42nd day, and the rest were killed at the termination of the experiment. Upon autopsy, the degree of tuberculous involvement in the lungs, omentum, liver, spleen, and peritoneum and kidneys was evaluated on the basis of a possible maximum of 20 as previously described.[3] The control group showed a tuberculosis index of 9.0 as compared with an index of 6.6 for the group inoculated with promin-treated bacilli. From the differences in the weight curves as well as from the tuberculosis index of the autopsied animals it appears that the results of inoculation of the treated and untreated strains into guinea pigs parallel the results obtained when the same strains were implanted on the chorio-allantoic membrane of the chick embryo.

Conclusions. 1. Experiments are described to show that prolonged cultivation of tubercle bacilli on a promin-containing medium attenuates their virulence. 2. Under the experimental conditions the reduction in virulence in the promin-treated strain persisted after the strain was returned to control media. 3. The change in virulence can be demonstrated both by implantation on the chorio-allantoic membrane of the chick embryo as well as by inoculation into guinea pigs. 4. The attenuating action of promin is slight, this being probably related to the relatively low bacteriostatic action of this drug against the tubercle bacillus. It seems likely that more promising results might be obtained by the application of this method to more active bacteriostatic agents.

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Action of Alkaline Acriflavine Solution on *Bacterium salmonicida* and Trout Eggs.*

WINSLOW WHITNEY SMITH. (Introduced by Anson Hoyt.)

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Trout furunculosis is a costly, widespread, epizoötic bacteremia, that annually destroys millions of salmon and trout.[1] The disease occurs in trout hatcheries and rivers in at least 8 European countries, in Canada, and in more than one-fourth of the United States. Trout furunculosis is caused by *Bacterium salmonicida* Lehmann and Neumann, a gram-negative non-spore-forming rod which shows deeply-staining, bipolar bodies; the taxomic position of this bacterium has not been determined.

Among the agencies by which furunculosis may be disseminated are trout ova, which in practical fish culture are freely transported from contaminated hatcheries and spawning beds to clean hatcheries. In fish hatcheries, eggs are usually incubated in headwaters; and thus may be a source of infection to fish downstream. The interior of living trout eggs is believed to be bacteriologically sterile.[2,3] Only the dead eggs and the exterior of living ones can be considered as sources of infection. The former may readily be removed manually. If, therefore, the exterior of the living ova can be disinfected, eggs may be placed in headwaters in hatcheries, without danger to fish downstream.

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1 Mackie, T. J., and Menzies, W. J. M., *J. Comp. Path. and Ther.*, 1938, **51**, 225.

2 Plehn, M., *Centl. Bakt.* (etc.), Abt. Orig., 1911, **60**, 609.

3 Williamson, I. J. F., *Fisheries, Scotland, Salmon Fish*, 1929, No. 1, His Majesty's Stationery Office, Edinburgh, 1929.

Blake[4] compared a number of disinfectants as to their toxicity for living trout ova and *Bact. salmonicida*. She recommended a 20-min dip in a solution of 500 parts per million (p.p.m.) neutral acriflavine (2,8-diamino 10 methyl acridinium chloride) as an effective disinfecting treatment which was harmless to trout eggs and lethal for *Bact. salmonicida*. Browning *et al.*,[5] Michaelis and Hayashi,[6] and Eggerth,[7] however, show that the bactericidal action of acriflavine is greatly reduced in neutral and acid solution. Gee and Sarles[8] recently compared the action of 19 disinfectants as to their toxicity for trout eggs and to *Bact. salmonicida*. They calculated a prophylactic index for each disinfectant, *i.e.* the ratio of germicidal power to the toxicity to eggs. They found that when used as herein described, acriflavine surpasses every product tested except sulfomerthiolate.

Experimental. Disinfection studies were made by means of the United States Food and Drugs Administration[9] method for testing disinfectants modified as follows: The medium was made by treating 500 g of ground, whole, fresh carp with 1 l of tap water for 2 hr at 70°C. The infusion was cooled, and the fat was swept off with gauze. The supernatant fluid was poured or pressed through several thicknesses of gauze and made up to 1 l. The infusion was autoclaved to precipitate the heat labile proteins and the fluid again filtered through gauze and restored to volume. One per cent of peptone (Parke Davis) was added and the pH adjusted electrometrically to 7.7. For semi-solid medium 0.4% of agar-agar powder and for solid medium 1.5% agar-agar was added. Agar media were used for subculturing the bacterium after treatment with acriflavine because of the marked adsorptive effect of agar on acriflavine.[7]

Stock cultures of *Bact. salmonicida* were carried on fish infusion agar slopes at 15°C and were transferred monthly. Work cultures were made up fresh each month from the month-old agar slope and were grown in tubes of 5 ml of fish infusion broth at 15°. These were transferred daily by pipetting 0.1 ml of 24-hr culture to a fresh tube. In resistance to acriflavine, this broth culture was shown to be equal to that grown in the standard methods broth made with special Armour's peptone. Except where otherwise designated, 24-hr broth cultures containing approximately 100,000,000 bacteria per milliliter were used in the disinfection tests. All tests were run at 10°.

Solutions of 500 p.p.m. neutral acriflavine U.S.P. were prepared with a naturally acid water (pH 6.2) with soft water in which carbon dioxide had been bubbled (pH 5.7), and soft water in which trout eggs had respired (pH 6.4). In all these solutions *Bact. salmonicida* survived longer than 30 min. Next, using Clark[10] buffer, 500 p.p.m. acriflavine solutions were prepared at pH 5.0, 5.5, 6.0, etc. to pH 8.5. Only those solutions at pH 7.0 and above killed *Bact. salmonicida* in 20 min; at pH 5.5 the bacteria survived for over 50 min.

A study was made of the action of 500 p.p.m. acriflavine solution at pH 7.7 on 23 cultures of *Bact. salmonicida*. The cultures used had been in stock in the laboratory from one week to 13 years when tested. They were isolated from 7 different epizoötics of trout furunculosis and from 4 species of trout in England and the United States. Every culture of bacterium was killed in less than 20 min. The various phases of the growth curve were also studied. Broth cultures that had been incubated from one hour to 20 days were tested, the tests being made on cultures varying by one day in age. The one-hour cultures were made from 12-day-old cultures and represented the lag phase. In no test did a culture survive longer than 20 min in

[4] Blake, Isobel, *Fisheries, Scotland, Salmon Fish*, 1930, No. 2, His Majesty's Stationery Office, Edinburgh, 1933.

[5] Browning, C. H., Gulbransen, R., and Kenneway, E. L., *J. Path. Bact.*, 1919, **23**, 106.

[6] Michaelis, L., and Hayashi, J., *Z. Immunitäts.*, 1923, **36**, 518.

[7] Eggerth, A. H., *J. Infect. Dis.*, 1926, **38**, 440.

[8] Gee, Lynn L., and Sarles, W. B., *J. Bact.*, 1942, **44**, 111.

[9] Ruehl, G. L. A., and Brewer, C. M., U. S. Dept. Agri., Circ. No. 198, 20 pp., Washington, D.C., 1931.

[10] *Manual of Methods for Pure Culture Study of Bacteria*, Biotech Publication, Geneva, N.Y., 1940.

500 p.p.m. acriflavine solution at pH 7.7.

To test the action of the disinfectant in the presence of trout eggs, ova were aseptically taken from ripe rainbow *(Salmo irideus)* and brown trout *(S. fario)*. They were charged with *Bact. salmonicida*, by placing 100 eggs in 100 ml of saline solution containing approximately 10,000,000 bacteria per milliliter. The number of bacteria adsorbed on an egg is not known, but they clouded broth medium more rapidly than eggs removed from contaminated trout abdomens. This is suggestive but is not clear cut evidence that the laboratory contaminated eggs were more heavily charged than naturally contaminated ova. Eggs were then treated for 20 min at 10° in the alkaline acriflavine solution, and removed to semi-solid, carp-infusion agar. After one week's incubation all tubes were negative for *Bact. salmonicida*. Eggs that had been exposed to the disinfectant for shorter periods carried viable *Bact. salmonicida* to the culture medium.

Next, it was desirable to know the effect of 500 p.p.m. of acriflavine at pH 7.7 on trout eggs under actual treatment conditions. Therefore, 7,500,000 ova from rainbow, brown, and brook trout *(Salvelinus fontinalis)* were treated for 30 min with the disinfectant with a total loss of only 3.65% of these eggs in the period between treatment and hatching. As a control, the records of 10,900,000 untreated eggs of the same species at the same hatcheries showed a loss of 7.92% for a similar period. Obviously, the treatment is not harmful; rather, it seems actually to benefit the eggs.

From these data, it is concluded that *Bact. salmonicida* on the shell of trout eggs is killed by a 500 p.p.m. acriflavine solution at pH 7.7 in 20 min and that the treatment of eggs for one-half hour with this solution is harmless to the eggs. Several hundred thousand trout from disinfected eggs were kept under observation for more than one year. No abnormalities in the trout were observed.

The efficacy of the disinfection in practical use cannot yet be judged because it is only one of several quarantine measures which are necessary to protect a disease-free stock. However, in one trout hatchery where egg disinfection has been combined with other quarantine measures, there has been no recurrence of trout furunculosis for 2 years.

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Assay of the Immunizing Effect of Scarlet Fever Toxin in the Rabbit.

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Evaluation of agents for immunization against scarlet fever has been restricted to the direct experience in human beings, the criteria of success being (1) the Dick test, (2) antitoxin determination in the serum, (3) clinical protection.[1-3] The latter will, of course, always remain the ultimate criterion of success. However, it was felt that for the development of an optimal immunizing agent for scarlet fever it would be of considerable help if a method could be found that would allow an experimental evaluation in the animal.

The assay of the scarlet fever toxin and its antitoxin in the skin of the rabbit has been successfully employed for some time.[4,5]

[1] Toomey, J. A., *Ann. Int. Med.*, 1941, **15**, 959.

[2] Kolchin, B. S., and Klein, J. F., *J. Immunol.*, 1941, **41**, 429.

[3] Veldee, M. V., Peck, E. C., Franklin, J. P., and DuPuy, H. R., *Public Health Reports*, 1941, **56**, 957.

[4] Buttle, G. A. H., and Lowdon, A. S. R., *J. Path. and Bact.*, 1935, **41**, 107.

[5] Plummer, H., *Br. J. Exp. Path.*, 1934, **15**, 80.