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Title: THE ISOLATION AND IDENTIFICATION OF A MICROBIAL INHIBITOR FROM PACIFIC HAKE (MERLUCCIUS PRODUCTUS).

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Allen F. Anglemier

Studies on the isolation and identification of a compound showing antimicrobial activity in the body tissues of Pacific hake (Merluccius productus) were completed. Verification of formaldehyde as the component in hake responsible for microbial inhibition was obtained. Of the five fractions of hake press fluid separated by gel filtration (Sephadex G-10), only one fraction exhibited antimicrobial activity. This fraction (fraction D) also gave a positive Schiff's reaction. The 2, 4-dinitrophenylhydrazine (DNP) derivative of the active fraction had the same maximum absorbance value at 348 mμ as the standard formaldehyde derivative. Paper chromatography of 2, 4-DNP derivatives of fraction D and of the original hake press fluid both had an R_f value of 0.32 which was identical to that obtained with the standard formaldehyde derivative.

The 3-methyl-2-benzothiazolone hydrazone method for determining water soluble aliphatic aldehydes was modified and adapted

for analyzing formaldehyde contents of hake extracts. Initial concentrations of formaldehyde in fresh fish, 30 hours post-mortem, ranged from 20 to 85 ppm. Grinding, freezing and frozen storage (-10° C) for 20 days increased formaldehyde levels in most tissues. The most marked rise occurred in backbone samples which averaged 53 ppm formaldehyde initially but increased to a mean value of 210 ppm after 20 days of storage at -10° C. When ground hake muscle was mixed with other carcass components prior to freezing, a two to three-fold increase in formaldehyde formation was evident after 20 days of frozen storage.

Exhaustive lyophilization of the hake press fluid greatly reduced microbial inhibition. Heating for one hour at 85° C in open or closed systems resulted in complete loss of antimicrobial activity in the press fluid extracts. Formation of a dark amber pigment was noted during heating. Loss of antimicrobial activity was thought to be due to a heat induced chemical reaction between formaldehyde and ninhydrin positive materials of the extracts.

The Isolation and Identification of a Microbial Inhibitor from Pacific Hake (*Merluccius productus*)

by

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THE ISOLATION AND IDENTIFICATION OF A MICROBIAL INHIBITOR FROM PACIFIC HAKE (MERLUCCIUS PRODUCTUS)

INTRODUCTION

Food preservation is in one respect the process of minimizing microbial contamination and preventing or retarding growth of spoilage organisms. In this regard, the food industry is constantly searching for new procedures and/or substances that can be used effectively to control microbial growth.

While considerable advancement has been made in the drying, heating and freezing technologies of food preservation, only one new method of preservation, namely irradiation, has been forthcoming in the past 25 years (Chichester and Tanner, 1968). However, this method is being critically examined by the Food and Drug Administration (FDA), and currently its application to food processing is in abeyance (Jamison, 1968). Since current food preservation processes cannot be applied to a number of foods, or are only partially effective, the use of chemical preservatives or food additives either alone or in conjunction with other accepted procedures is essential to meet the ever increasing demands for food (Chichester and Tanner, 1968).

The actual definition of a food additive, as contained in the Food Additives Amendment enacted in 1958 to the Federal Food, Drug and Cosmetic Act of 1938, is quite lengthy and complicated (Luckey,

1968). The following description is clearly more meaningful for the purposes of this discussion. "A food additive," according to the Food Protection Committee of the National Academy of Sciences - National Research Council (1959), "is a substance or a mixture of substances, other than a basic food stuff, which is present in food as a result of any aspect of production, processing, storage or packaging."

This definition includes both intentional and nonintentional additives (Sanders, 1966a). Intentional or direct additives are purposely added in relatively low concentrations to perform specific functions in accordance with legal requirements. Additives may make food more nutritious and flavorful or may inhibit decomposition and thus retard food deterioration. Nonintentional or incidental additives serve no purposeful function but usually become a part of food due to unavoidable contamination. Equipment lubricants, packaging plasticizers, cleansing residues, etc., are examples of incidental additives. Materials of this nature are considered harmless and are permitted in minute amounts since it is almost impossible to prevent their occurrence in foods (National Research Council, 1959; Luckey, 1968; Sander, 1966a).

The 1958 Food Additives Amendment provides that before any substance can be added to food, the safety of the proposed use of the additive must be established by the manufacturer and a regulation

permitting its specific usage must be obtained from the Food and Drug Administration. Moreover, the manufacturer must also satisfy FDA that the additive will accomplish the function for which it is claimed so that, if a limit is necessary, the tolerance may be set at a level no higher than required to obtain the intended effect (National Research Council, 1959).

As a result of the enactment of the 1958 Food Additives Amendment, food additives are now closely scrutinized and regulated with the philosophy of protecting the public without discouraging free enterprise (Luckey, 1968). Often times, compounds effective in restricting microbial growth have deleterious effects on humans. For instance, β-propiolactone has many desirable antimicrobial properties but its possible carcinogenicity has ruled out its use as a food additive (Chichester and Tanner, 1968).

While some food additives (propionic and benzoic acids) are derived from various food sources, many are synthesized chemically for specific purposes or functions. In the latter category, butylated hydroxytoluene (BHT) is produced commercially as a food grade antioxidant. Propionic acid is produced naturally in Swiss cheese where it exerts preservative effects. Various propionates are used extensively to inhibit mold growth in bakery products, particularly bread (Sanders, 1966a, b). Another preservative, benzoic acid, occurs naturally in cranberries and prunes. In the form of sodium salt, it

has long been used as an antimicrobial additive for carbonated beverages and fruit juices (Chichester and Tanner, 1968). These few examples of some common food additives indicate that regardless of the source of origin, they are chemical in nature.

Since the enactment of the Food Additives Amendment, emphasis in food additive research has shifted toward investigation of naturally occurring compounds already present in foods or are chemically related to such compounds. The rationale of this trend probably stems from a reluctance on the part of FDA to approve totally new compounds and/or greater willingness to recognize naturally occurring additives that people have been consuming for years. FDA safety-testing requirements are more stringent for totally new synthetic compounds than for those which occur naturally (Luckey, 1968; Sanders, 1966a, b).

Although a wide array of food additives (> 2000) are currently available (National Research Council, 1965), these substances do not entirely satisfy the requirements of the food industry. Obviously, any single preservative which could be safely used for any product or groups of products to control microbial growth would be of considerable importance (Sanders, 1966b).

The presence of a seemingly potential antimicrobial factor in Pacific hake (Merluccius productus), which might be applicable for use in food, was observed during the early 1960's by Dr. F. M. Stout, Department of Animal Science, Oregon State University. This

observation evolved from mink nutrition studies. Stout, Oldfield and Adair (1960) reported that the feeding of frozen hake to mink resulted in loss of fur pigmentation and hypochromic anemia. In subsequent studies, Stout (1967) noted that when frozen Pacific hake were thawed, ground and allowed to stand at room temperatures, putrefactive spoilage was delayed. Water extracts of frozen, thawed hake, when added to ground beef or fluid milk, were effective in controlling microbial growth to the extent that normal spoilage did not readily occur at room temperatures. Moreover, water extracts showed an inhibitory effect against a broad spectrum of microorganisms including those associated with food spoilage. Stout also indicated that the antimicrobial factor could be inactivated in 20-30 minutes at a temperature of 68-70° C.

The above observations raised considerable interest in our laboratory, since this substance apparently possessed the desired prerequisites of an excellent antimicrobial food additive. It was present in water extracts from hake (a food source), exhibited a broad spectrum of antimicrobial activity, and was inactivated by heat. While there has been some question concerning hake as a food source, the Food and Drug Administration issued a regulation in early 1967, substantiating the wholesomeness of Fish Protein Concentrate (FPC) prepared by isopropanol extraction procedure from whole hake and hake-like species. This regulation does, however, restrict the use of FPC

to that of a food additive, intended for use in the home as a protein supplement (Roels, 1969). Furthermore, there is some indication that the Russians process Pacific hake for human consumption. Their fishing fleet collected 130, 000 metric tons of hake from the coastal waters of Oregon and Washington in 1966 (U. S. S. R. . . . , 1967).

In recognition of the need for new additives, particularly those for the preservation of food, and taking into account the rigorous requirements to be met in obtaining FDA approval for such compounds, several approaches to this investigation were originally considered. While studies concerning the evaluation of the antimicrobial factor in Pacific hake for the preservation of food were quite appealing, the main objective of this study was the isolation and identification of the factor _per se_. Hence, research described herein is concerned mainly with the latter aspect.

EXPERIMENTAL PROCEDURE

Source of Material

Pacific hake (Merluccius productus) were obtained from commercial fishermen based at the Yaquina Bay and Astoria regions of the Oregon coast. The migratory habits of Pacific hake contributed to their limited availability. Thus, fish were obtained between the months of May and October when they migrated into coastal fishing areas.

Fish were transported under ice in insulated containers to the Department of Food Science and Technology Laboratories in Corvallis. They were either weighed and analyzed in the fresh state or weighed, frozen and held at -10° C for subsequent study. The average weight of the hake was 980 gm. Although sex was not included as a criterion of selection, approximately equal numbers of male and female fish were analyzed.

Grinding and Pressing

Initial information (Stout, 1967) concerning the possible presence of an antimicrobial factor in Pacific hake indicated that the factor was most prevalent in the fluid obtained from grinding and pressing ground whole fish. Hence, much of the experimental work

described hereafter involves the use of the press fluid.

Frozen fish thawed at room temperature or fresh unfrozen fish were ground in an Enterprize meat chopper (1/3 h. p.) having plate holes 5 mm in diameter. Approximately 400-500 gm of ground tissue were placed in 6 x 10 inch double-layered cheesecloth bags. Bags were tied and stacked alternately between slotted pressing boards before being placed on a 6 x 12 x 1 inch plastic collecting tray. Filled bags were subjected to 900 p. s. i. for 10 minutes in a Carver Laboratory Press (Figure 1). The press fluid in the plastic collection tray was transferred to a two liter flask by connecting tygon tubing between the drain of the tray and the flask.

Dialysis and Lyophilization

Collected press fluid (one liter volumes) was dialyzed against equal volumes of deionized water for 24 hours at 2° C. Regenerated cellulose dialysis tubing (3.5 inch flat width), having an average pore size of 24 Å, was used for this separation. Dialysates were concentrated to 200 ml by lyophilization in Virtis filter seal freeze-drying flasks on a batch-type freeze-drier operated at a pressure of 0.3 mm of mercury. Concentrated dialysate was stored in stoppered, amber-colored bottles at 5 - 7° C.

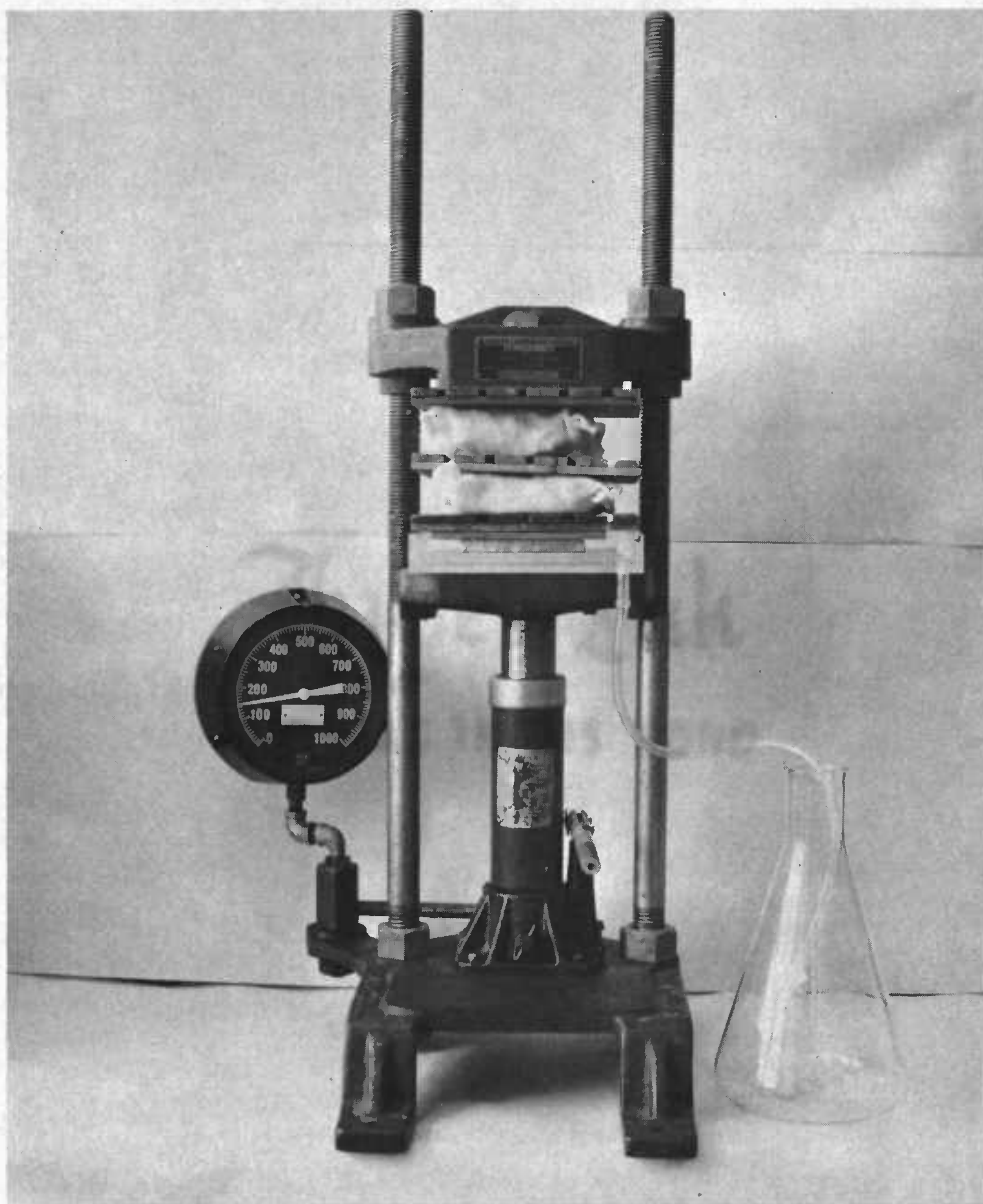

Figure 1. Carver Laboratory press and collection apparatus.

Fractionation of Press Fluid

Gel filtration was employed to purify and fractionate the antimicrobial factor in the concentrated dialysate. A Sephadex K25 laboratory column (2.5 x 100 cm), having a bed volume of 485 ml, was packed to a height of 80 cm with Sephadex G-10 gel. The gel was allowed to swell in an excess of deionized water prior to packing. The column was packed and operated in a cold room at 2 - 4° C.

Five milliliters of concentrated dialysate were placed on top of the column and eluted with deionized water at a flow rate of 2 ml/minute. The effluent emerging from the column was carried by polyethylene tubing to the flow-through cell of a Gilson UV absorption meter where transmittance of the solution was continuously monitored at 254 mμ and recorded with a Beckman Potentiometric linear recorder. After passing through the absorption meter, the effluent was collected in 10 ml fractions with an LKB fraction collector. Each fraction was assayed for antimicrobial activity by the modified agar plate assay method described later.

Descending paper chromatography was employed to determine the purity of the concentrated dialysate and of the tubes showing the highest absorption at 254 mμ of each fraction separated by gel filtration. Appropriate samples were spotted on Whatman No. 1 chromatographic paper and developed in a closed glass chamber for eight hours

with a solution of methanol:butanol:water (10:10:5 v/v/v). After completion of a run, papers were thoroughly dried and the spot patterns developed with ninhydrin reagent spray (Moore and Stein, 1948).

Microbial Assay Method

A qualitative method, utilizing a modification of the disc-plate technique for screening antibiotics (Arret and Kirshbaum, 1959), was used to detect antimicrobial activity of the hake press fluid. Bacterial cultures were prepared by inoculating 10 ml of nutrient broth with colonies from agar slants of pure *Bacillus cereus* 569R obtained from Dr. Dorothy K. Fraser, Department of Microbiology, Oregon State University. Cultures were incubated for 24 hours at 30° C. Two milliliters of this inoculum were added to 250 ml of standard plate count agar (Difco) and thoroughly mixed. Inoculated agar was poured into Petri dishes to a height of 7 - 9 mm and allowed to cool. Holes, 15 mm in diameter, were bored in the agar to serve as wells for the test solutions. Portions (0.5 ml) of test solutions were pipetted into separate wells and the plates were incubated at 30° C for eight hours. The antimicrobial activity of the solutions was estimated by measuring the width of the inhibition zone surrounding the well. Similarly, 1 gm samples of hake tissue were placed on the surface of other agar plates to evaluate tissue samples for antimicrobial activity prior to extraction.

Bacillus cereus 569 R was used as the test organism since similar bacterial strains are routinely used for detecting antibiotics in milk. Moreover, preliminary studies conducted by Stout (1967) indicated that this organism was quite sensitive to hake press fluid.

Qualitative Methods of Identification

The presence of hydrogen peroxide in extracted hake dialysate was determined by procedures developed in this laboratory. Five milliliters of concentrated dialysate were added to 0.5 ml of catalase (EC 1.11.1.6) at 25° C. Specific activity of the enzyme was 47,000 I.U./mg with a protein content of 20 mg/ml. The antimicrobial activity of the dialysate was determined using the modified agar plate assay after treatment with catalase for one hour at pH 6.9.

The reagent for the anthrone test for detecting carbohydrates was prepared by dissolving 2 gm of anthrone (9-oxyanthracine) in one liter of 95% sulfuric acid. Five milliliters of 5% trichloroacetic acid were added to 3 ml of concentrated dialysate and to the eluant of the active fraction separated by gel filtration. Subsequent solutions were filtered through Whatman No. 42 paper to remove precipitated protein. Five milliliters of the filtrates were added to 10 ml of the reagent. The absorbance was determined with a Beckman DB recording spectrophotometer at 620 mμ using a reagent blank (Morris, 1948).

Ninhydrin reagent was prepared according to the method of Moore and Stein (1948) by dissolving 20 gm of ninhydrin and 3 gm of hydrindatin in 750 ml of peroxide-free methyl cellosolve and diluting to 1 liter with sodium acetate buffer, pH 5.5. One milliliter of reagent was added to 2 ml of concentrated dialysate and to fraction eluants obtained from gel filtration. Solutions were heated in a boiling water bath for 15 minutes. A purplish-blue color developed when free amino groups were present.

Rosaniline hydrochloride (200 mg) was dissolved in 120 ml of warm deionized water and cooled with stirring (Jacobs, 1958). Two grams of anhydrous sodium bisulfite were dissolved in the initial solution followed by the addition of 2 ml of concentrated hydrochloric acid. The entire mixture was diluted to 200 ml and allowed to stand overnight. Five milliliter aliquots from the tubes of the active peaks from the elution curves were mixed with 1 ml of the reagent and allowed to stand at ambient temperature for approximately 30 minutes. Pink to deep violet colors develop in the presence of aldehydes.

Derivatives of 2, 4-Dinitrophenylhydrazine (DNP)

Hydrazones of aldehydes in the concentrated dialysate and the active fraction separated by gel filtration were prepared using the procedure described by Day, Bassette and Keeney (1960). Aliquots of the dialysate and active eluant were mixed with equal volumes of

5 N hydrochloric acid saturated with 2, 4-DNP. Subsequent to a 12 hour reaction period, formed hydrazones were extracted with chloroform and evaporated to dryness by vacuum. Hydrazone derivatives were dissolved in methanol and recycled through a 4-5 gm Dowex-50 cation exchange resin column until colorless. To determine maximum absorbance, aliquots of the colorless eluate were scanned from 700 to 230 mμ on a Beckman DB recording spectrophotometer.

Chloroform extracts containing the hydrazones were spotted on 8-1/2 x 19-1/2 inch Whatman No. 1 chromatograph paper. Chromatograms of the unknown derivatives and known standards were developed according to the method of Huelin (1952) using methanol:heptane (1:2 v/v).

Quantitative Assay for Formaldehyde

The amount of formaldehyde in a series of standard formaldehyde solutions was determined by measuring the absorbance of the 2, 4-dinitrophenylhydrazones in methanol at 348 mμ and calculating the mg of formaldehyde by the formula described by Day, Bassette and Keeney (1960):

$$\frac{A_s \cdot MW \cdot 1000}{A_m \frac{(1000)}{X}} = \text{mg of the carbonyl}$$

where

A_s = absorbancy,

MW = molecular weight of the carbonyl,

A_m = molecular absorbancy index,

X = volume of solution containing the DNP hydrazone for A_s measurement.

Assays were also performed on water extracts of fish tissues. Five grams of ground tissue were homogenized with 20 gm of deionized water and centrifuged at 15,000 x G for 20 minutes at 2° C. One milliliter aliquots were assayed as described above.

3-Methyl-2-benzothiazolone Hydrazone Test

Sawicki *et al*. (1961) developed the 3-methyl-2-benzothiazolone hydrazone test for the rapid determination of aliphatic aldehydes of auto exhaust fumes and polluted air. Lindsay and Day (1965) modified this method for the spectrophotometric determination of diacetyl in lactic acid starter cultures.

Standard formaldehyde solutions were prepared ranging from 1 to 50 ppm. One milliliter of each standard was added to 5 ml of a 0.4% aqueous solution of 3-methyl-2-benzothiazolone hydrazone hydrochloride. Each solution was mixed and allowed to stand at room temperature for 25 minutes. Twenty milliliters of 0.2% ferric chloride in 1 *N* hydrochloric acid were added with mixing, allowed to stand for an additional 25 minutes and then diluted to 100 ml with

deionized water. A standard curve was prepared by plotting concentration versus absorbance with a Beckman DB recording spectrophotometer at 670 mμ.

Assay Method for Hake Tissues

Five grams of tissue were homogenized with 20 gm of deionized water. The slurry was transferred to a 25 x 100 mm polyethylene centrifuge tube and centrifuged at 15,000 x G for 20 minutes at 2° C. Appropriate aliquots were taken for dilution. One milliliter of diluted sample was added to 5 ml of a 0.4% aqueous solution of 3-methyl-2-benzothiazolone hydrazone hydrochloride. The solution was mixed well and allowed to react for 25 minutes at room temperature. Twenty milliliters of 0.2% ferric chloride in 1 N hydrochloric acid were added with mixing and allowed to stand for 25 minutes. The mixture was diluted to 100 ml with deionized water. The absorbance at 670 mμ was determined within 40 minutes after the final dilution by reading against a similarly prepared reagent blank with a Beckman DB recording spectrophotometer.

RESULTS AND DISCUSSION

Experiments of an exploratory nature were initially carried out to gain some insight about the characteristics of the antimicrobial factor present in Pacific hake. In the early part of November of 1967, several hake which had been frozen intact and stored for five months were obtained from Dr. F. M. Stout. These fish were taken from the same lot of frozen hake that were previously found to possess antimicrobial activity (Stout, 1967).

Subsequent to grinding of the frozen fish, press fluid was collected and dialyzed against an equal volume of distilled water. Two to three hundred milliliters of dialysate were concentrated by lyophilization and the resulting dry orange-colored powder rehydrated to 40-60 ml volumes with deionized water. Using _B. cereus_ as the test organism, microbial assays of the dialysate prior to concentration, resulted in zones of inhibition 3 mm in width beyond the agar wells. Zones of inhibition increased to only 5 mm when the dialysate was lyophilized and rehydrated which in effect should have resulted in a five-fold concentration of the antimicrobial factor. Hence, considerable inhibitory activity apparently was lost during lyophilization.. Furthermore, no microbial inhibition was detected in lyophilized dialysates which were held under freeze-drying conditions (300μ of Hg vacuum) for 8-12 hours beyond the initial stage of dryness, rehydrated

and tested for antimicrobial activity. A color change from orange to deep amber also accompanied the complete loss of inhibitory activity. By controlling lyophilization time so that freeze-drying was not allowed to proceed beyond the point where ice crystals were no longer visible seemed to increase recovery of the antimicrobial factor. When dialysate concentrated by controlled lyophilization times was rehydrated and tested against B. cereus, zones of inhibition 10 mm wide were observed. Moreover, formation of the deep amber color was not evident. These preliminary observations indicated that the antimicrobial factor was extremely volatile, particularly in the absence of water. Another possible explanation, however, was that the factor became chemically bound to other dialysate components during the removal of water.

This work was continued further by inserting a cold-finger trap containing dry ice-ethanol mixture (-72° C) between the vacuum pump and the sample drying flask where the vapors being liberated from the frozen dialysate during the lyophilization were trapped and refrozen. When the melted condensate from the cold-finger trap was tested for antimicrobial activity, inhibitory zones 3 mm wide were noted. These results confirmed the volatility of the inhibitory factor in hake extracts. In all subsequent preparations, lyophilization was stopped prior to the disappearance of the ice crystals.

No attempt was made to account for total loss of the

antimicrobial factor during lyophilization since a reliable and sensitive quantitative assay procedure had not been developed. Measurement of zones of inhibition was considered only as a rough estimate of antimicrobial activity.

To determine the direct effect of heat upon the stability of the antimicrobial factor in hake extracts, 5 ml samples of the concentrated dialysate were heated in open test tubes in an 85° C water bath. At designated intervals, aliquots of samples were removed from the water bath, cooled to room temperature in an ice bath and subjected to microbial assay. A curve of the heat inactivation data was prepared by plotting widths of the inhibitory zones against time (Figure 2). After two to three minutes of heating, the light yellow colored dialysate turned deep amber accompanied by a decrease in the width of zones of inhibition. At the end of 60 minutes of heating, no inhibitory activity could be detected by the microbial assay procedure. In view of indications that the inhibitory factor might be lost by volatilization during heating in open tubes, several 5 ml portions of the concentrated dialysate were sealed in glass tubing and heated in an 85° C water bath for 60 minutes. Upon removal from the water bath, samples were cooled and analyzed for inhibitory activity. Samples thus treated showed no inhibitory capacity. This line of investigation was discontinued since the results, other than showing heat lability, were relatively meaningless without positive identification of the

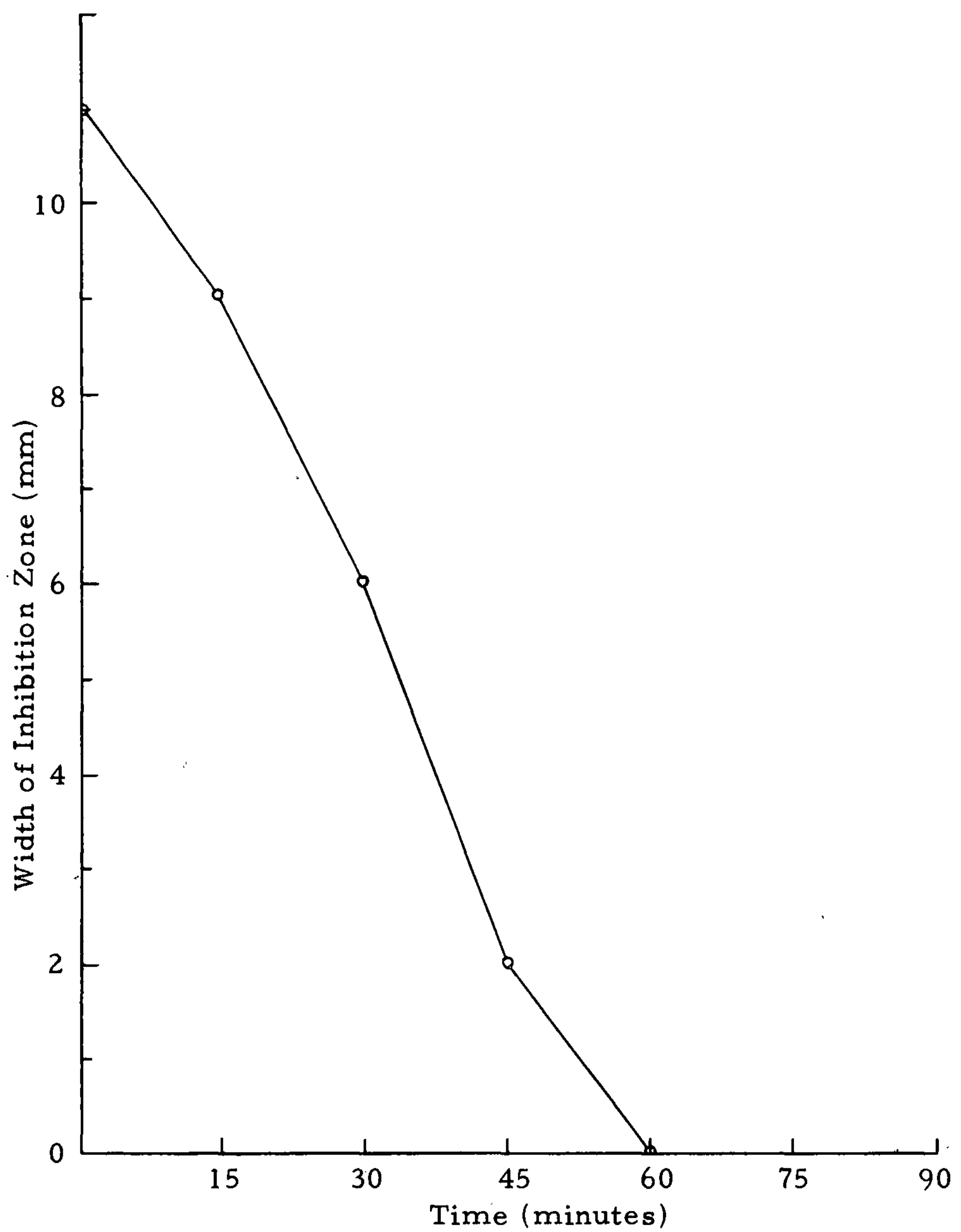

Figure 2. Effect of heat (85° C) on antimicrobial activity of concentrated dialysate as determined by the modified agar plate assay procedure.

antimicrobial factor.

Another area of preliminary interest was that concerned with the absence or presence of the antimicrobial factor in tissues and press fluid of fresh Pacific hake. In all of the previous work, frozen hake was the source of raw material. Concurrent with the initial studies described previously, fresh hake were obtained from the Astoria Seafoods Laboratory within 30 hours after being caught. Four average sized hake were carefully dissected into the following designated samples: muscular, skeletal, digestive, reproductive, nervous, stomach contents, and parasitic worms from the liver. One gram of each of the above described samples was placed on agar plates for assay. None of these samples inhibited the growth of _B. cereus_. These results suggested that fresh hake did not possess an active microbial inhibitor _per se_ and that factors other than death were responsible for its development, formation and/or liberation from a bound complex.

In view of these data, the effect of freezing of hake in relation to the formation of the antimicrobial agent was investigated. Tissues remaining from the previous experiment were combined, ground and thoroughly mixed. One-half of the ground sample was held at room temperature while the remainder was frozen at -10° C and stored for five days. Occurrence of putrefactive spoilage in the sample stored at room temperature was noted within 48 hours by the appearance of

considerable microbial growth plus the development of a foul, putrid odor. When 1 gm samples of frozen material were placed on agar plates and incubated for eight hours, zones of inhibition ranging from 10 to 15 mm in width were evident. Hence, freezing was apparently a key factor involved with the formation of the antimicrobial component. Although the exact contribution of freezing to the development of the inhibitory factor was not clear, freezing retarded growth of normal spoilage microorganisms present, and perhaps, allowed other organisms to grow and possibly produce the antimicrobial factor. Another effect of freezing was that of ice crystal formation which was extremely damaging to the integrity of hake muscle. Examination of hake muscles used in the previous experiments revealed "gapping" in the frozen tissue as well as a very porous and sponge-like structure. In this regard, an antimicrobial compound may have been liberated as the result of extensive tissue damage caused by freezing. In spite of the intriguing possibilities connected with the formation of the microbial inhibitor, further study in this area was not considered essential at this time because the isolation and identification of the antimicrobial factor was the chief objective of this study.

Distribution of the Antimicrobial Factor

Before proceeding to other areas of research, it was considered pertinent at this point to determine which tissues of the hake possessed

the highest concentrations of the antimicrobial factor. Such knowledge would be invaluable for isolating sufficient amounts of the inhibitor for identification studies as well as reducing contamination from other water-soluble constituents. Since results of the work previously described indicated that fresh, unfrozen hake did not contain the active microbial inhibitor, studies were designed to monitor its formation in various hake tissues during frozen storage.

Sixteen fresh hake obtained from commercial fishermen at Yaquina Bay were transported under ice to the Corvallis laboratory. They were dissected in a cold room (4° C) by opening the ventral surface and removing the viscera. The abdominal cavity was thoroughly rinsed with water. Muscle tissues were removed from the backbone and head in the manner of normal commercial fillet preparation. Viscera, head, and backbone were combined after grinding and thoroughly mixed. This mixture was designated as the ground viscera sample. Fillets from the right side of the fish were left intact while those from the opposite side were ground, mixed and designated as whole muscle and ground muscle samples, respectively. These samples were placed in one pound plastic freezer cartons with snap-on lids and were assayed for microbial inhibition using the B. cereus modified agar plate procedure after 0, 15, 21, and 30 days storage at -10° C.

In the following discussion, tissues inhibiting the growth of the

test organism will be classed as active while those designated as inactive showed no microbial inhibition. Microbial inhibition was not evident in any of the samples prior to freezing which concurred with previous data that the factor was not present or not active in fresh fish tissue. Inhibition was readily observed in samples of both the viscera and ground muscle after a storage period of 15 days.

A graph, depicting microbial inhibition, was prepared (Figure 3) by plotting the percentage of active samples versus storage time (days). The maximum percentage of active samples occurred at approximately 20 days of storage suggesting that the element of time was involved in the formation of the antimicrobial compound. Grinding of muscle also influenced the development of the inhibitor since 50% of the ground muscle samples showed antimicrobial activity after 20 days of storage whereas analagous whole muscle samples were completely inactive even after storage of 50 days.

In reviewing the data obtained thus far, such factors as freezing, grinding, length of storage and type of hake tissue appeared to be directly involved in the formation of the microbial inhibitor. Since all of the viscera samples were found to be active after 20 days of storage, it was necessary to separate this sample further to determine the most active component of the hake carcass.

An additional 30 fresh hake obtained from Yaquina Bay were prepared and assayed in a manner similar to that described above

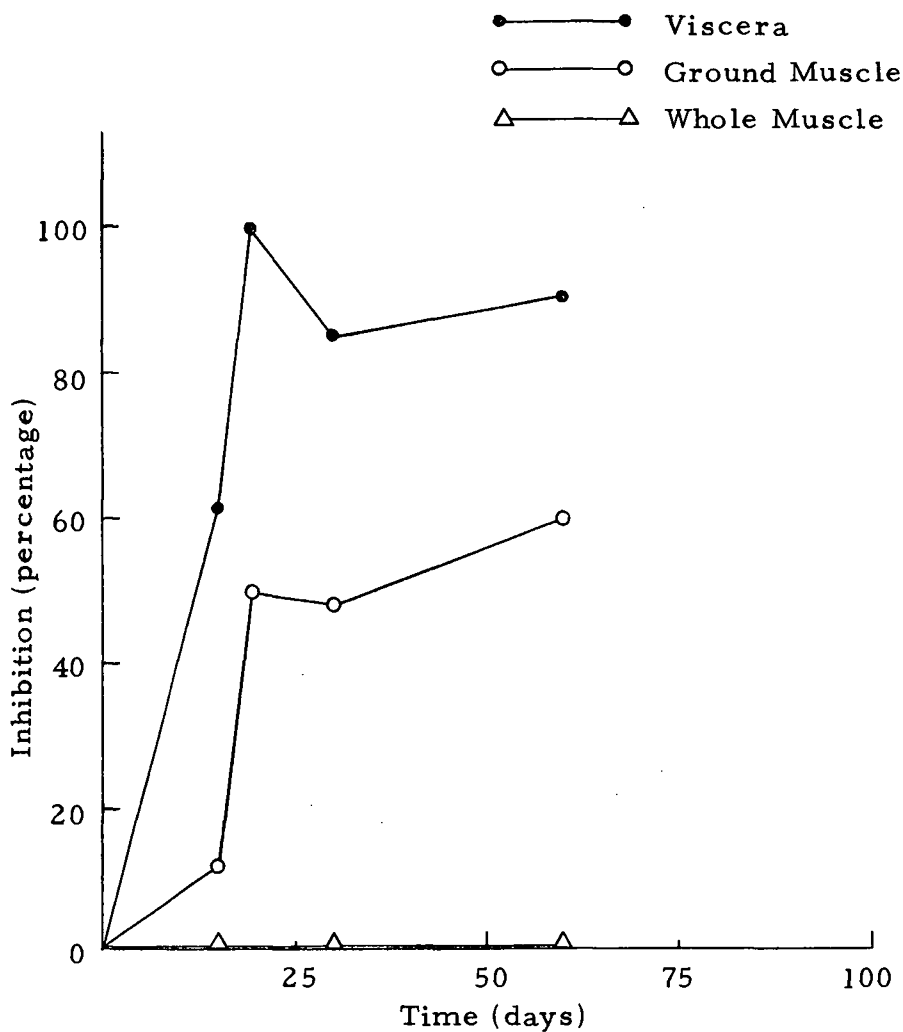

Figure 3. Percentage of total samples of various tissues from Pacific hake showing antimicrobial activity during storage at - 10° C. as determined by the modified agar plate assay.

with the following exception. The head and backbone portion was separated from the viscera sample prior to grinding. Thus, in this trial, antimicrobial activity was determined on the following samples: whole muscle, ground muscle, head and backbone, and viscera. Results of this trial were plotted similarly to those of the previous experiment and are graphically shown in Figure 4. All samples containing the head and backbone were active after 25 days of frozen storage. Since none of the viscera samples were active prior to a storage time of 50 days, much of the inhibition shown by this sample in the previous trial might well be attributed to the head and backbone portion of the sample. Data shown in Figure 4 also reiterate the influence of storage time upon the formation of the antimicrobial factor. In Figure 3, 50% of the ground muscle samples showed microbial inhibition after 20 days of storage as compared to less than 10% of similar samples stored for 100 to 180 days in Figure 4. Other than the possibility of inherent variation between the hake samples used, this degree of difference cannot be readily explained nor is there an obvious reason for the decrease in the number of active head and backbone samples after 25 days of storage.

Gel Filtration of Concentrated Dialysate

Gel filtration techniques were used to further fractionate and purify the microbial inhibitor contained in concentrated hake dialysate.

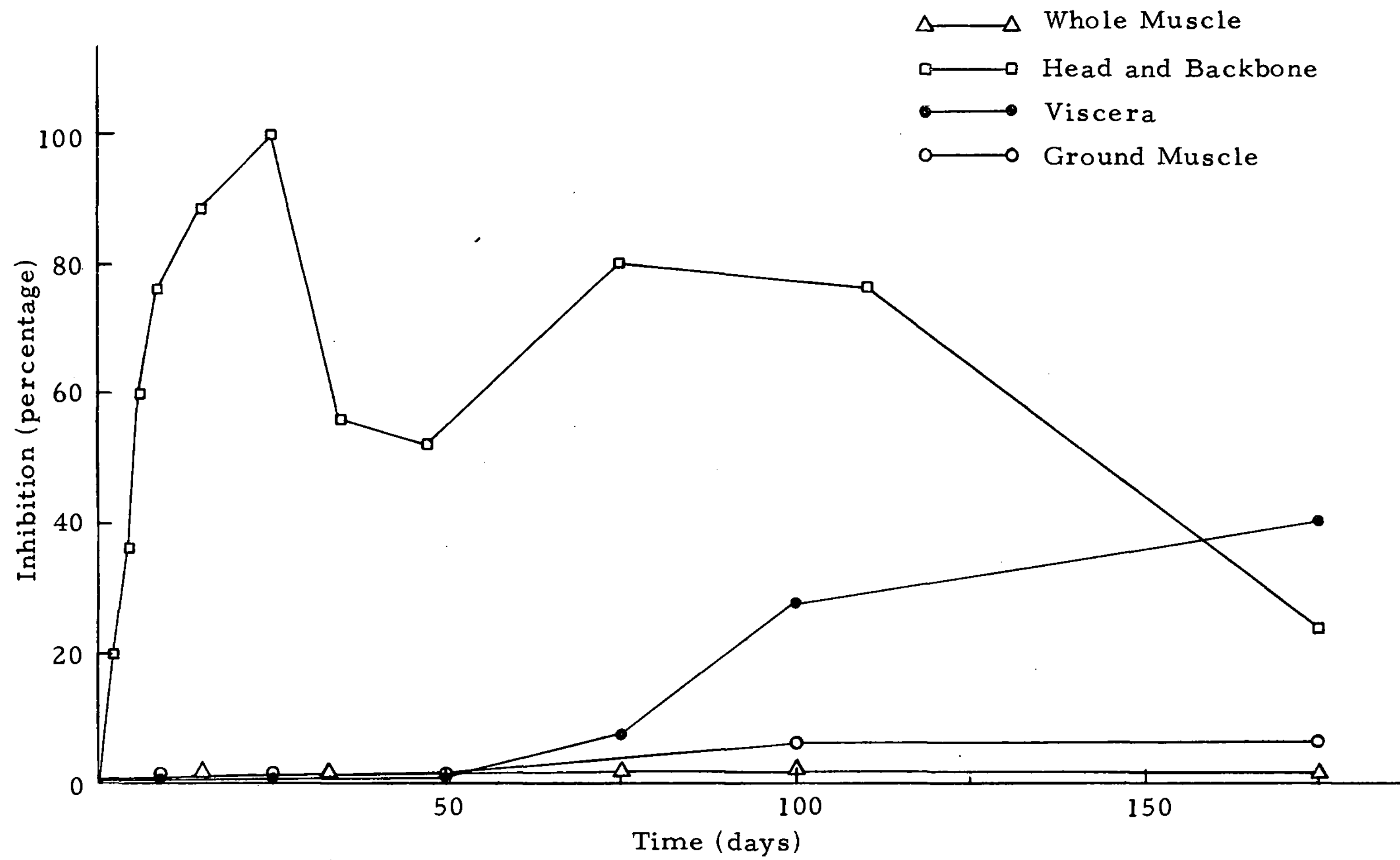

Figure 4. Percentage of total samples of various tissues from Pacific hake showing antimicrobial activity during storage at -10° C. as determined by the modified agar plate assay.

Columns were preapred as previously described using Sephadex G-10 gel having exclusion limits near a molecular weight of 700 (Pharmacia, 1966). This gel was chosen because in previous experiments, Stout (1967) estimated the molecular weight of the microbial inhibitor to be less than 700 and preliminary results obtained in this laboratory indicated that Sephadex gels (G-15 and G-25) having higher exclusion limits were not effective in separating the inhibitor from other dialysate components.

Prior to utilization of columns for separation of the concentrated dialysate, 10 ml of an aqueous solution of Blue Dextran 2000 (Pharmacia, 1966) were eluted through each column to determine the homogeneity of the column bed. Only columns having an elution pattern similar to that shown in Figure 5 were employed. From this pattern, the elution volume of a typical Sephadex G-10 column was calculated to be 260 ml.

Spectrophotometric scans from 700 to 230 mμ were made on concentrated dialysates with a Beckman DB recording spectrophotometer. As shown in Figure 6, a single peak of maximum absorbance was obtained at a wavelength of 250 mμ. Eluants of columns were monitored at 254 mμ due to limitations of the absorbtion meter used.

A typical elution pattern of 5 ml of concentrated dialysate on Sephadex G-10 columns is presented in Figure 7. Elution volume is shown in 10 ml fractions on the abscissa and absorbance of the eluant

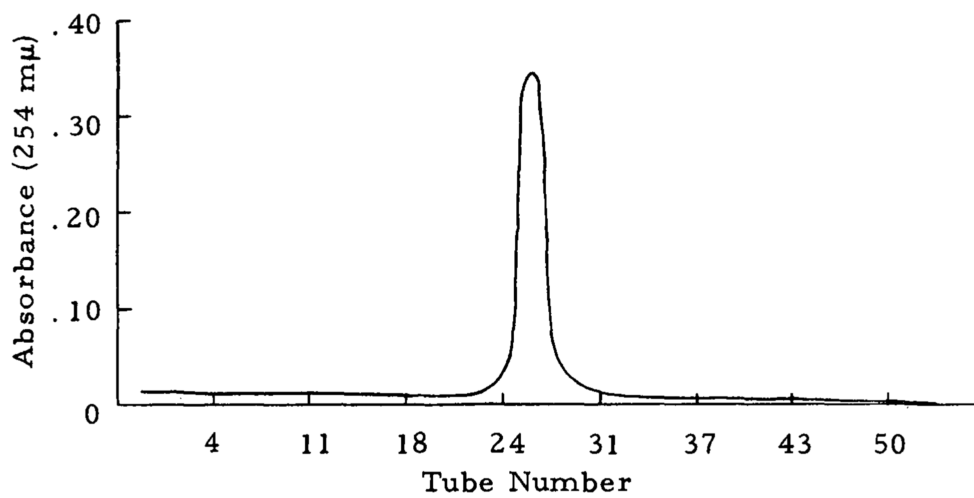

Figure 5. Elution pattern of Blue Dextran 2000 on a Sephadex G-10 column.

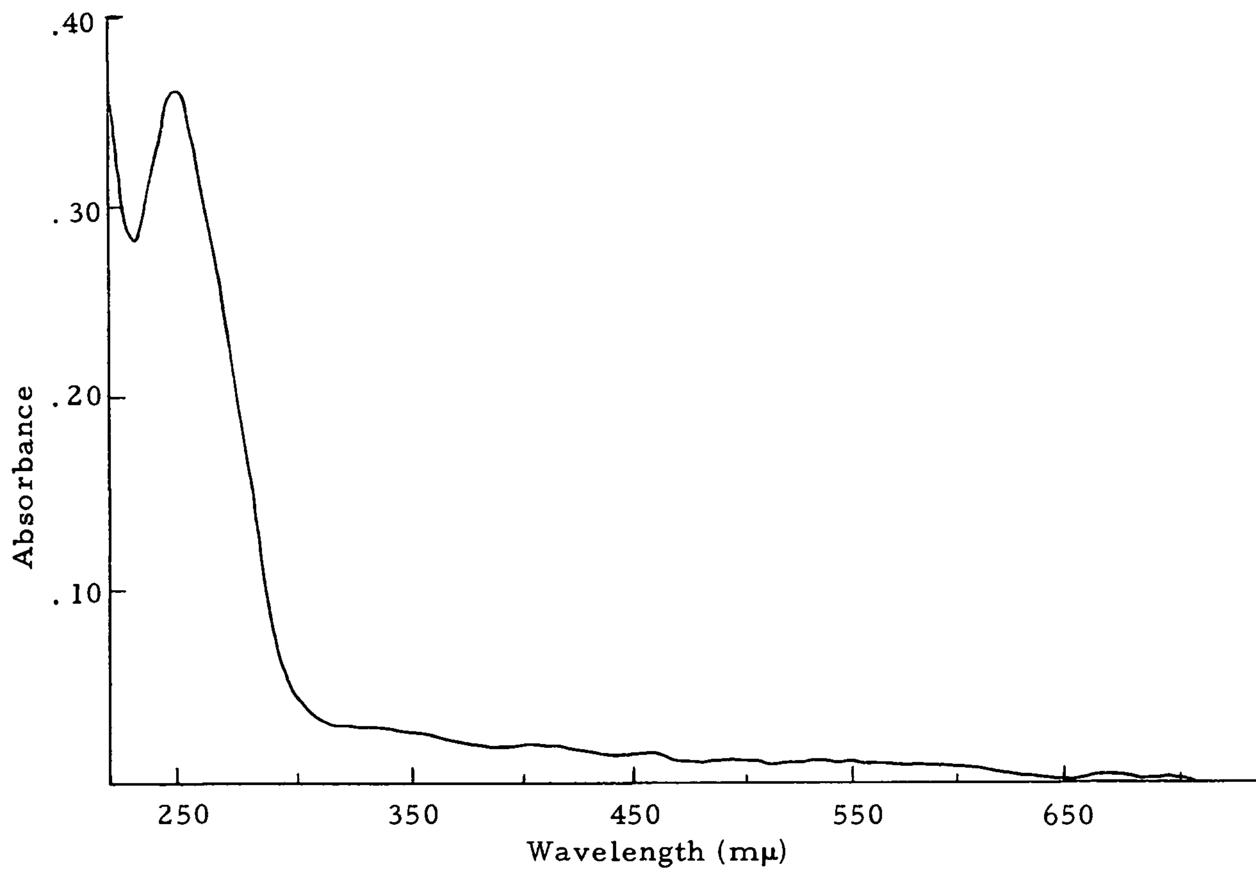

Figure 6. Absorbance spectrum of the concentrated dialysate.

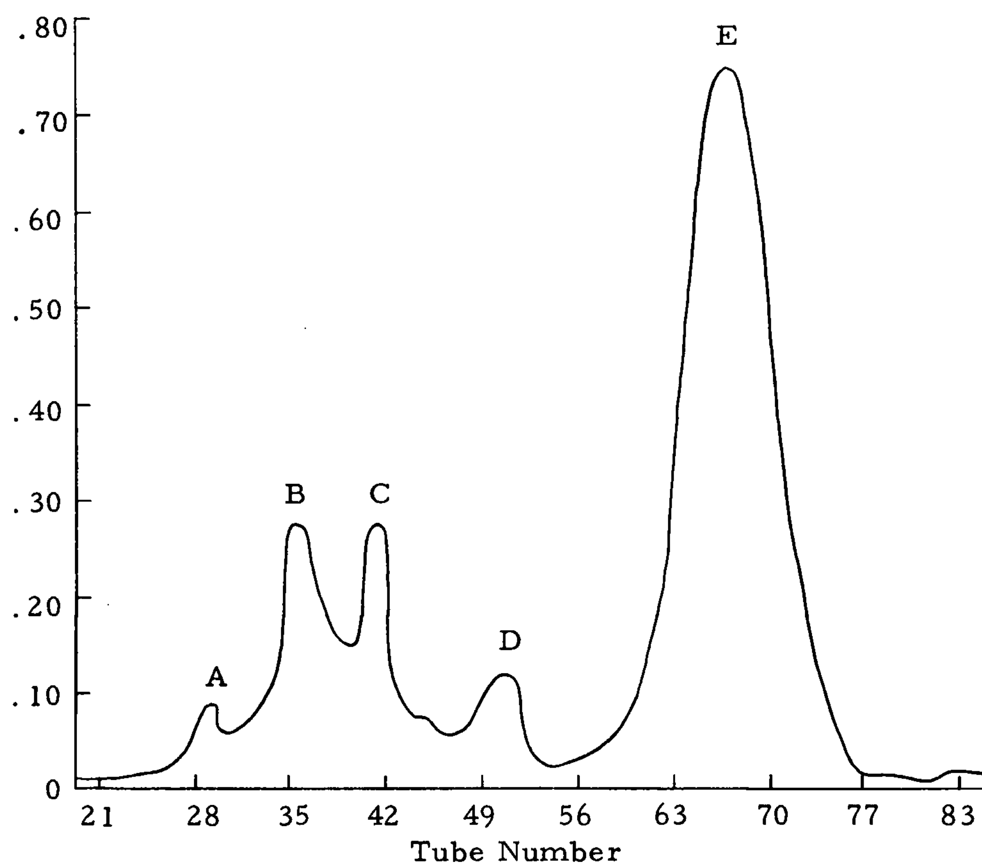

Figure 7. Elution pattern of concentrated dialysate separated on a Sephadex G-10 column.

read at 254 mμ on the ordinate. The dialysate was separated into five major fractions which were arbitrarily labelled A through E, for convenience, in their order of elution from the column.

Eluant from each of the 10 ml fractions was screened for antimicrobial activity by the modified agar plate procedure. Tubes 49 and 50 from peak D each showed inhibition zones of 3-4 mm as compared to a 10 mm zone for the concentrated dialysate. Five replications of gel filtration were carried out in which 5 ml of concentrated dialysate were separated. In each of these separations, only the eluant from peak D showed microbial inhbition when tested by the modified agar plate procedure. Eluants of peaks B and C had a light yellow color similar to that observed in the concentrated dialysate while those of peaks A, D and E were essentially colorless. In subsequent work, eluant from peak D was collected from gel filtration of the active concentrated dialysate whenever needed.

Paper Chromatography

Results of the paper chromatographic separation of the concentrated dialysate and of aliquots from tubes 29, 36, 41 and 50, which showed the highest absorbance for peaks A, B, C and D, respectively, plus tubes 57, 66 and 74 from peak E, are shown in Figure 8. This chromatogram reveals that the concentrated dialysate contained nine different ninhydrin-sensitive spots. So far as gel filtration

Tube	29	36	41	57	66	74	50	Concentrated Dialysate
Peak	A	B	C	E	E	E	D	

Figure 8. Chromatogram of the concentrated dialysate and aliquots of eluant from the Sephadex G-10 column.

fractions are concerned, ninhydrin positive components were located in the eluants of peaks B and C. Results of the chromatographic separation of eluants of the remaining peaks (A, D and E) failed to show the presence of ninhydrin-sensitive materials. From these results, plus the fact that the antimicrobial factor was found only in peak D of gel filtration patterns, it was concluded that the microbial inhibitor did not contain free amino groups.

Since heat destroyed or inactivated the microbial inhibitor and caused the formation of a dark amber color, the effect of heating the concentrated dialysate at 85° C for 60 minutes was also determined by paper chromatography. Chromatograms are not presented since patterns of separation of the ninhydrin-sensitive spots were identical to that shown in Figure 8 for the non-heated concentrated dialysate although the intensity of some of the spots of the heated samples seemed to increase. Since data in Figure 8 indicate that all components of the concentrated dialysate were present in the eluant of gel filtration peaks B and C, components responsible for the yellow color of the dialysate and the dark amber color formed upon heating were assumed to be the influence of the same compounds although present in larger amounts after heating. When 5 ml of heat-inactivated dialysate were eluted through gel filtration columns, the dark amber color was always found in the eluants of peaks B and C. Studies of the nature of formation of amber discoloration in conjunction with those on the heat

inactivation of the microbial inhibitor were not continued although such studies should be investigated from the standpoint of the Maillard or browning reaction which commonly occurs in many foods during heating (Hodge, 1953).

Qualitative Methods of Identification

On the basis of the results thus far obtained, the absence of ninhydrin-sensitive material in the gel filtration fraction showing antimicrobial activity was a finding of considerable importance. The lack of free-amino groups in this fraction rules out the possibility of the inhibitor being a protein- or peptide-like compound. However, formation of a deep amber color upon heating of the concentrated dialysate was considered as strong evidence for the presence of carbohydrate-like materials. Thus, subsequent investigations were directed toward the identification of a carbohydrate component.

Catalase Test

Since several families of microorganisms (i. e. Lactobacillaceae) are capable of forming hydrogen peroxide in amounts sufficient to inhibit the growth of other bacteria in culture media (Dahiya and Speck, 1968), the catalase test was conducted on the concentrated dialysate to determine whether it contained hydrogen peroxide.

Catalase was added to samples of concentrated dialysate which

had produced a zone of inhibition 10 mm wide when tested for antimicrobial activity by the modified agar plate technique. After sufficient time had elapsed, this mixture was tested for microbial inhibition by the modified agar plate method and an inhibition zone of 10 mm was still evident. Also, the release of oxygen in the form of bubbles was not apparent in this mixture as it was in a control sample containing only hydrogen peroxide and catalase. These results indicated that hydrogen peroxide was not present in the concentrated dialysate nor involved with microbial inhibition.

Anthrone Test

The formation of a dark amber color during heating and lyophilization suggested the possible presence of reducing sugars or carbonyls which may have reacted with the amino groups as evident in the results of chromatographic separation of peaks B and C eluted by gel filtration. When 3 ml of concentrate dialysate were mixed with the anthrone reagent, a blue color developed indicative of the presence of carbohydrates such as saccharides, dextrans, dextrins, starches, gums and glucosides. Addition of anthrone to 3 ml of the eluant from peak D produced a dark amber color considerably different from that obtained with the concentrated dialysate.

Ninhydrin Test

Chromatographic results of fractions B and C eluted during gel filtration of the concentrated dialysate indicated the presence of ninhydrin-sensitive materials. When ninhydrin was added directly to the concentrated dialysate and to the eluants of fractions B and C, a purple color developed confirming the presence of ninhydrin-sensitive materials. A purple color did not develop when ninhydrin was added to eluants of gel filtration fractions A, D, and E, indicating that free amino groups were absent from these fractions.

Schiff Test

Amano and Yamada (1965) reported the formation of formaldehyde in cod tissues and also mentioned its presence in other species including the Alaskan pollack. Stout (1969) reported that when frozen Alaskan pollack were fed to mink, physiological symptoms developed similar to those noted previously when mink were fed frozen Pacific hake. Moreover, Stout isolated and identified formaldehyde in Pacific hake. In view of these developments, investigation of the aldehyde content of hake tissues was initiated.

Addition of Schiff's reagent to both the concentrated dialysate and the eluant of fraction D obtained by gel filtration produced a pink color indicative of the presence of aldehydes. These results indicated

that further tests were necessary to determine which aldehydes were present.

2, 4-Dinitrophenylhydrazine (DNP) Derivatives

Maximum absorbance values of 348 mμ for formaldehyde, and 356 mμ for acetaldehyde, differ enough from each other and from high m.w. aldehydes (> 358 mμ) that absorbance spectra can be used as criteria for aldehyde identification (Day, Bassette, and Keeney, 1960). The 2, 4-DNP derivatives of both the concentrated dialysate and eluant from fraction D showed maximum absorbance at 348 mμ which were identical to that for the 2, 4-DNP derivative of formaldehyde. The absorbance spectra for the above derivatives are presented in Figure 9. These findings strongly indicate that formaldehyde was responsible for microbial inhibition in both the concentrated dialysate and in eluant of fraction D.

Results of paper chromatographic separation of the 2, 4-DNP derivatives of (1) formaldehyde, (2) benzaldehyde, (3) furfural, (4) butanal, (5) concentrated dialysate, and (6) eluant of fraction D, are presented in Figure 10. These data show that derivatives of standard formaldehyde solution, concentrated dialysate and eluant from fraction D had identical R_f values of 0.32. These results support the data described above concerning the identification of formaldehyde by maximum absorbance spectra.

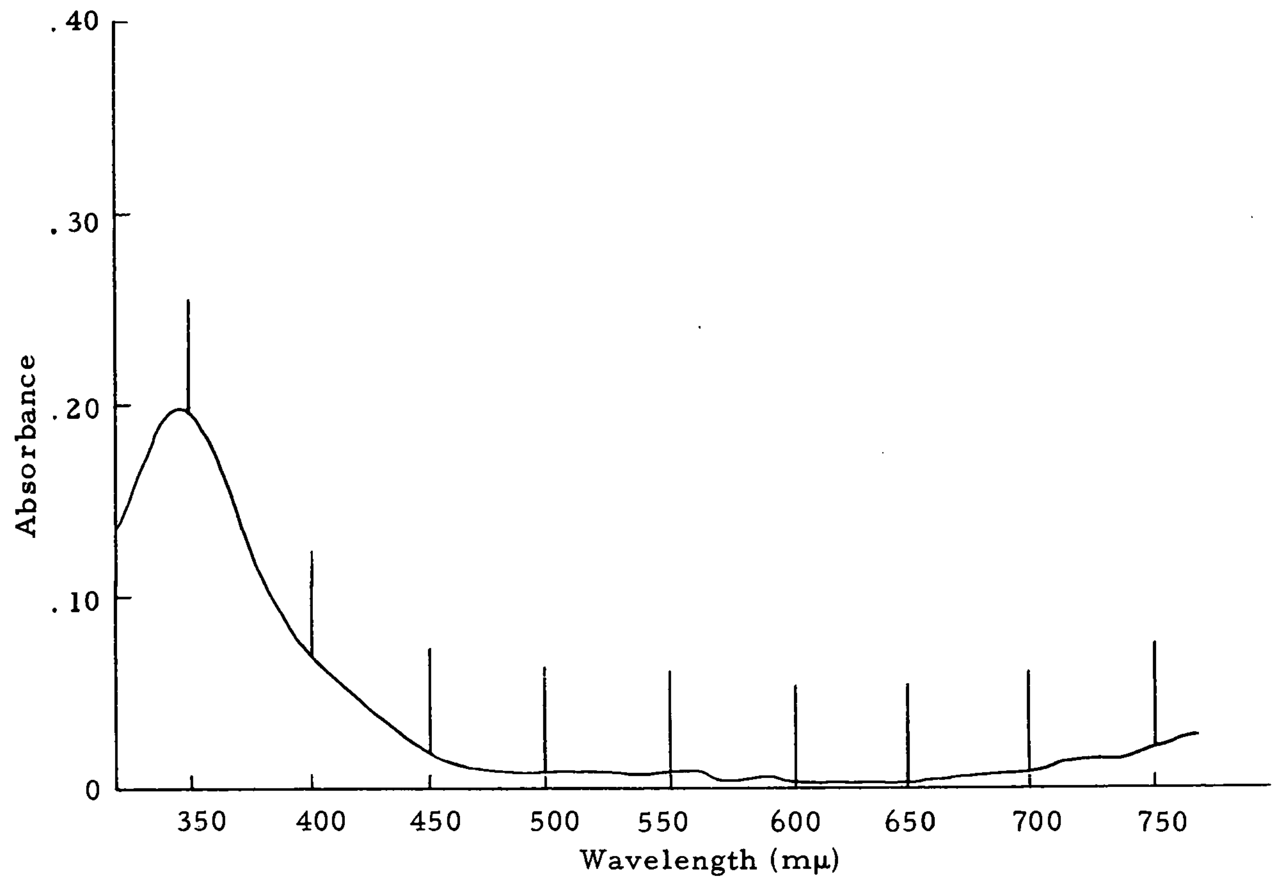

Figure 9. Absorbance spectra of the 2, 4-DNP derivatives prepared from the eluant of peak D, concentrated dialysate, and standard formaldehyde solutions.

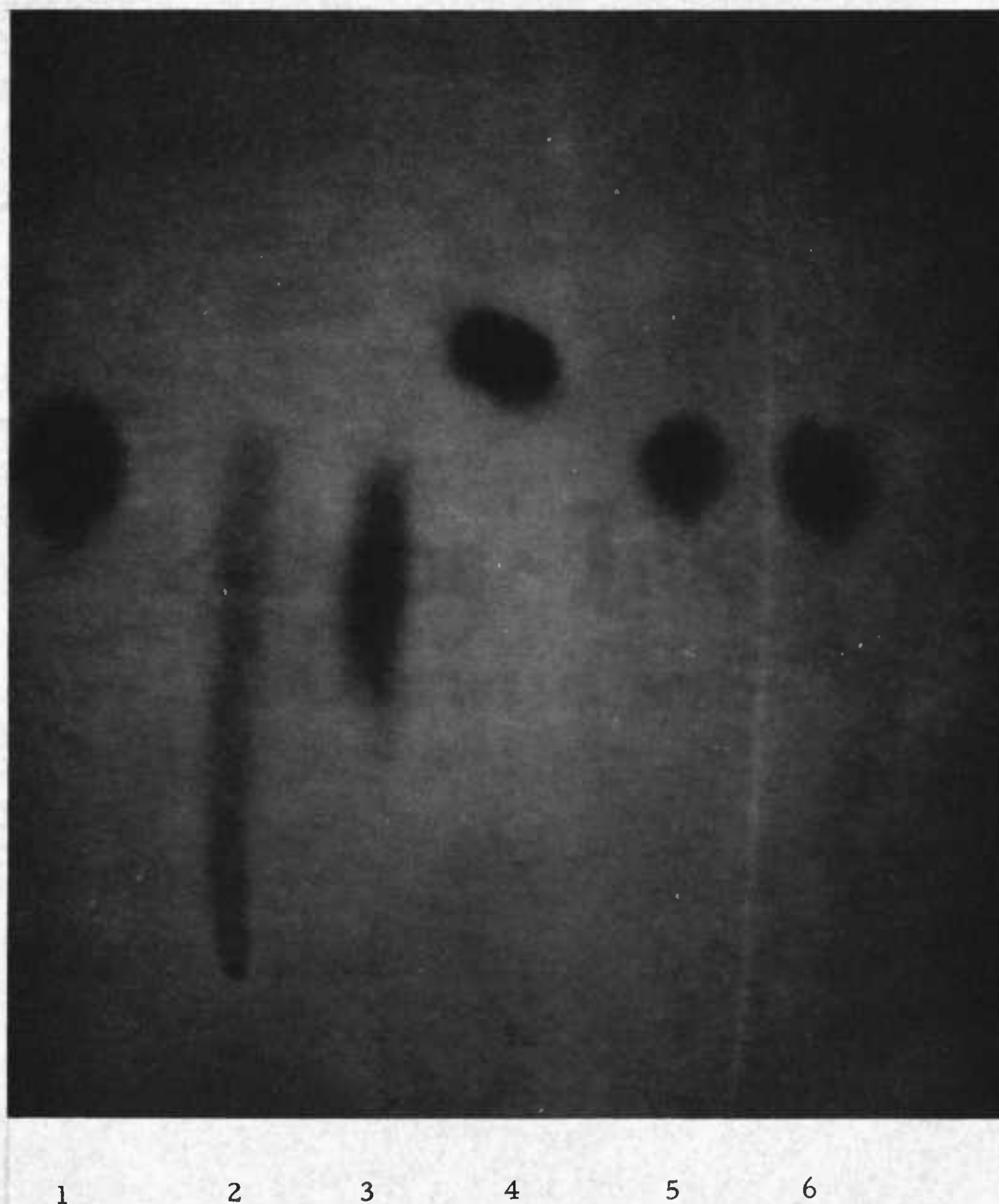

Figure 10. Chromatogram of 2, 4-DNP derivatives of (1) formaldehyde, (2) benzaldehyde, (3) furfural, (4) butanal, (5) eluant from peak D, and (6) concentrated dialysate.

Standard formaldehyde solutions did not abosrb at 250 mμ which suggested that the absorbance of the peak tube of fraction D was due to other compounds. Addition of 3 ml of formaldehyde to the concentrated dialysate prior to its separation by gel filtration resulted in no detectable differences in the elution pattern although the initial concentration of formaldehyde had been increased. In subsequent work, aliquots of a standard formaldehyde solution were passed through gel filtration columns. Each of the 10 ml fractions eluted was tested for formaldehyde by Schiff's reagent. Results of these trials indicated that the elution volume of the standard formaldehyde solution was identical to that for peak D.

Upon obtaining evidence for the positive identification of formaldehyde, a quantitative method for determining concentrations of this component in hake tissues was deemed necessary. Quantitative assays could then be used to evaluate the previous work with the modified agar plate assay method and to determine if concentrations of formaldehyde formed in hake tissues were sufficient to inhibit the growth of the test organism, _B. cereus_.

Quantitative Assays of Formaldehyde in Hake Tissue

The 2, 4-DNP method, as described previously, was used to good advantage by Day, Bassette and Keeney (1960) to estimate amounts of carbonyl compounds in cheddar cheese extracts. Amounts of

formaldehyde in standard solutions, as determined by this method, were in fair agreement with the actual contents of the standards as shown in Table 1. Although this method was applicable for determining the formaldehyde content of fish tissues, it required excessive sample manipulation which was considered to be too time consuming to be of practical value. In terms of simplicity, rapidity and sensitivity, the 3-methyl-2-benzothiazolone hydrazone procedure developed by Sawicki *et al*. (1961) for measuring aliphatic aldehydes appeared to be an alternative method. Subsequently, this procedure was investigated to determine whether it would adequately measure the formaldehyde contents of hake tissue.

Standard formaldehyde solutions were prepared from reagent grade formaldehyde (36-38% w/w) in a series of concentrations ranging from 1 to 50 ppm. Aliquots of these solutions were reacted with 3-methyl-2-benzothiazolone hydrazone and absorbance was measured at 670 mμ. In Figure 11, these results are presented graphically by plotting absorbance versus concentration. Since absorbance of formaldehyde concentrations, ranging from 0 to 20 ppm, fell on a straight line following Beer's law, this plot was used for hake samples which had been diluted to a concentration less than 20 ppm prior to analysis.

The 3-methyl-2-benzothiazolone hydrazone method as developed by Sawicki *et al*. (1961) included the addition of acetone after

Table 1. Comparison of results of standard formaldehyde solutions with those of pressed fluid extracts as determined by the 2, 4-DNP method.

Actual Formaldehyde Content (μg)	Absorbance at 348 mμ	Formaldehyde Content by Calculation (μg)[a]
Blank	0.04	2
5	0.08	6
10	0.14	11
15	0.20	16
20	0.30	23
25	0.36	28
Whole Muscle (1:5 dilution)	0.06	5[b]
Ground Muscle (1:5 dilution)	0.16	14[b]

[a] Average of duplicate determinations

[b] μg/ml extract

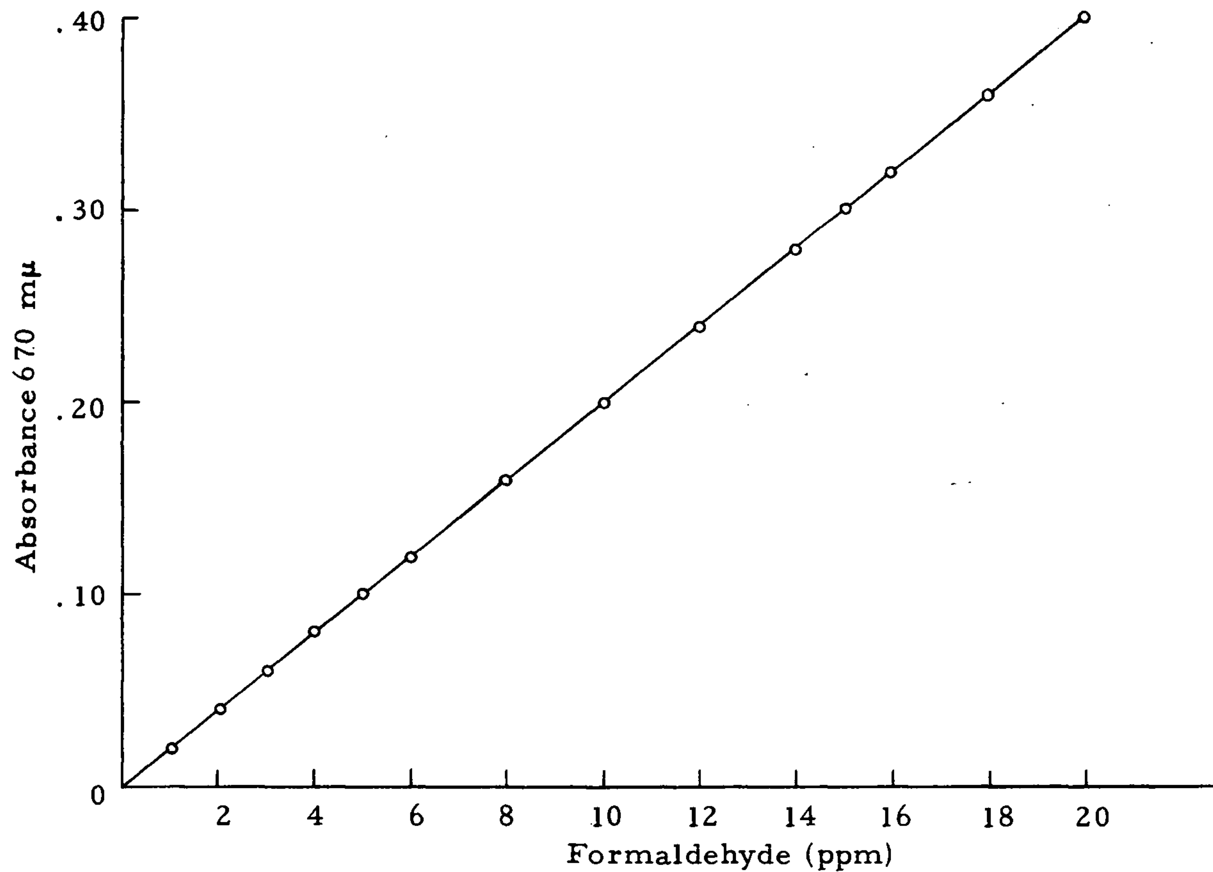

Figure 11. Standard curve for formaldehyde as determined by the 3-methyl-2-benzothiozolone hydrazone method.

development of the colored ion. Acetone apparently dissolved an impurity causing persistent turbidity as well as terminating the oxidation by ferric chloride. When acetone was added to extracts from hake homogenates, the solutions turned cloudy as a result of protein precipitation. In an attempt to eliminate this defect, the use of trichloroacetic acid (TCA) was studied. In preparation of the hake extracts, 20 ml of TCA were used in place of deionized water during homogenization. Although this change in procedure prevented the formation of a precipitate when acetone was added as the final diluent, TCA interferred with the measurement of absorbance. As shown in Table 2, the addition of 1 ml of TCA to the reagent blank, as would be present in the actual assay of hake extracts, increased the absorbance fivefold over that of the reagent blank without TCA. These results ruled out the use of TCA in this procedure. With further experimentation, it was found that distilled water could be substituted for acetone in the final dilution without the formation of turbid or cloudy solutions.

The effect of oxidation time upon the color development of the reaction of formaldehyde with 3-methyl-2-benzothiazolone hydrazone was studied. As indicated in Table 2, absorbance values were reproducible after 25 minutes and remained constant up to 65 minutes. A minimum oxidation time of 25 minutes was found to be necessary for maximum color development of the reaction of acetaldehyde with 3-methyl-2-benzothiazolone hydrazone (Lindsay and Day, 1965).

Table 2. Effect of various conditions and reagents on absorbance of formaldehyde as determined by the 3-methyl-2-benzothiazolone hydrazone method.

Trial No.	Sample	Variable	Absorbance at 670 mμ[a]
1	Reagent Blank	None	0.020
2	Reagent Blank + 1 ml 5% TCA	1 ml 5% TCA	0.096
3	10 μg Formaldehyde	15 min Oxidation Time	0.215
4	10 μg Formaldehyde	25 min Oxidation Time	0.220
5	10 μg Formaldehyde	45 min Oxidation Time	0.220
6	10 μg Formaldehyde	65 min Oxidation Time	0.223

[a]Average of duplicate samples of three replications.

Subsequent analyses were timed so that length of oxidation was maintained within a range of 25 to 65 minutes.

Sensitivity limitations of the agar plate microbial assay were not known at the time it was being used in preliminary studies to detect the presence of the antimicrobial factor and/or formaldehyde in various hake tissues. With the adaptation of a colorimetric method suitable for determining the formaldehyde contents in hake tissues, it became possible to evaluate the modified agar plate assay procedure. Such an evaluation would allow for a more meaningful interpretation of data obtained in earlier work. Aliquots (0.5 ml) of various hake tissue extracts and of standard formaldehyde solutions were placed in the wells of agar plates and incubated at 30° C for eight hours. Results of this study are tabulated in Table 3. Comparisons of the actual formaldehyde contents with widths of zones of inhibition indicate that levels of formaldehyde less than 25 μg did not prevent the growth of _B. cereus_ 569 R. Upper limits of the agar plate assay appear to be about 200 μg of formaldehyde since higher levels did not increase the zone of inhibition beyond that observed for 200 μg. Although limited diffusion of samples having higher levels of formaldehyde was apparent, volatilization of formaldehyde during incubation at 30° C may also contribute to the lack of increase of inhibitory zones. The test organism was not inhibited by 0.5 ml aliquots of 15% methanol although this concentration was present in the reagent formaldehyde used

Table 3. Comparsion of known formaldehyde levels with inhibition zones of the modified agar assay.

Actual Formaldehyde[a] Content (μg)	Width of Inhibition[b] Zone (mm)	
15	0	
20	0	
25	3	
50	5	
100	8	
200	10	
250	10	
300	10	
350	10	
75	5	Backbone Extract
55	5	Viscera Extract
30	3	Muscle Extract

[a]Determined by 3-methyl-2-benzothiazolone hydrazone method.

[b]Duplicate determinations of two replications.

to prepare the standard solutions. The modified agar plate assay was not as sensitive nor quantitative as originally hoped. However, it was a useful tool for screening various samples for antimicrobial activity in the early stages of this study when little else was known.

Formaldehyde Contents of Hake Tissues

Hake used in much of the earlier work had been stored for varying lengths of time under dubious conditions. Since these factors may have had considerable influence upon the rate of formaldehyde formation and on the ultimate levels obtained in hake, a more systematic study was carried out to determine the effects of storage time, type of tissue, freezing, and grinding on the amount of formaldehyde formed.

Forty-five fresh hake, designated as group 1, were randomly divided into three equal subgroups labelled A, B, and C. Fish of each subgroup were grossly dissected according to the following eight categories: head, gill, brain, ground muscle, whole muscle, backbone, viscera and liver. Components of each of the 15 fish per subgroup were pooled, ground and mixed to form one composite sample per category with the exception of whole muscle which was left intact. Samples from subgroups A, B and C were stored at -10° C and analyzed for formaldehyde content by the 3-methyl-2-benzothiazolone hydrazone method intermittently during 20 days of storage. Initial

formaldehyde determinations were performed prior to freezing, approximately 24 hours after the fish had been caught. These results and those subsequently completed during the 20-day storage period are listed in Table 4. Initial formaldehyde contents of all samples ranged from 20 to 85 ppm with the average being 42 ppm. The most obvious increase in formaldehyde occurred in the backbone. After five days of storage, the average formaldehyde content of these sampleas more than tripled, increasing from 53 to 184 ppm. Compared to the average content of the eight composite samples, the backbone sample showed the highest increases in formaldehyde (Figure 12). Ground muscle showed a similar magnitude of increase although both initial and five-day contents were considerably lower than those of the backbone. At the end of 20 days storage at -10° C, backbone samples showed a four-fold increase (from 53 to 210 ppm) whereas ground muscle had a final formaldehyde content about 2.5 times that found initially. With the exception of appreciable increases in formadehyde of head and brain samples, remaining tissues showed little, if any, build-up during 20 days of frozen storage.

Data presented above indicate that the backbone had greater ability and/or materials to produce formaldehyde than did the other sample categories. Conversely, backbone samples not only contained the spinal column and accompanying nervous tissue but also undoubtedly included some portions of muscle and air bladder. In considering

Table 4. Formaldehyde content of various tissues of subgroups A, B, and C of group 1 as determined by the 3-methyl-2-benzothiozolone hydrazone method.

Sample	Time (days)							
	0	1	5	7	8	13	17	20
	Formaldehyde (ppm)[a]							
Subgroup A								
Gills	55	30	40	25	25	25	50	25
Ground Muscle	25	30	50	65	60	65	60	60
Liver	85	35	90	65	60	40	65	50
Backbone	40	40	125	165	150	175	190	165
Head	20	15	25	15	20	25	50	40
Brain	25	30	40	25	25	40	50	50
Viscera	80	30	75	75	90	65	75	75
Whole Muscle	25	25	20	15	--	15	--	20
Mean	44	29	58	56	61	56	77	61
Subgroup B								
Gills	35	25	50	25	25	25	40	35
Ground Muscle	25	50	90	65	60	65	100	60
Liver	35	50	75	50	60	25	90	50
Backbone	65	135	240	200	175	140	315	265
Head	30	25	50	25	20	40	50	50
Brain	65	90	100	100	100	75	100	75
Viscera	50	45	95	65	75	75	90	60
Whole Muscle	25	20	20	20	--	20	--	25
Mean	41	55	90	69	74	58	112	77
Subgroup C								
Gills	55	35	50	25	40	25	50	40
Ground Muscle	25	40	50	50	40	65	80	40
Liver	50	40	75	65	50	90	90	90
Backbone	55	130	185	165	150	190	215	200
Head	30	25	65	25	50	40	40	40
Brain	25	25	100	90	75	75	100	100
Viscera	75	65	100	75	50	75	125	115
Whole Muscle	25	--	25	20	25	20	--	20
Mean	43	51	80	64	60	72	100	81
Overall Mean	42	45	76	63	65	62	96	73

[a]Average of duplicate determinations.

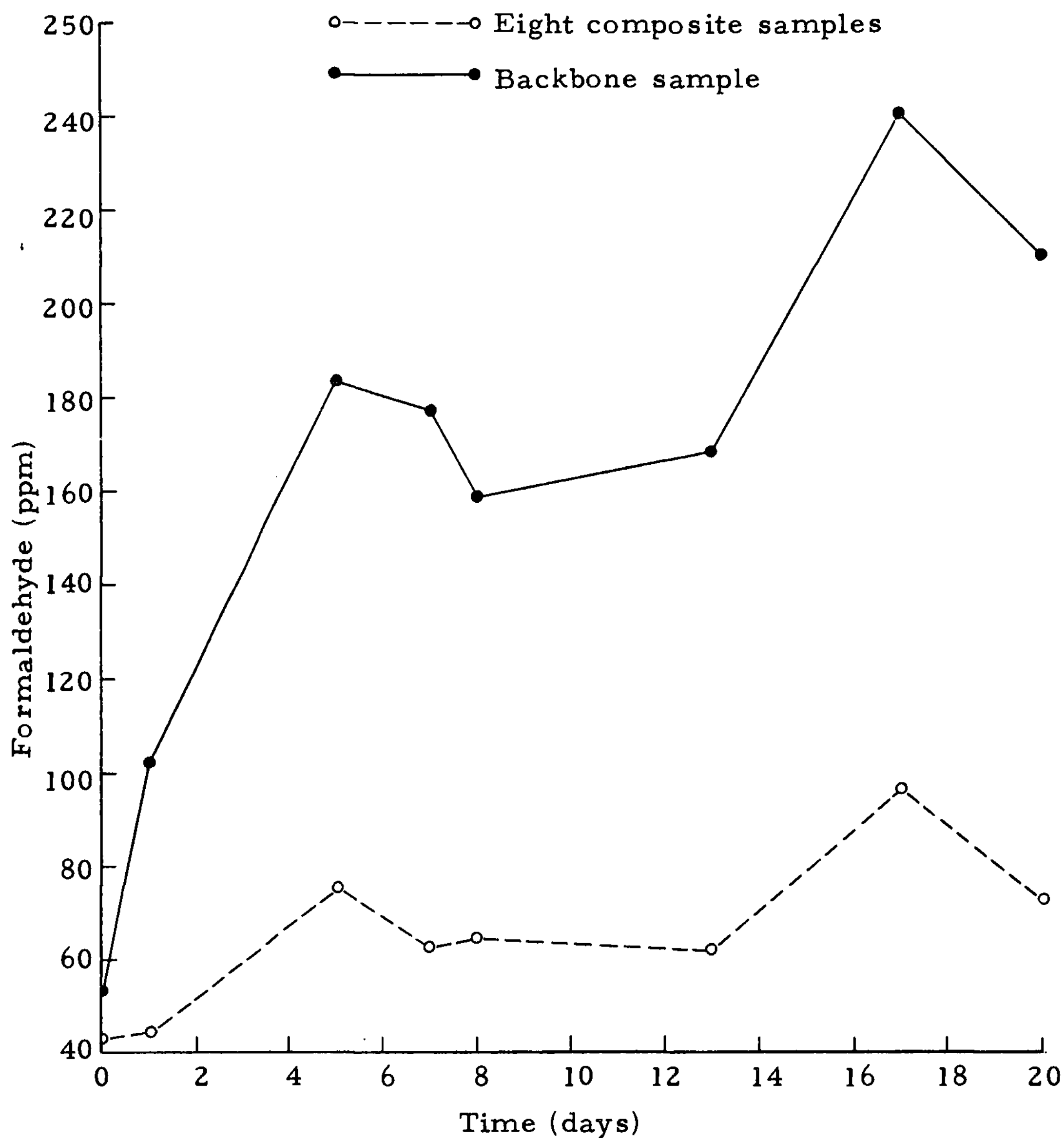

Figure 12. Average formaldehyde content of the eight composite and backbone samples from group 1 during storage at -10° C as determined by the 3-methyl-2-benzothiozolone hydrazone method.

these aspects, it became apparent that mixtures of tissues might yield higher concentrations of formaldehyde than any particular tissue studied individually. Experiments to test this assumption are presented later.

Samples of subgroup C were removed from frozen storage after seven days. Appropriate amounts were taken from each category for formaldehyde analysis. Remaining portions were thawed at room temperature for four hours and then held at 4° C for 24 hours after which, formaldehyde determinations were completed for the eighth day storage interval. Remainder of samples were frozen and thus held for sampling after 13, 17 and 20 days of storage. These time-temperature conditions simulated those occurring in the pressing and dialysis steps during the initial preparation of the concentrated dialysate in earlier studies. In comparing the formaldehyde contents of the various tissue categories with those of subgroups A and B (Table 4), it would appear that the time-temperature changes specified above had no influence on formaldehyde production.

Effect of grinding of tissues on formaldehyde formation was evaluated by comparing ground muscle data with that of whole muscle during the 20 day storage period. These were selected for this comparison since they were the most homogeneous of the eight sample categories and because of the interest in formaldehyde levels in the edible flesh. Formaldehyde concentrations of muscle samples were

averaged from data of subgroups A, B and C (Table 4) and are presented in Table 5. Grinding had no effect on the initial formaldehyde contents since both ground and whole muscle samples had identical levels of 25 ppm. After one day of storage at -10° C, however, ground muscle contained 2.5 times as much formaldehyde as whole muscle. For the remainder of 20 days storage, formaldehyde contents of ground muscle were about three times that of whole muscle. These findings indicate that components necessary for formaldehyde production were released during grinding. Since formaldehyde concentrations of whole muscle remained constant during frozen storage, separating or filleting hake muscle from the carcass prior to frozen storage would minimize the accumulation of this compound in the flesh used for food.

Table 5. Average formaldehyde content of muscle samples of subgroups A, B and C as determined by the 3-methyl-2-benzothiazolone hydrazone method.

Sample Description	Time (days)							
	0	1	5	7	8	13	17	20
	Formaldehyde (ppm)[a]							
Ground Muscle	25	55	75	65	65	65	85	60
Whole Muscle	25	20	20	20	--	20	--	25

[a]Average of duplicate determinations.

Fifteen fresh hake comprising group 2 were covered with an ice glaze and frozen intact at -10° C. After one year of frozen storage they were thawed, grossly dissected and appropriate parts pooled in the following six categories: gill, ground muscle, backbone, head, brain and viscera. These samples were ground and mixed prior to initial formaldehyde analysis after which they were refrozen, stored at -10° C and analyzed at intervals similar to those described for group 1. Data concerning formaldehyde contents of this experiment are given in Table 6. Initial average formaldehyde content for all samples of this group was 168 ppm, almost four times higher than an initial average of 42 ppm for group 1 samples. Formaldehyde concentrations listed in Table 6 show that considerable amounts of this compound had formed in all sample categories during a storage period of one year. Although the viscera had a relatively high level of formaldehyde (265 ppm) at the initial sampling, its contents decreased after grinding, refreezing and subsequent frozen storage. In contrast, backbone had an initial formaldehyde content of 175 ppm which increased two-fold during the 20 day storage period. These findings indicate that the viscera contained necessary components and/or was subject to conditions favorable for the accumulation of high levels of formaldehyde in the intact fish during one year of -10° C storage. Pattern of formaldehyde build-up in the backbone in this experiment followed that observed for samples of group 1. Apparently, conditions

Table 6. Formaldehyde content of various tissues of group 2 during storage at -10° C as determined by the 3-methyl-2-benzothiazolone hydrazone method.

Sample	Time (days)						
	0	5	7	8	13	17	20
	Formaldehyde (ppm)[a]						
Group 2							
Gills	175	150	100	125	100	150	100
Ground Muscle	125	125	100	75	15	130	110
Backbone	175	425	350	300	250	415	340
Head	100	125	50	60	75	160	75
Brain	165	190	190	125	40	215	165
Viscera	265	240	215	175	150	240	175
Mean	168	210	168	143	105	218	161

[a]Average of duplicate determinations.

of grinding and refreezing released components from the backbone necessary for production of large amounts of formaldehyde during subsequent frozen storage.

Since the backbone was generally higher in formaldehyde than other samples and since some of the initial press fluid extracts obtained from a mixture of tissues showed high antimicrobial activity, effect of mixing various dissected carcass portions on formation of formaldehyde was investigated. Muscle tissue, the largest component of the carcass, was mixed with each of the other six tissue categories. Concurrent with the preparation of individual samples of groups 1 and 2, 40 gm of ground muscle were mixed with 40 gm each of gill, viscera, head, backbone, brain and liver, stored at -10° C. These

mixtures were analyzed for formaldehyde contents by the 3-methyl-2-benzothiazolone hydrazone method during the 20day storage period (Table 7). Mixing of ground muscle with other carcass components accelerated the formation of formaldehyde. Individual tissue samples of subgroups A, B and C of group 1, previously shown in Table 4, had an initial average formaldehyde content of 42 ppm as compared to an initial mean value of 116 ppm for the mixed samples in Table 7. After 20 days of frozen storage, mixed samples averaged 159 ppm as contrasted to a mean content of 73 ppm for the individual tissues.

Data in Table 8 were obtained from hake which had been frozen and stored intact for one year at -10° C, then thawed and dissected into the six categories listed. After grinding, 40 gm of each tissue category were mixed with an equal amount of muscle, refrozen and stored for an additional 20 days. With the exception of the muscle-head mixture, remaining samples showed increases in formaldehyde with storage time. Muscle-viscera mixture had the highest level of formaldehyde throughout the test period, starting at 200 ppm and reaching a level of 325 ppm after 20 days of storage.

In view of the results thus far obtained concerning formation of formaldehyde in various hake tissues, certain data are somewhat confusing. With fresh hake, backbone samples always developed higher formaldehyde levels over a 20-day storage period when various tissue categories were tested individually. When fresh ground muscle was

Table 7. Formaldehyde content of mixed tissue samples from subgroup A, B, and C of group 1 during storage at -10° C as determined by the 3-methyl-2-benzothiazolone hydrazone method.

Sample	Time (days)						
	1	2	4	10	14	17	20
	Formaldehyde (ppm)[a]						
Subgroup A							
Muscle + Gills	100	75	100	90	120	100	150
Muscle + Viscera	140	140	125	90	125	125	150
Muscle + Head	50	75	40	25	50	25	65
Muscle + Brain	100	90	150	165	190	240	190
Muscle + Liver	150	140	140	90	170	125	175
Muscle + Backbone	75	75	100	90	160	175	175
Mean	103	99	109	92	136	132	151
Subgroup B							
Muscle + Gills	115	175	165	140	175	175	175
Muscle + Viscera	165	210	165	115	175	150	140
Muscle + Head	65	25	40	40	75	50	75
Muscle + Brain	75	225	190	240	300	250	250
Muscle + Liver	175	225	175	100	225	190	225
Muscle + Backbone	165	75	50	75	250	225	215
Mean	127	156	131	112	202	173	180
Subgroup C							
Muscle + Gills	140	140	165	125	185	140	140
Muscle + Viscera	190	140	165	125	125	165	165
Muscle + Head	40	25	25	25	50	40	60
Muscle + Brain	50	75	75	75	125	140	165
Muscle + Liver	200	150	200	150	200	225	190
Muscle + Backbone	90	100	140	190	160	150	150
Mean	118	101	101	115	141	143	145
Overall Mean	116	119	114	106	160	150	159

[a] Average of duplicate determinations.

Table 8. Formaldehyde content of mixed tissue samples from group 2 during storage at -10° C as determined by the 3-methyl-2-benzothiazolone hydrazone method.

Sample	Time (days)						
	1	2	4	10	14	17	20
	Formaldehyde (ppm)[a]						
Group 2							
Muscle + Gills	150	125	150	125	190	190	175
Muscle + Viscera	200	290	325	325	440	325	325
Muscle + Head	110	75	75	50	100	90	65
Muscle + Brain	150	125	140	125	190	200	165
Muscle + Backbone	150	135	165	150	240	200	190
Mean	190	150	171	155	232	201	184

[a]Average of duplicate determinations.

mixed with equal amounts of other fresh tissue components, formaldehyde contents of backbone-muscle mixture were equalled or exceeded by muscle-brain and muscle-liver mixtures. In using hake frozen for one year, muscle-viscera mixtures developed considerably higher levels of formaldehyde than any of the others studied.

Omitting ground muscle, the effect of mixing and storing portions of various tissues together on production of formaldehyde was also studied. Twenty grams each of gill, brain and backbone portions of subgroup C were mixed with 20 gm each of viscera and liver. In addition, a composite sample containing 20 gm each of brain, liver, gill and backbone was prepared. After the initial formaldehyde contents were determined, samples were stored at -10° C and analyzed at

seven-day intervals during a 21-day storage period. Results of this study are presented in Table 9. Formaldehyde levels exceeding 100 ppm were evident only in samples containing equal amounts of backbone. The mixing together of non-edible components had little influence on formaldehyde contents as was apparent when the same samples were mixed and stored with ground muscle (Table 7). These data also show that muscle tissue is conducive to the development of high levels of formaldehyde in hake.

Table 9. Formaldehyde content of the mixed tissues of subgroup B in the absence of ground muscle during storage at -10° C.

Sample	Time (days)			
	0	7	14	21
	Formaldehyde (ppm)[a]			
Subgroup B				
Gill + Liver	50	55	50	60
Gill + Viscera	65	50	50	50
Brain + Liver	65	90	75	90
Brain + Viscera	65	65	50	50
Backbone + Liver	100	75	100	100
Backbone + Viscera	165	125	100	125
Brain + Liver + Viscera + Gills + Backbone	90	100	90	100
Mean	86	80	74	79

[a]Average of duplicate determinations.

Further investigations concerning the mechanism and conditions involved in formation of formaldehyde in Pacific hake need to be carried out. Amano, Yamada and Bito (1963) found high formaldehyde contents (5.7-7.5 mg/100 gm) in the pyloric caeca of cod and Alaskan pollack. Under *in vitro* conditions, ground pyloric caeca of cod was reported to be capable of converting trimethylamine oxide to formaldehyde and dimethylamine at 20° C (Amano and Yamada, 1964). These workers also indicated that an unidentified enzyme was responsible for this reaction. More recently, they have reported that an unidentified heat-tolerant cofactor is present in tissue of cod-like fish which enhances the enzymatic conversion of trimethylamine oxide to formaldehyde and dimethylamine (Yamada, Harada and Amano, 1969).

Identification of formaldehyde in Pacific hake has some important practical considerations. Hake is currently considered a trash fish mainly because of poor texture attributes, especially when frozen. Textural changes such as "gapping" and sponge-like structure which develop in hake during frozen storage may be the result of protein-formaldehyde reactions since free amino groups react readily with formaldehyde (Conn and Stumpf, 1963). Controlling such reactions may improve the physical properties. Carbonylamine type of browning reactions between aldehydes and amino groups occur in many foods and often result in adverse flavor formation (Hodge, 1967). If similar reactions occur in hake, particularly during heating, then a

variety of adverse flavors might result in view of the formaldehyde contents found in fish of this study.

SUMMARY AND CONCLUSIONS

Studies concerning the isolation and identification of an antimicrobial factor in both fresh and frozen Pacific hake (Merluccius productus) were completed. Confirmation of formaldehyde as the microbial inhibitor was obtained through the use of gel filtration, maximum absorbance spectra, and paper chromatography.

In preliminary work, frozen hake were thawed, ground and the resulting mixtures were subjected to 900 p. s. i. for ten minutes to obtain the press fluid containing the microbial inhibitor. After dialysis against distilled water, the modified agar plate assay procedure utilizing B. cereus 569 R as the test organism was employed to monitor dialysates for antimicrobial activity. Limits of microbial sensitivity to this assay procedure ranged from 25 to 200 ppm of formaldehyde. Lyophilization was used to concentrate those dialysates showing microbial inhibition. Considerable antimicrobial activity was lost when dialysates were lyophilized to a dry powder. Heating concentrated dialysates in open or closed systems to a temperature of 85° C for one hour resulted in complete destruction of the inhibitor accompanied by the formation of a dark amber pigment.

Gel filtration (Sephadex G-10) was used to fractionate concentrated dialysates. Five fractions were separated according to elution patterns obtained at 254 mμ. Antimicrobial activity was found only in

fraction D. Negative results were obtained when eluants of fraction D were reacted with ninhydrin. However, eluant of this fraction gave positive responses when tested for aldehydes by Schiff's reagent. Maximum absorbance value, 348 mμ, of the 2, 4-dinitrophenylhydrazine (DNP) derivatives of the eluant of fraction D was the same as that for the standard formaldehyde derivative. Paper chromatographic separation of 2, 4-DNP derivatives of eluant from peak D and of the original press fluid both gave an R_f value of 0.32 which was identical to that obtained for the standard formaldehyde derivative. On the basis of these results, it was concluded that formaldehyde was the compound exerting antimicrobial effects in Pacific hake.

The 3-methyl-2-benzothiazolone hydrazone method of Sawicki et al. (1961) was modified and adapted for the spectrophotometric determination of formaldehyde in hake tissue extracts. With the use of this method, formaldehyde contents of various hake tissues were found to range from 20 to 85 ppm at 30 hours post-mortem. Grinding, freezing and subsequent frozen storage for three weeks effectively increased formaldehyde levels of most tissues. Formaldehyde contents of backbone samples increased four fold during 20 days of frozen storage, starting at an initial average level of 53 ppm and ultimately reaching a mean value of 210 ppm. Grinding of hake muscle prior to frozen storage resulted in a three-fold increase in formaldehyde levels as compared to that for whole muscle. Additions of ground muscle to

other carcass components before freezing increased formaldehyde levels of such mixtures during storage of 20 days at -10° C. Relatively high formaldehyde contents (100-265 ppm) were found in hake stored intact at -10° C for one year.

Some of the possible effects of formaldehyde formation in hake tissues are discussed in terms of deleterious textural and flavor changes observed in these fish when stored in the frozen state.

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