury phosphate) was toxic for chinook at concentrations beyond 2 ppm (Figure 15). The LD_{50} is between 2-3 ppm and LD_{100} occurs at 4 ppm. PMA (pyridylmercuric acetate) is toxic for chinook at concentrations above 10 ppm (Figure 16). The LD_{50} is between 20-25 ppm, while the LD_{100} occurs at 35 ppm. Figure 14 shows that malachite green is toxic at concentrations over 2 ppm. The LD_{50} is between 3-4 ppm and the concentration at which LD_{100} occurs is 5 ppm. In Figure 15 it can be seen that Lignasan is toxic at over 2 ppm. The LD_{50} is between 4-5 ppm, with an LD_{100} of 6 ppm. PMA (Figure 16) was toxic at concentrations over 14 ppm, with an LD_{50} between 16-18 ppm. LD_{100} was observed at 30 ppm.

Juvenile Experiments

Figure 17 shows the toxic effect of malachite green begins at a concentration above 1 ppm for juvenile coho and above 2 ppm for juvenile chinook. The LD_{50} for coho is approximately 3 ppm and 4.5 for chinook. LD_{100} for both species was reached at a concentration of 6 ppm. Lignasan (Figure 18) was toxic at concentrations greater than 2.5 ppm for chinook and 3.0 ppm for coho. The LD_{50} is approximately 4 ppm for both species, while LD_{100} was reached at a concentration of 4.5 ppm for chinook but not until 6 ppm for coho. Figure 19 shows the toxic effect of PMA on chinook begins at concentrations over 8 ppm and on coho at

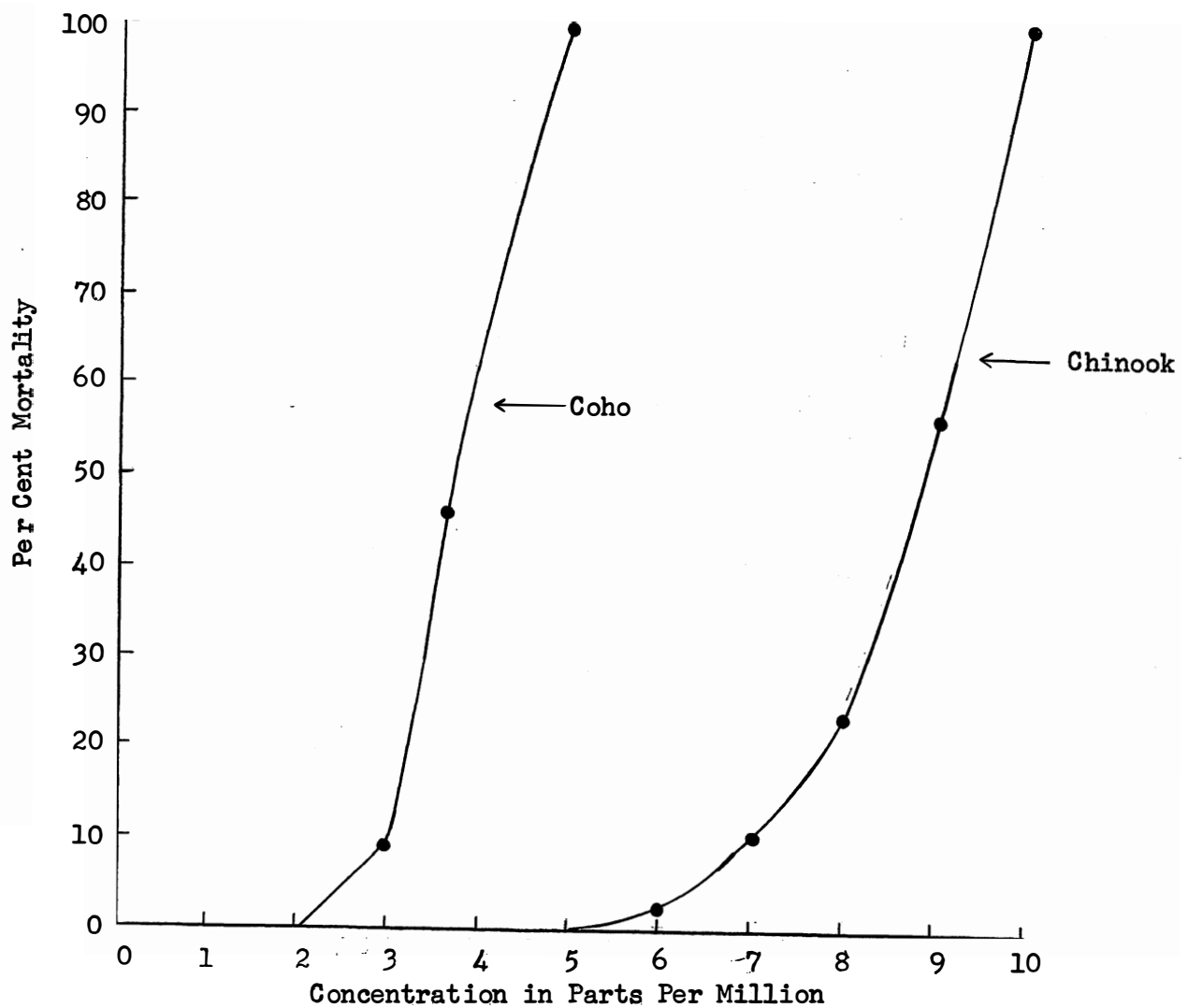

Figure 14. Toxicity of Malachite Green to Adult Chinook and Coho.

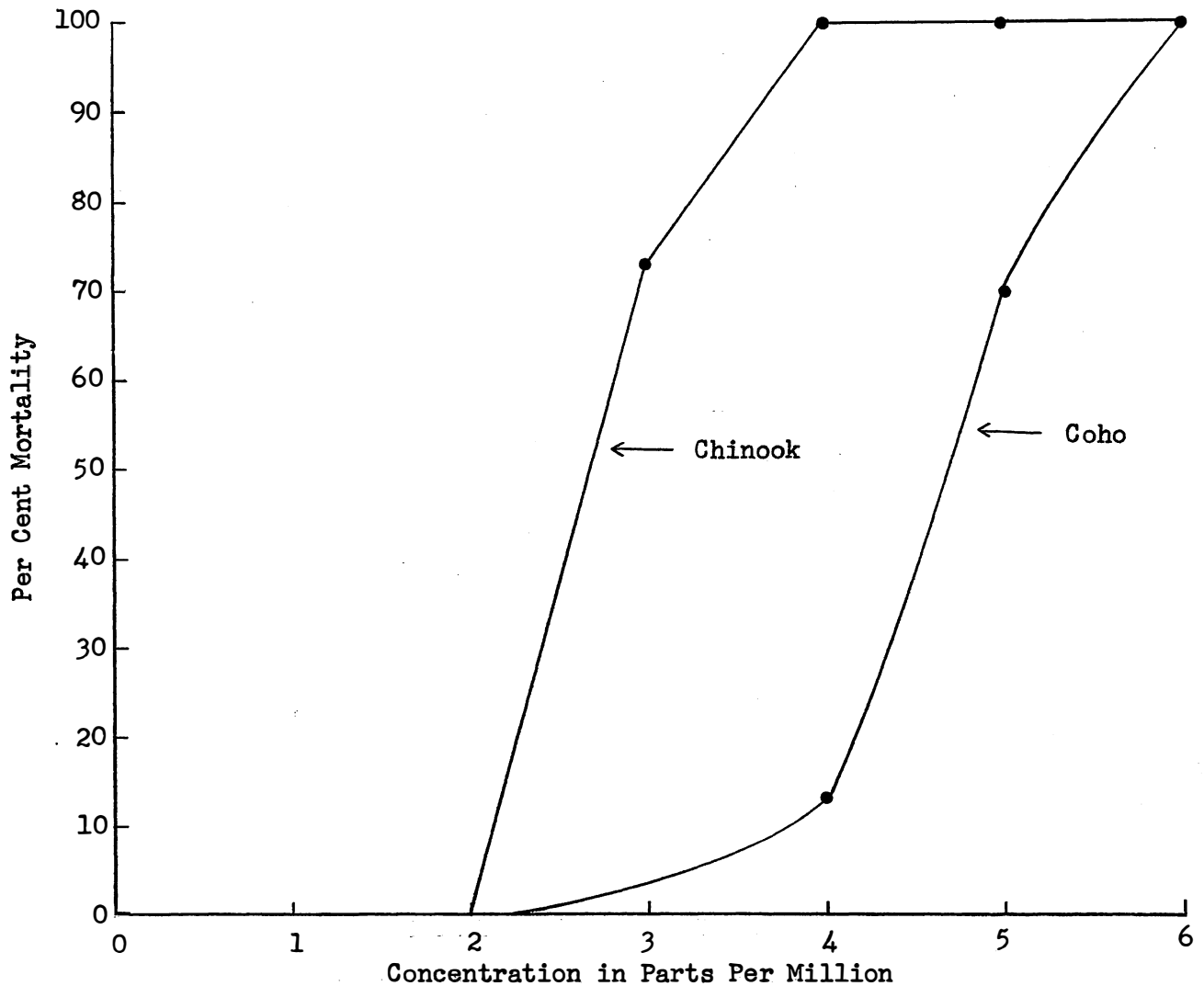

Figure 15. Toxicity of Lignasan to Adult Chinook and Coho.

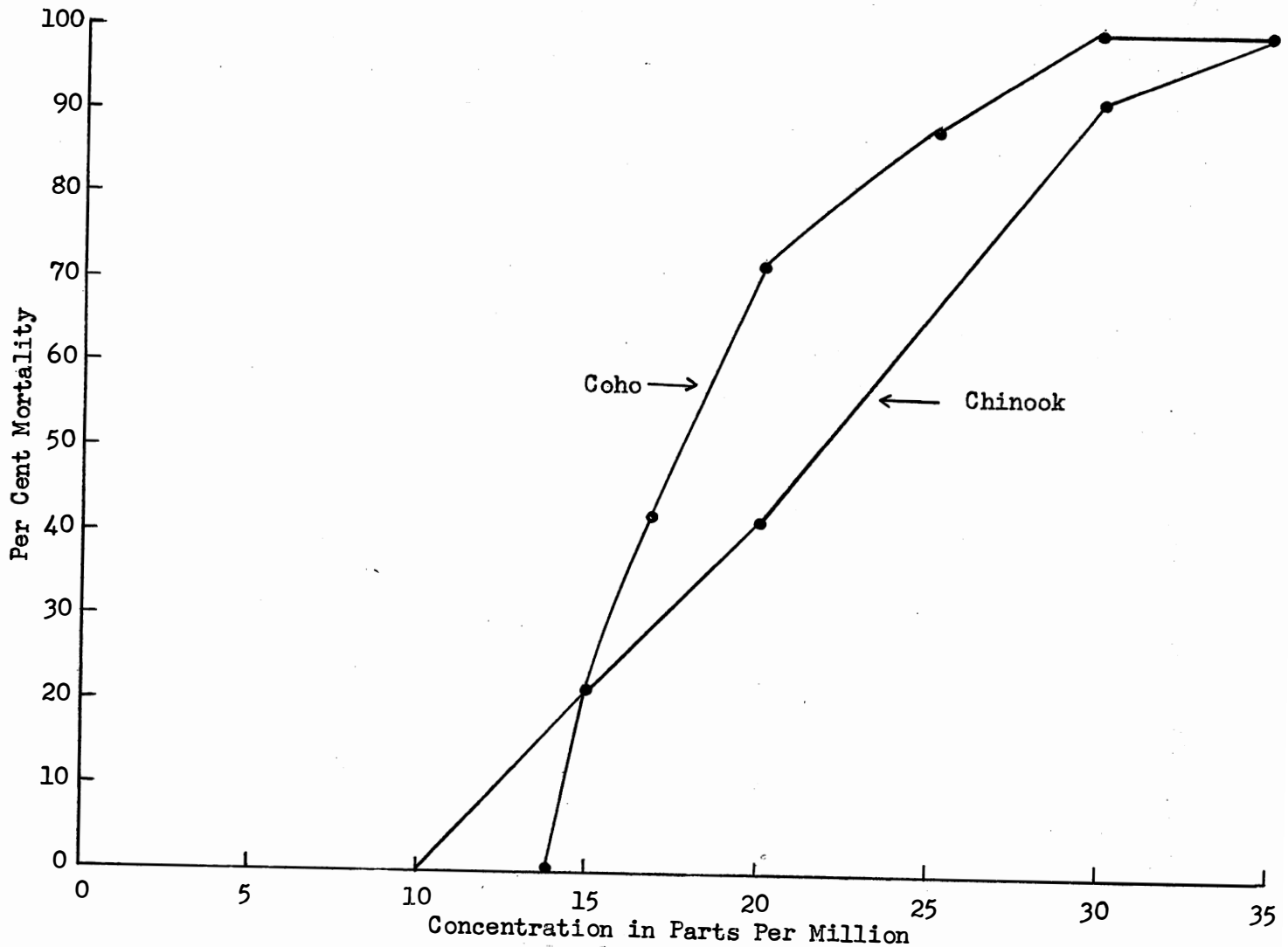

Figure 16. Toxicity of P.M.A. to Adult Chinook and Coho.

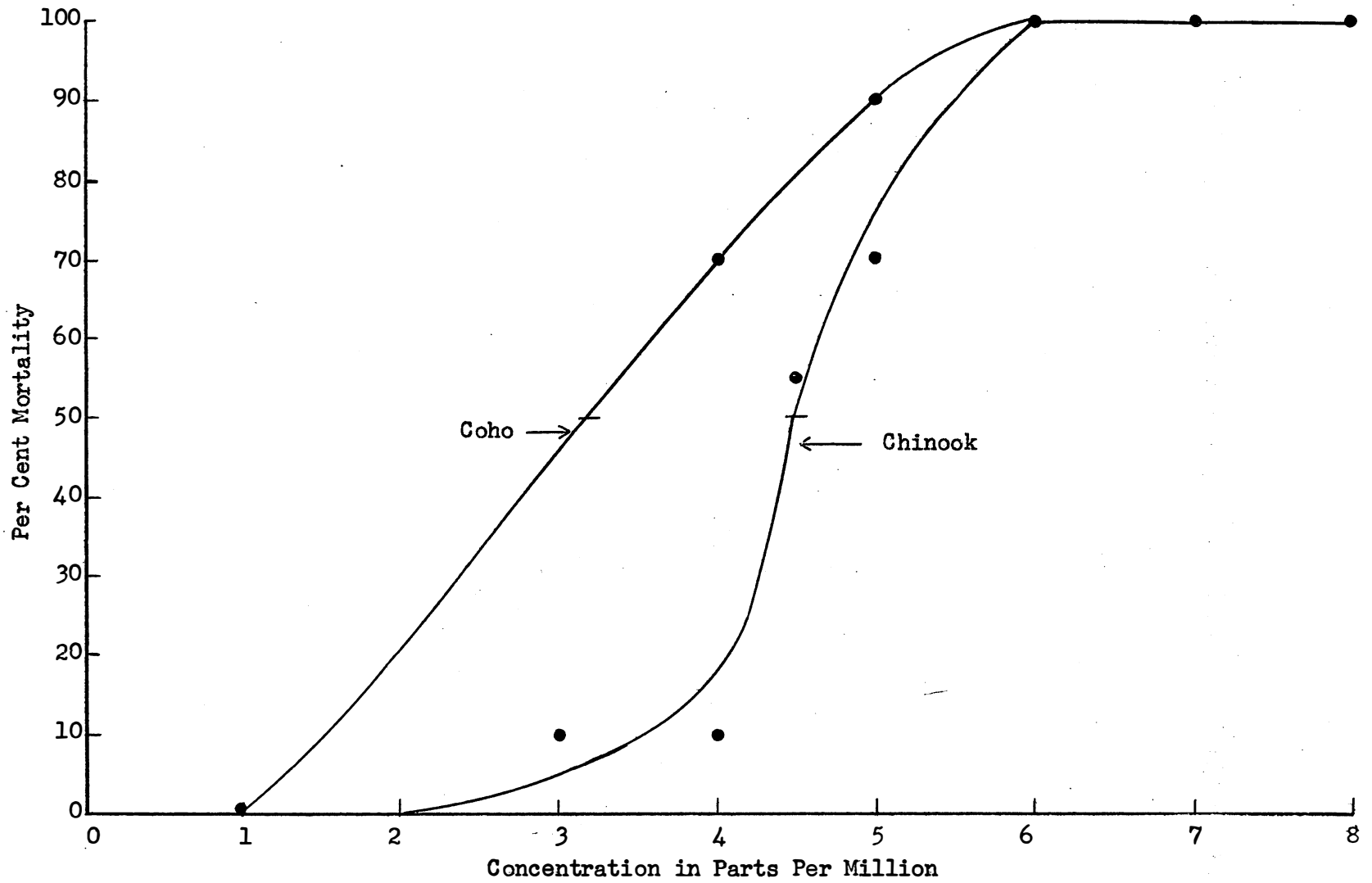

Figure 17. Toxicity of Malachite Green to Yearling Chinook and Coho.

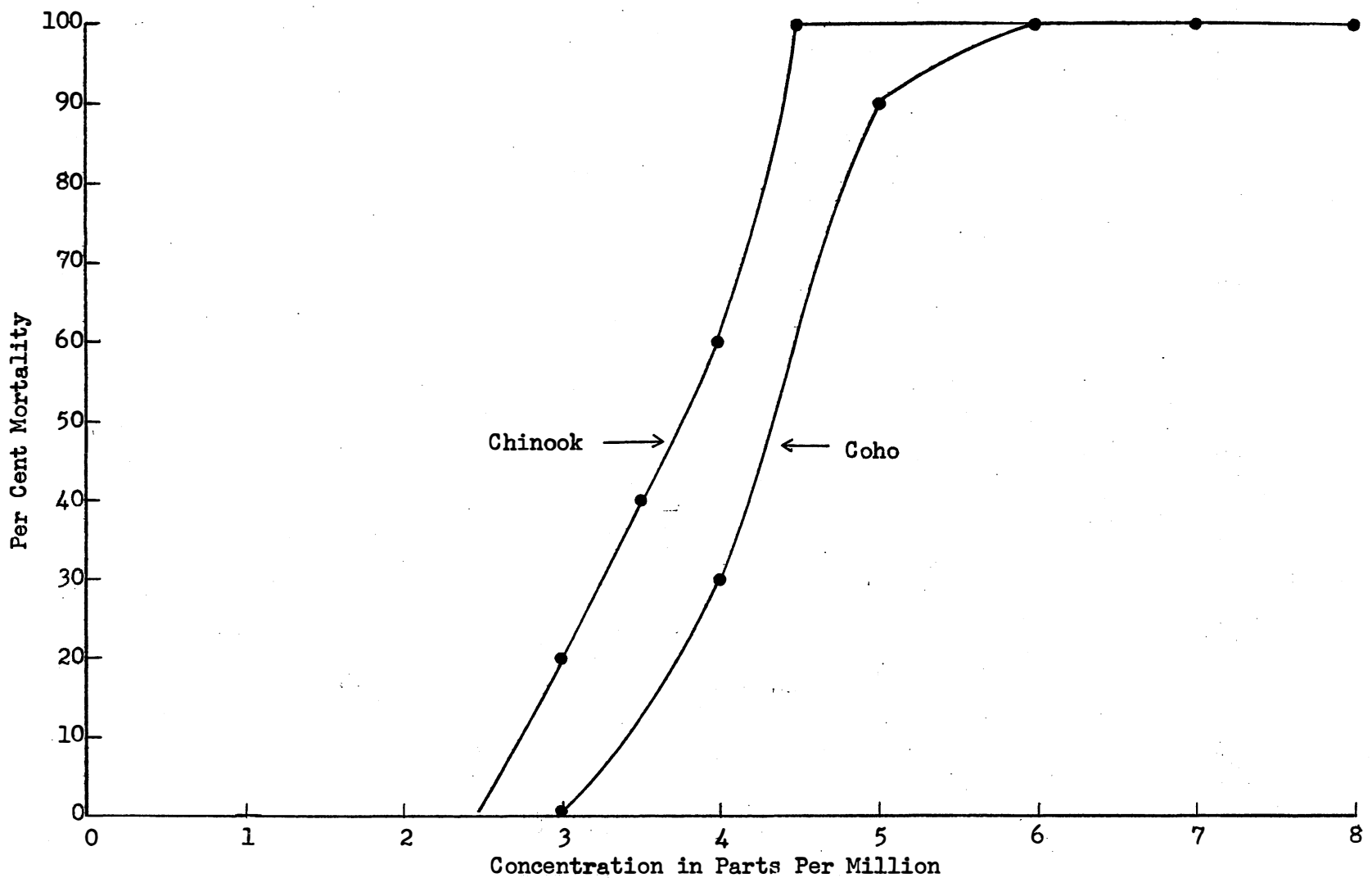

Figure 18. Toxicity of Lignasan to Yearling Chinook and Coho.

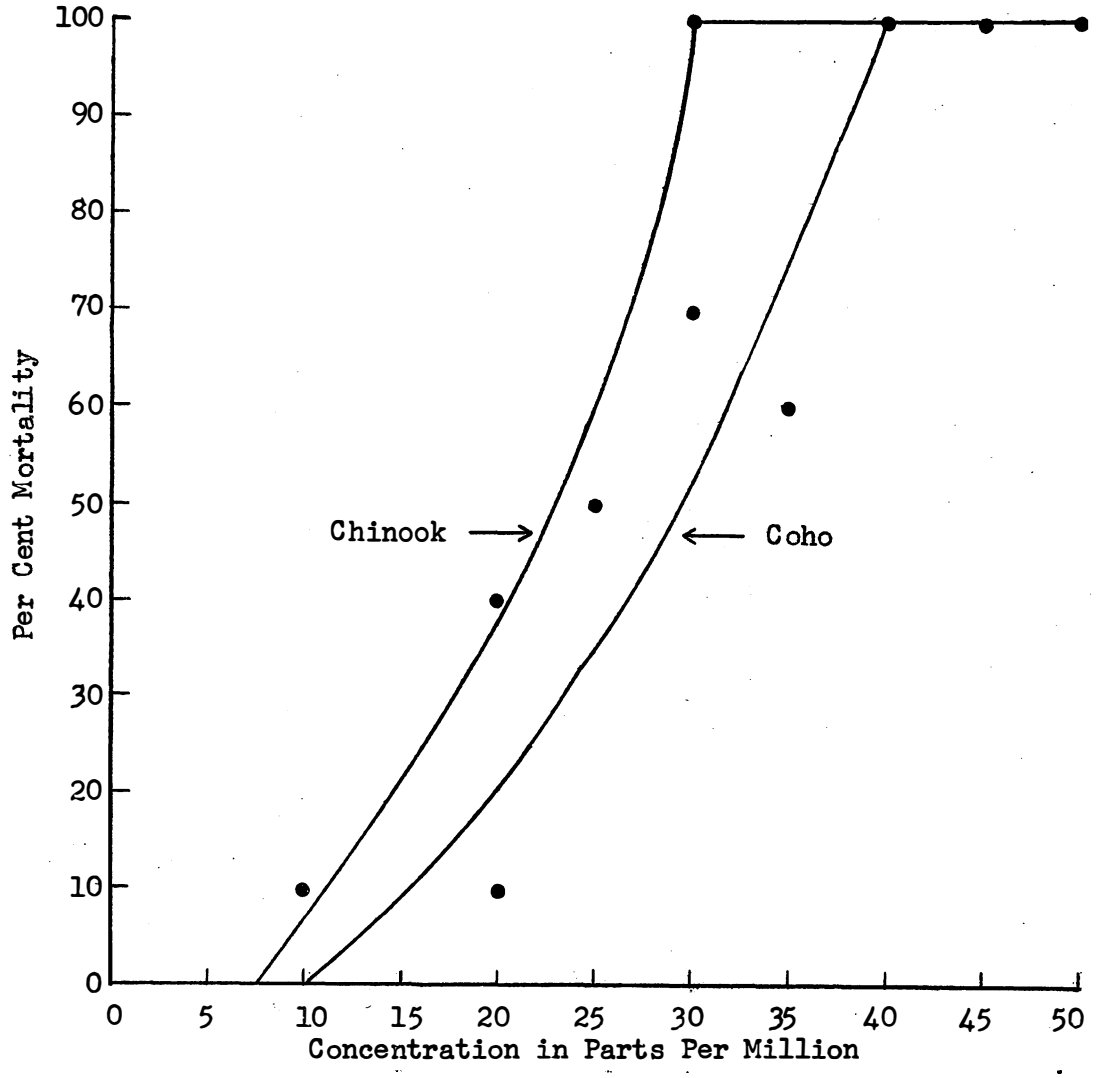

Figure 19. Toxicity of P.M.A. to Yearling Chinook and Coho.

greater than 10 ppm. The LD_{50} for chinook is approximately 23 ppm and for coho approximately 30 ppm. The LD_{100} for chinook was 30 ppm, and for coho, 40 ppm.

CONCLUSIONS

A study of these results indicates that there are differences in the ability of coho and chinook salmon to withstand the toxic effect of the chemicals tested. However, such comparison should not be made as the experiments were carried out at two different sites, at different times, and at different water temperatures. Variations in experimental results caused by water quality differences was clearly shown by two experiments, not reported here, in which the water was slightly turbid. In the turbid water the toxic level of malachite green for adult coho dropped to less than 1 ppm, as compared with greater than 2 ppm in non-turbid water at the same site. Thus under adverse water conditions possibly all water treatment chemicals should be used with great caution.

The experiments with juvenile salmon can be compared because identical experimental conditions were imposed on both species. It can be seen that chinook yearlings are more resistant to the toxicity of malachite green than coho. The reverse is true with Lignasan where coho yearlings are slightly more resistant than chinook. The experiments with PMA show coho are again more resistant to the chemical's toxic properties.

The variations in experimental results caused by water quality indicates that in order to establish accurate guidelines for use with various waters, experiments should be performed at each hatchery or holding facility involved.

MISCELLANEOUS

I. Area Survey Reports

The Middle Fork of the Willamette River and the Snake River were selected as study areas, since a comprehensive knowledge of the two groups of fish (viz. spring chinook in the Willamette and fall chinook in the Snake) would allow a comparison with information gained prior to this study. Periodic disease surveys were made in these areas and laboratory and other information gathered in Special Reports as follows:

Udall Disease Investigations, Hatchery Examinations of Adult Chinook, Summer 1963

Udall Disease Investigations, Hatchery Examination of Adult Chinook, Oxbow Hatchery, September-October 1963

Udall Disease Investigations, Hatchery Examinations of Adult Chinook, Dexter Holding Ponds, September-October 1963

In review, information contained in these reports leads us to the following conclusions:

1. While Chondrococcus columnaris was isolated from between 30-80% of the dead fish examined, it was not considered a primary cause of death. In many cases, columnaris lesions appeared to be in an advanced state of healing.

2. Secondary invaders, viz., A. liquefaciens and other opportunists of the Pseudomonad type, appeared to be a principle cause of death. In most cases of death of this type, there appeared to be a correlation between abrasions and other external injuries and generalized infection. Deaths due both to primary pathogens, i.e., A. salmonicida, and to secondary invaders appear to be enhanced by high holding pond temperature.

3. Malachite green treatment of holding pond water is beneficial as an antifungal agent and as a general preventative in disease transmission.

4. Parasites of the myxosporidian group, identified as Ceratomyxa sp., contribute to a marked degree to deaths in the study areas. (While not a part of those surveys summarized here, findings from autopsies of fish from other areas of the state bear out the same conclusion). If Ceratomyxa sp. are not a primary cause of death, they are important in establishing a route of entry for secondary invaders. They were, in several instances, encountered in sufficient numbers in fish examined to be considered generally debilitating.

Additional reports are being prepared for the current season's operations, 1964.

II. A Synthetic Medium for the Growth of *Aeromonas salmonicida*

INTRODUCTION

Since a well-defined study of pathogenicity, drug resistance, susceptibility, or the physiology of an organism requires a chemically-defined, or synthetic medium, it was thought advisable to undertake the development of such a medium for *A. salmonicida*, a fish pathogen of importance in this study.

MATERIALS AND METHODS

Cultures included *A. salmonicida* 5000H isolated from Snake River adult fall chinook and *A. salmonicida* 5-14174 (ATCC 14174) obtained from American Type Culture Collection were used as test organisms.

Chemicals: All amino acids, vitamins, and nucleic acids were obtained from Calbiochem, California. All other chemicals were reagent grade chemicals.

RESULTS

Through culturing of 5000H and 5-14174 on various media lacking in all but one compound the following medium was found to adequately support the growth of these two organisms:

NH_4Cl	2.0	gm/liter
$MgSo_4 \cdot 7 H_2O$	0.1	" "
KH_2Po_4	1.5	" "
NA_2HPO_4	3.5	" "
Glucose	50.0	" "
Arginine	100	mg/liter
Methionine	100	" "
Aspartic Acid	100	" "
Leucine	100	" "
Glutamic Acid	100	" "
Histidine	100	" "
Serine	100	" "
Glycine	100	" "
Tyrosine	100	" "
Tryptophan	100	" "
Phenylalanine	100	" "
Lysine	100	" "
Alanine	100	" "
Pyridoxal Phosphate	0.0006	gm/liter
Pantothenate	0.001	" "
Riboflavin	0.001	" "
Niacin	0.002	" "
Para Amino Benzoic Acid	0.002	" "
Biotin	0.000002	gm/liter
Folic Acid	0.00002	" "
Uracil	0.02	" "
Adenine	0.02	" "

Distilled water	1000 ml/liter
For solid medium add:	
Agar	15 gm/liter
pH	7.0
Autoclave	15 minutes at 15 lbs.

DISCUSSION

It was found that most of the amino acids could be individually deleted from the medium without completely inhibiting A. salmonicida growth. Growth was, however, retarded. A similar effect was noted when individual vitamins were deleted. Removal of tyrosine, tryptophan, and histidine had little effect on the growth.

CONCLUSIONS

A. salmonicida has the ability to form most of the biosynthetic intermediates necessary for its growth (either completely from simpler components, or by alteration of already-supplied amino acids), however in most instances these are produced in minimal amounts not sufficient to support more than limited growth.

III. The Pathogenicity of a Phage-sensitive Strain of *Aeromonas liquefaciens*

INTRODUCTION

A gram negative rod-shaped bacterium was isolated from the kidneys of over 70% of the adult fall chinook mortalities occurring at the Oxbow Dam Hatchery on the Snake River in 1962. The frequency of its occurrence in culture from these mortalities prompted its identification. It was later classified as *A. liquefaciens*.

Work was undertaken to determine if *A. liquefaciens* is a pathogen, and also if it can be considered an important cause of adult chinook mortalities.

With the isolation of *A. liquefaciens* from the mortalities, a phage was also isolated. The indicator strain was designated 2035H as a stock culture number and the phage numbered 2035H Ø. This strain proved useful in determining the pathogenicity of *A. liquefaciens*.

MATERIALS AND METHODS

Cultures

Cultures were isolated and maintained on Meuller-Hinton (Difco) medium and transferred weekly. Incubation was at room temperature. Liquid cultures were maintained in a broth containing 11.5 gm/liter Bacto Casitone (Difco), 50 gm/liter brain-heart infusion (Difco), and 1.5 gm/liter soluble starch, (pH is adjusted to 7.0).

Cells of 2035H used for injection were centrifuged from liquid culture and washed twice with sterile saline. Cells were injected as a saline suspension. Cells for feeding experiments were harvested by centrifugation, and the packed cells added to the thawed food and mixed. The mixture was then refrozen until fed.

Fish

Juvenile spring chinook from Willamette Hatchery and juvenile coho from the Sandy River Hatchery were used as experimental fish. They were weighed and distributed 10 fish per group.

Autopsy and Reisolation of Test Organism

The phage indicator strain was used in all experiments of infectivity. When a mortality occurred, cultures were made from the kidney onto Meuller-Hinton agar, and the resulting growth tested for phage sensitivity by the agar overlay method (1). This proved to be a rapid and concise method for identification of the isolated organism. This phage was found to be specific for the indicator strain 2035H. This was shown by absence of plaque formation when 2035H Ø was tested against 20 other isolates of *A. liquefaciens* from the same area.

Feeding Experiments

Oregon pellets were thawed, and the cells from an 18-hour broth culture, plus other test ingredients were added, (such as ground glass). New pellets were made by

re-pelleting through a home food chopper, and refreezing. Fish were fed at the rate of 1.5% of their body weight per day.

RESULTS

Table 14 lists the experimental routes of infection which were tried. When the organism was injected intraperitoneally or subcutaneously the fish began to die about 9 hours after injection, and all were dead by 15 hours. The infection which resulted was systemic; the test organism could be isolated from the liver, heart, blood, and kidney of the mortalities. These methods of infection are, of course, quite severe and do not demonstrate any invasive powers on the part of the organism.

The next experiment was designed to determine if the organism could enter by way of an abrasion of the skin. The slime layer was first scraped away from the side of a fish and the skin lightly abraded with fine sand paper. This area was "painted" with a 5-hour culture. 1/ Figure 20 presents the results of this experiment. The first mortalities occurred at 65 hours after infection and the rate was nearly constant until 81.2% of the test fish were dead (140 hours after infection). Four control fish also died between 135 hours and 140 hours, however, the test bacterium could not be isolated from them.

It is apparent that a fatal infection with A. liquefaciens can be produced by abrasion or some similar trauma. In this laboratory fatal infections caused by A. salmonicida could be produced in this same manner. The method consistently was found to give uniform and reproducible measurement of infectivity (9).

Ground Glass Feeding Experiments

Wolfe and Dunbar (12) attempted to establish a route of entry through the intestine for the agent of kidney disease by using ground glass in the food, plus the bacterium. A similar experiment was designed using four lots of fish. One lot received the bacterium incorporated into the diet. The second lot received the bacterium plus 5% (by weight) ground glass in the diet. Corresponding control groups received the normal diet only, and diet plus ground glass but no bacteria. The rationale behind this was: if Ceratomyxa sp. infection of the intestinal tract had provided the route of entry of the bacterium under natural conditions, then the ground glass in the diet should experimentally reproduce a similar intestinal lesion and fatal systemic infection.

Slide preparations were made from organs of the mortalities at the Snake River for Ceratomyxa sp. identification. In the limited number of slides, Ceratomyxa sp. was determined as being present. It is known, from the work of Wood (13) at Washington hatcheries on the Columbia River, the incidence was quite high during the fall of 1962. This information promoted a search for some method whereby an infection with the isolate from the Snake River could be established by way of the intestinal route.

Table 15 gives the results of this experiment. It can be seen that the group receiving the ground glass plus the bacterial suspension contracted a fatal infection. The fish which received the bacterial suspension only in the food did not contract the infection. The group that received the ground glass but no bacteria

1/ In the modified Meuller-Hinton broth A. liquefaciens grows very rapidly and a moderately turbid culture could be grown in this time.

Table 14. Experimental Routes of Infection for A. liquefaciens.

Route of Inoculation	Per Cent Mortality End of 24 hr.	Number of Organisms per ml Injected or Infinial Concentration
Intraperitoneally	100	100 x 10^7
Subcutaneously	100	100 x 10^7
Feeding 1/	0	10.9 x 10^7
Control (SP)	0	0
Control (Sub Q)	0	0
Control (Feeding)	0	0

1/ The experimental fish were fed the diet containing the bacterium for a total of two weeks at the end of which all experimental fish were sacrificed and cultured.

incurred some mortalities, however, the infection was not caused by the test organism 2035H susceptibility. Again, the phage sensitivity of 2035 H was of practical value.

DISCUSSION

The bacterium which is discussed here was identified as a member of the Aeromonas genus by the Presumptive Identification Chart Published by Bullock (2). The bacterium was then classified as use of Bergy's Manual of Determinative Bacteriology and by use of published results of Omers (3, 4, 7, 8, 10, and 11). Several isolates of this same bacterium were all classified as A. liquefaciens and later compared to other members of the Aeromonas genus obtained from the American Type Culture Collection.

From the evidence which has been presented it can be seen that this bacterium causes death of juvenile salmon if it is interperitoneally or intramuscularly injected. When the organism is fed to fish in a food containing ground glass, the infection can be established, however, no infection occurred by general water contact alone in these experiments. The results point to the conclusion that A. liquefaciens lacks power of invasiveness and to establish an infection. It must be introduced into the tissue by some other means.

These conclusions are further supported by field observations of a group of adult spring chinook salmon during the summer of 1963. Again, Ceratomyxa sp. infection was observed in the majority of these fish from which A. liquefaciens was isolated.

CONCLUSIONS

A. liquefaciens is able to establish a fatal infection in fish if given a portal of entry.

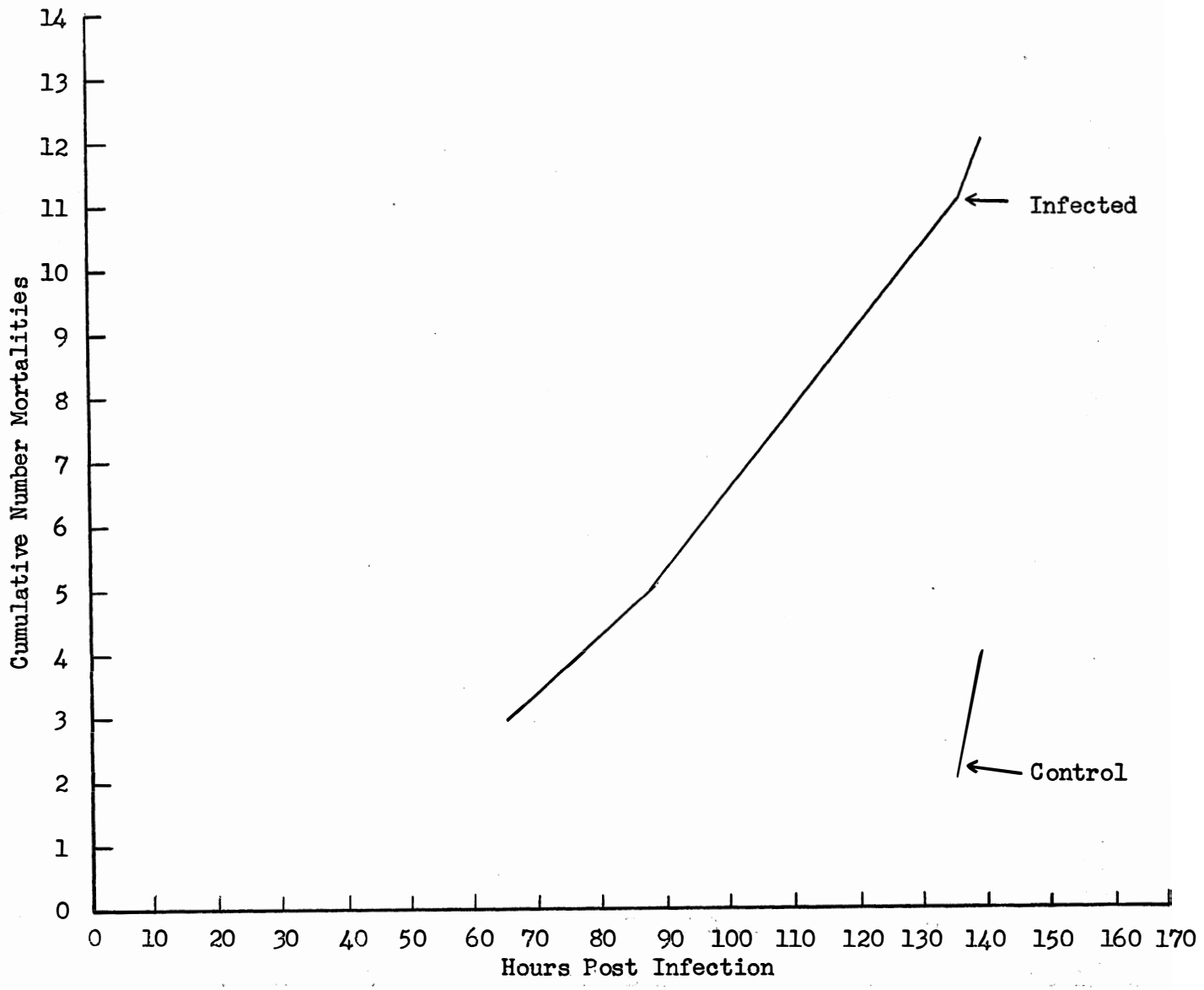

Figure 20. Cumulative Mortalities of Abraded Juvenile Coho After Infection with A. liquefaciens 2035H at 20° C.

Table 15. Thirty-Day Feeding 1/ Experiment Using the Isolated Bacterium from the Snake River.

Diet	Number of Mortalities 2/ Diagnosed as 2035H Infection
Control (Oregon Pellet)	0
Oregon Pellet plus Bacterium	0
Oregon Pellet plus Ground Glass	0
Oregon Pellet plus Ground Glass and Bacterium	5

1/ Rate of 1.5% of body weight per day.
2/ The organism used in this experiment was a phage sensitive strain, therefore the isolates from the mortalities were tested for the sensitivity to the phage.

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