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ANTIBIOTICS IN FOOD PROCESSING

Experimental Preservation of Fish and Beef with Antibiotics

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The need for a simple inexpensive method of reducing losses of flesh foods due to bacterial spoilage prompted an investigation of the value of antibiotics. Spoilage of whole eviscerated fish was retarded markedly by ices containing 1 to 4 p.p.m. of chlorotetracycline (Aureomycin hydrochloride), by holding 6 days at −1° C. in sea water containing 2 p.p.m., or by 1-minute immersion in solutions containing 50 or 100 p.p.m. of the antibiotic prior to icing. The effect of different conditions on stability of chlorotetracycline in flesh foods was studied. These treatments have value in protecting fish from bacterial degradation. Owing to presence of interfering substances, it was not possible to detect, by the usual microbiological assay procedure, chlorotetracycline in flesh of fish so iced.

PRESERVATION OF FISH AND MEATS with antibiotics was reviewed recently, and it was shown that chlorotetracycline was more effective in preserving such foods than any of fourteen other antibiotics studied (*5*). Another investigation, conducted at about the same time, led to similar conclusions regarding the relative effectiveness of chlorotetracycline (*1*). The present report is concerned with possible practical methods of application of chlorotetracycline in retarding bacterial spoilage of fish, and its stability in flesh foods in the presence and absence of certain additives and on application of heat. Data concerning the antimicrobiological activity of puromycin and thiolutin in flesh materials are also included.

Materials and Methods

Ices containing chlorotetracycline or chlorotetracycline plus potassium dihydrogen phosphate were prepared by continuous addition of freshly prepared solutions to water which was frozen in a North Star type of flake ice machine. The resulting ices, in which the chlorotetracycline was uniformly distributed, were stored at about −20° C. until required. The fish were iced in 30 × 17 × 17 inch galvanized tanks equipped with drain spouts, four tanks being enclosed in a large box with an approximately 2-inch thickness of glass wool as insulation. The fish were sampled under clean conditions by removing a center steak about 1 inch thick from each fish, and blending a representative 200-gram portion with 600 ml. of water.

Experience in this laboratory has indicated that direct determinations of the total bacterial populations of samples of similar fish stored under comparable conditions are usually a good indication of their general state of preservation as judged organoleptically. For this reason total bacterial counts of the fish have been used arbitrarily throughout the present work as an indication of their comparative keeping quality. In the first experiment with iced fish the skin was removed from the steaks before blending with three volumes of water for a direct bacterial count, and in the second the skin was included. Bacterial counts were made by a direct method (*4*) using aniline methylene blue stain (*3*). Chlorotetracycline was determined by a pad-plate assay procedure (*2*). The pads containing the solutions (usually 0.1 ml.) were dried in vacuo over concentrated sulfuric acid before being placed on the assay agar. In some experiments vegetative cells of *Bacillus cereus* ATCC 10702 were used in the seed layer of agar. However, with this inoculum a response was not obtained with less than about 0.03γ of chlorotetracycline per pad. A similar assay, using spores of *Bacillus mycoides* (Lederle No. PCI 213) instead of vegetative cells, was found to be somewhat more sensitive, exhibition zones being obtained with between 0.005 and 0.01 γ of chlorotetracycline per pad. This assay was used in attempts to demonstrate chlorotetracycline in flesh of whole fish which had been stored in ices containing chlorotetracycline.

For determination of chlorotetracycline in flesh samples a standard curve was obtained by adding the required

Table I. Comparative Keeping Quality of Lingcod Stored in Ice

Days in Ice	Chlorotetracycline Concentration in Ice, γ/Gram: 0	1	2	2 + 200 KH_2PO_4	4
	Bacteria × 10^6 per gram				
Expt. 1					
11	128	24	9	...	12
15	187	20	37	...	15
Expt. 2					
9	62	12	18	9	...
14	420	65	68	75	...

level of the antibiotic to fish flesh (usually 40 mg. per 100 grams), promptly blending 1 part of this flesh with 9 parts of acid acetone solution (*2*), centrifuging at high speed, making appropriate dilutions in acid acetone, and carrying out the usual pad-plate assay. In flesh samples which contained very small or negligible amounts of chlorotetracycline 1 part of flesh was blended with 2 parts of acid acetone before a pad-plate assay was carried out with *B. mycoides* spores as inoculum. In such assays several separate additions of 0.1-ml. amounts of acetone extracts were added to the pads with intermediate drying in attempts to concentrate the probable minute amounts of the antibiotic present.

Tests with Ices Containing Chlorotetracycline

Lingcod were employed in two experiments in which the bacteriostatic effect of ices containing chlorotetracycline was determined. The fish used had been eviscerated and beheaded on the fishing boat and landed within 24 hours of capture. They could be considered as strictly fresh and some were still in *rigor mortis*. These fish were promptly and thoroughly iced with control and chlorotetracycline-containing ices, stored in the insulated containers, and sampled and reiced at appropriate intervals. In the first experiment six fish averaging about 5 pounds were iced with each of the experimental ices, while in the second experiment four fish were used. Individual fish, or in certain instances two fish, were removed at intervals, and direct bacterial counts and in some instances chlorotetracycline assays were performed. Unfortunately, acid acetone extracts of the flesh of normal fresh fish gave zones of inhibition in the pad-plate assay for chlorotetracycline, and no distinction was found between fish iced with ordinary ice and with ices containing the antibiotic. Thus, one set of assays carried out on fish iced with ices containing 1, 2, and 4 γ per gram of chlorotetracycline, and with ordinary ice, indicated that the fish flesh contained 0.15, 0.26, 0.15, and 0.15 γ per gram of chlorotetracycline, respectively.

Figure 1 shows the results of a typical assay for tetracycline in lingcod flesh. There was no apparent difference in results with fish iced with ordinary ice and with ices containing chlorotetracycline. Aqueous extracts of ordinary fish flesh obtained by removing the bulk of muscle proteins by isoelectric precipitation at pH 4.6 have exhibited antibiotic activity similar to that obtained with acid acetone extracts. It is evident from these results that the microbiological assay method now available is probably inadequate for determining this antibiotic in fish flesh. The nature of the substances in fish flesh which cause bacterial inhibition in the above assay procedure has not yet been determined.

The results of bacterial counts obtained at two intervals during storage in two different experiments with iced lingcod are given in Table I. As the direct count used is readily applicable only when fairly large numbers of organisms are present, the results obtained after a fairly extensive storage period are given. They indicate that all the ices containing chlorotetracycline, or chlorotetracycline plus potassium dihydrogen phosphate markedly suppressed bacterial growth, no important difference being observed with the various chlorotetracycline levels used. Even more marked differences in bacterial counts between fish iced with ordinary ice and ices containing chlorotetracycline might have been obtained if the fish had been exposed to the germicidal ices immediately after capture and evisceration.

Table II. Keeping Quality of Red Spring Salmon

(Stored on boat for 6 days in ordinary sea water and in sea water containing 2 γ per ml. of chlorotetracycline and maintained at −1° C.)

Days Stored in Ice after Landing	Bacteria, Millions per Gram: Stored in ice	Stored in chlorotetracycline soln.
6	25	0
9	62	0.12
14	212	12

Table III. Comparative Keeping Quality of Red Spring Salmon Stored on Boat for 5 Days

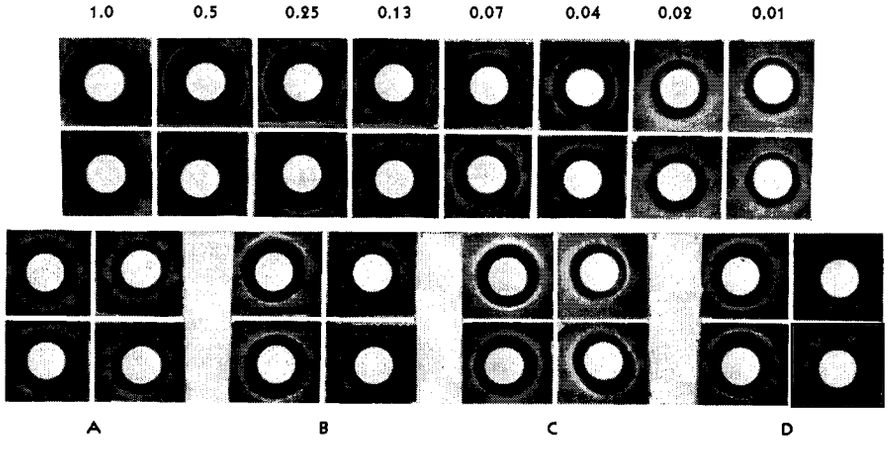
Days Stored in Ice after Landing	Bacteria, Millions per Gram: Stored in ice	Stored in −1° C. sea water
7	125	14
11	171	31

Immersion in Solutions Containing Chlorotetracycline

Red spring salmon weighing about 4 to 5 pounds were eviscerated and washed

Figure 1. Pad-plate assay for chlorotetracycline in lingcod flesh using *B. mycoides* spore technique

Two top lines represent a control assay with from 1.0 to 0.01 γ of chlorotetracycline per pad. *A*, *B*, *C*, and *D* are results obtained with fish stored in ices containing, respectively, 2 γ/g. tetracycline; 2 γ/g. tetracycline + 200 γ/g. KH_2PO_4; 4 γ/g. tetracycline; and no additive. Large and small inhibition zones were obtained with acid acetone extracts of the fish representing 100 and 66 mg. of flesh per pad, respectively



in the usual manner on a commercial fishing troller. Several fish were iced carefully, while a comparable number were stored in about one third their weight of sea water to which 2 γ per ml. of chlorotetracycline had been added, and which was maintained at approximately −1° C. by mechanical refrigeration. The fish were stored on the boat for 6 days under these conditions and were then re-iced on shore in the experimental holding tanks. Direct bacterial counts were made on these fish at stated intervals. Table II shows that the fish stored for 6 days at −1° C. in sea water containing 2 γ per ml. of chlorotetracycline spoiled very much more slowly than those iced in the usual manner. In a comparable experiment similar fish were stored in ice and in sea water at −1° C. without added chlorotetracycline. Table III shows that bacterial spoilage was less rapid in chilled sea water than in ice, but that the differences were in no instance comparable to those obtained in the preceding test in which 2 γ per gram of chlorotetracycline had been added to the sea water.

A further experiment was conducted on another trolling boat under somewhat different conditions. Coho salmon weighing about 7 pounds were eviscerated promptly after capture, the gills removed, and the visceral cavities washed thoroughly with clean sea water. Two fish were iced without further treatment and two were dipped for 1 minute in each of three different solutions containing 5 and 10 γ per ml. of chlorotetracycline, and 5 γ per ml. of chlorotetracycline plus 200 γ per ml. of potassium dihydrogen phosphate dissolved in sea water. The fish were promptly iced in the usual manner. Two separate lots of fish were so treated during the trip, and all fish were re-iced carefully after delivery to the laboratory. They were sampled at intervals, direct counts being made in the usual manner. The bacterial counts obtained with one fish from each treatment, recorded in Table IV, show that only a slight improvement in keeping quality resulted from the chlorotetracycline treatment.

Table IV. Effect of Immersing Coho Salmon for 1 Minute in Sea Water Containing Chlorotetracycline on Subsequent Keeping Quality of Iced Fish

	Bacteria × 10^6 per Gram			
Days in Ice	Controls	Chlorotetracycline, 5 γ/ml.	Chlorotetracycline, 10 γ/ml.	Chlorotetracycline, 5 γ/ml., + KH_2PO_4, 200 γ/ml.
13	8	0.8	1.0	0.5
18	180	120	90	75

In another similar test three lingcod, obtained while in pre-*rigor mortis* condition, were immersed for 1 minute in each of the following chilled solutions: 3% sodium chloride, 50 γ per ml. of chlorotetracycline plus 3% sodium chloride, and 100 γ per ml. of chlorotetracycline plus 3% sodium chloride. The fish were promptly iced with ordinary ice and stored and sampled as in the foregoing experiments. At intervals center steaks from each fish weighing roughly 500 grams were removed and incubated in covered glass containers for 24 hours at 10° C. in order to accelerate bacterial spoilage. The bacterial counts of these samples (Table V) show that brief immersion in the chlorotetracycline solutions occasioned a marked improvement in keeping quality.

Table V. Effect of Immersing Lingcod for 1 Minute in 3% Sodium Chloride Solutions on Subsequent Keeping Quality of Iced Fish

	Bacteria × 10^6 after Incubation of Excised Samples for 1 Day at 10°C.		
Days Stored in Ice	3% NaCl	3% NaCl + 50 γ/ml. chlorotetracycline	3% NaCl + 100 γ/ml. chlorotetracycline
3	1	0	0
6	130	<1	<1
10	280	6	<1

Stability of Chlorotetracycline In Flesh Materials

Three 95-gram portions of minced fresh Pacific brill flesh were prepared and 5-ml. portions of an aqueous chlorotetracycline solution of concentration sufficient to yield 5 γ of the antibiotic per gram of flesh were added to each. The apparent chlorotetracycline content of these samples was determined after different treatments with the following results: untreated, 6 γ per gram; stored 2 days at 4° C., 6.5 γ per gram; and heated in boiling water to an internal temperature of 80° C., 3 γ per gram.

In a further test minced red spring salmon and beef samples were stored for 2 days at 4° C. with 2 and 5 γ per gram of added chlorotetracycline. Assays of the stored samples yielded the following results:

Chlorotetracycline Added, γ/G.	Chlorotetracycline Found, γ/G.	
	Salmon	Beef
5	4.8	5.5
2	1.8	2.5

When ground lingcod or coho salmon flesh containing 5 γ per gram of added chlorotetracycline was heated at 100°C. in boiling water in covered glass containers, there was a progressive destruction of the antibiotic, only about 10% of the initial concentration being recovered after 30 minutes (Table VI). The results of these experiments indicate that chlorotetracycline is fairly stable in flesh

Table VI. Effect of Heat on Chlorotetracycline Content of Fish Flesh Containing 5 γ per Gram of Antibiotic

	Chlorotetracycline Recovered after Heating at 100° C., γ/Gram				
	0 min.	10 min.	15 min.	20 min.	30 min.
Lingcod	5.1	2.4	1.5	0.6	0.45
Coho salmon	4.8	1.8	1.8	...	0.30

Table VII. Antimicrobiological Activity of Chlorotetracycline, Thiolutin, and Puromycin in Ground Beef and Fish

	Bacteria × 10^6 per G. after Storage at 1.5° C.		
	8 days	11 days	15 days
Lingcod			
Controls	9.4	820	
Chlorotetracycline, 1 γ/g.	<0.1	<0.1	17 (yeasts)
			0.02 (bacteria)
Chlorotetracycline, 1 γ/g.			12 (yeasts)
Thiolutin, 10 γ/g.	<0.1	<0.1	0.1 (bacteria)
Thiolutin, 10 γ/g.	0.88	280	[a]
Puromycin, 10 γ/g.	8.7	240	[a]
Puromycin, 1 γ/g.	9.4	820	[a]
Beef			
Controls	1.6	310	[a]
Chlorotetracycline, 1 γ/g.	<0.1	0.31	12 (yeasts)
Chlorotetracycline, 1 γ/g.			<0.1 (bacteria)
Thiolutin, 10 γ/g.	<0.1	0.28	3.7 (yeasts)
			1.3 (bacteria)
Thiolutin, 10 γ/g.	5.5	460	[a]
Puromycin, 10 γ/g.	3.5	200	[a]
Puromycin, 1 γ/g.	...	390	[a]

[a] Putrid.

materials stored for 2 days at 4° C., but it is readily inactivated by heating.

In a further similar experiment samples of lingcod flesh containing 5 γ per gram of chlorotetracycline, with and without 40 γ per gram of ascorbic acid or 200 γ per gram of sodium nitrite, were stored 2 days at 5° C. At the conclusion of this storage period the chlorotetracycline content of the various samples was: control 4.8, ascorbic acid–treated 3.6, and nitrite-treated 4.8 γ per gram. Thus, nitrite appears to have no effect and ascorbic acid a slight destructive effect on chlorotetracycline in fish flesh.

Antimicrobiological Effect of Chlorotetracycline, Puromycin, And Thiolutin

To test the antimicrobiological effect of added chlorotetracycline, puromycin, and thiolutin in ground beef and fish, a technique was used identical with that employed in previous work (*5*). Table VII shows that chlorotetracycline (1 γ per gram) suppressed bacterial development and permitted yeast growth, and that thiolutin (10 γ per gram) did not inhibit yeast development in the presence of chlorotetracycline to any important extent. In this respect thiolutin differed from rimocidin, which had been shown to exert a marked antiyeast activity (*5*). Puromycin was devoid of antibacterial activity.

Organoleptic Findings

During the investigation the experimental samples were subjected to occasional superficial organoleptic examinations. It was observed that fish iced with ordinary ice attained a state of obvious staleness about 4 or 5 days earlier than fish stored in the chlorotetracycline-containing ices. These organoleptic improvements in quality were even more obvious with fish stored in sea water containing chlorotetracycline, or briefly immersed in the stronger chlorotetracycline solutions.

No extensive tasting tests were made on the cooked fish, but the results of a few trials indicated that treated samples remained in an edible condition very much longer than those which were not treated.

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The authors are indebted to Charles Brumwell for carrying out the dipping experiments with coho salmon, and to the British Columbia Packers for kindly granting all facilities for preparing the chlorotetracycline ices. Thanks are due to the Lederle Laboratories Division of the American Cyanamid Co. for supplying cultures and the necessary data for conducting chlorotetracycline assays, and for liberal supplies of chlorotetracycline, tetracycline, and puromycin.

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Quicker Method for Determining Fat in Liver

DETERMINATION OF LIVER FAT

Comparative Studies of Different Methods

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Fat is usually determined in liver by the rather lengthy ethyl ether extraction technique of the Association of Official Agricultural Chemists. The Roese-Gottlieb or Mojonnier technique of wet ether extraction is widely used in the dairy and food industry. Because this is shorter, it has been modified and adapted to the determination of fat in fresh liver tissues. The fat values obtained using this technique are consistently higher than the values obtained by dry ethyl ether extraction. When a mixed extracting solvent of ethyl ether, petroleum ether, and ethyl alcohol was used in the dry Goldfisch extractor, liver fat values approximated those obtained by the Mojonnier extraction. Alcohol may play a role in making the fat more easily accessible to ether extraction.

LIVER FAT IS PROBABLY most often determined by the lengthy technique of the Association of Official Agricultural Chemists for meat and meat products (*3*), using extraction with dry ethyl ether.

Only a few methods have been reported specifically for the determination of liver fat. Leites and Odenov (*7*) reported success with an alkaline digestion of liver, followed by a sulfuric acid extraction with petroleum ether, but,