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TECHNOLOGICAL STATION

Vancouver, B. C.

H. L. A. TARR, Director



FISHERIES RESEARCH BOARD OF CANADA

TECHNOLOGICAL STATION
VANCOUVER, B.C.

INTRODUCTION

At the time of preparation of this Report a completely new building is being erected on the campus of the University of British Columbia, which will be the new quarters of the Vancouver Technological Station. This building will be the first new and fully complete physical plant which this Station has occupied since it first commenced operations in Prince Rupert in 1926, and it is sorely needed as the present quarters are seriously overcrowded.

During the present year the policy of maintaining a balance between fundamental and applied research at the Station has been continued. While in past years the main contributions which this Station has made have been in the fields of control of post-mortem bacterial spoilage of fish, refrigeration and cold storage, and various processing and reduction problems, this year a very significant proportion of the Station's activities has been concerned with various problems connected with the chemistry of living fish. Thus the most thorough chemical study which has ever been made on migrating salmon is now in progress at this Station. There is little doubt that many of the most pressing problems in deterioration of fish after capture may be concerned with the condition of the fish when they are caught.

H.L.A. Tarr,
Director.

GENERAL SUMMARY OF INVESTIGATIONS

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Biochemistry of Sockeye Salmon During Spawning Migration

Three groups of migrating sockeye salmon (216 fish) have been obtained from the Stuart Lake run; the fish, representative of both sexes, were caught at the mouth of the Fraser River, at Lillooet, and at the spawning grounds (Forfar). They were captured and stored under conditions which minimized, as much as possible, undesirable chemical changes.

Physical measurements have been made on all major organs and tissues. The data show that the population was sufficiently uniform in size that average weights and measurements performed on fish taken at the 3 locations on the migration route quite accurately represented a standard fish of both sexes. The results will enable chemical analyses to be interpreted as changes in the absolute amounts of a given tissue component rather than only on a percentage basis. The merits of this approach to an evaluation of changes in migrating salmon is demonstrated by the magnitude of the weight changes in many tissues during the 715-mile migration. A standard male lost only 11% of its body flesh whereas a standard female lost 30%. The alimentary tract of males and females decreased to 18% and 16.5%, respectively, of the initial weight. The milt of the male increased by 51% while the female roe increased by 272%. Thus, it is apparent that percentage composition data can not show quantitative changes in a fish as it moves up the river. The results are discussed in relation to the findings of others on Atlantic ocean Salmo salar, Columbia River Oncorhynchus tshawytscha and Amur River Oncorhynchus keta.

Plasma cholesterol, magnesium, calcium, sulphate and urea levels were measured on pooled blood samples from all 216 fish. Both males and females entered the river with cholesterol levels three times those found in human plasma. The cholesterol levels decreased 65% in females and 31% in males during the 715-mile swim to the spawning grounds. However, the losses were greater in the male over the first 250 miles. Magnesium levels increased significantly in the plasma of both sexes during the first 250 miles. Urea nitrogen levels were lower than human levels and somewhat higher than reported for fresh water teleosts. Urea levels decreased at points up

the river. Plasma inorganic sulphate sulphur levels were 12 times as high as the levels reported for human or carp serum. The sulphate levels decreased very significantly for the first 250 miles and then increased very significantly over the final 465 miles. Plasma calcium levels were high when compared to levels in humans and a fresh water teleost and decreased very significantly beyond the first 250 miles.

The changes in the flesh of the standard fish during migration, as reflected by proximate analyses, has been completed. The levels of both free and esterified cholesterol have also been determined in the flesh of the standard fish for both sexes. Data have been obtained which indicate that salmon blood contains both corticosteroids and 17-ketosteroids; corticosteroids other than 17-hydroxycorticosterone appear to be present.

The acid soluble phosphorus compounds of migrating salmon organs have been separated into groups representing inorganic phosphate, sugar phosphates and nucleotide phosphates. Creatine phosphorus has been determined separately. Fat soluble lipid-phosphorus is also being followed. Thus a balance sheet of acid-soluble phosphorus compounds is being obtained. Water soluble, fat soluble, and total inositol are being determined microbiologically. Inositol has been implicated as the storage form of hexoses by some workers who have noted the virtual absence of free-reducing sugars and the relatively high proportion of inositol in shark fins. It is hoped that some insight can be obtained into the fate of sugar and nucleotide compounds during migration.

A study was made of the changes in the nitrogenous compounds (protein and non-protein nitrogen) of the muscles of migrating sockeye salmon. When calculated on the basis of a "standard fish", the total nitrogen decreased very significantly during migration. This was largely due to a decrease in the actomyosin fraction and was more pronounced in females than males. During the early stages of migration the proteins extractable by salt solutions of low ionic strength did not decrease, but during the later stages decreased sharply. There was no change in the actual concentration of non-protein nitrogen constituents. A thorough study of these constituents

showed that they were the same in both male and female fish and were present in approximately identical amounts in both sexes. The histidine content of the muscle in all fish decreased to one-fifth of the original value during the early stages of the migratory journey and remained at the low level thereafter. Some of the other constituents changed to a smaller extent, usually with a slight increase during the later stages of the migration. This was especially noticeable in female fish.

Last year's investigations of the levels of Na^+ and K^+ in the flesh of migrating spring salmon have been extended to three runs of sockeye salmon. Some species differences were observed in the changes which occurred but the general pattern remained the same. A drop in Na^+ and rise in K^+ occurred at the point where the fish entered fresh water. The levels were then restored very nearly to those prevailing at sea until the fish neared the spawning grounds. Just prior to spawning a marked rise in Na^+ and fall in K^+ was observed. When males and females were differentiated on arrival at the spawning grounds the rise in Na^+ was found to have occurred in the male but not in the female fish.

Colour Changes in Sockeye Salmon on Canning

Colour and oil determinations of sockeye salmon samples representing 10 different areas of capture have been completed, the data analyzed statistically, and published. Since there is a definite agreement between the colour of fish prior to canning and that of the canned material, several polysterene resin-colour reproductions have been made to simulate the colour of sockeye salmon flesh, and these will be used in order to assess the accuracy of visual colour grading of raw salmon during the next year's sockeye salmon run. A formula has also been derived which may be used to predict the colour of the canned flesh from the colour and quantity of the fresh flesh oil.

Transportation and Storage of Fish in Refrigerated Sea Water

This year's work on refrigerated sea water was almost entirely in the field of consultation and observation of privately-built installations on ship and shore. The M.V. "Silver Viking" which was built and equipped two years ago with refrigerated sea

water was accompanied on two halibut trips by an engineering staff member. The performance of the equipment under actual open ocean fishing conditions further demonstrated the feasibility of this system. The vessel was one of the top halibut producers, to some extent because of her ability to make longer trips with this system.

A second large vessel, the 72-ft. steel combination fishing vessel "Pacific Ocean" was built and outfitted with refrigerated sea-water equipment. She has a unique hold arrangement permitting her to carry full loads of herring with no sacrifice of capacity due to the sea-water equipment. Her refrigeration equipment is hydraulically driven and this will permit assessment of this means of driving, which has many advantages.

A new steel vessel for the former owner of the "Silver Viking" has been built. Her unique features are: a new system of tank construction employing expanded styrene insulation and a Fiberglas reinforced plastic tank lining and a complete alternating current system for driving all auxiliary equipment, including refrigeration. This latter feature is a result of the success of the electrical system installed, on the advice of this Station, on the "Silver Viking".

Two shore refrigerated sea-water installations, one for salmon and one for halibut, were built and successfully operated. This is a new field in British Columbia and is one of the most promising for the exploitation of refrigerated sea water. While the halibut installation is of a pilot plant scale, the salmon installation is an integral part of a large new cannery and is capable of holding over 200,000 lb. of salmon.

In the field of smaller vessels studies and experiments have been made on means of more economical installation of insulated tanks, and in collaboration with a local refrigeration manufacturer development has continued on "packaged" refrigeration equipment for small vessels to provide a compact, efficient, low-cost unit which can be installed with a minimum of labour at the boat. A considerable number of these units have been sold in the past year.

Fish Muscle Enzymes

This investigation, which originated with a study concerning the post-mortem formation of the sugar ribose in fish muscle, and its relation to browning of the flesh during heat processing and dehydrative procedures, has now assumed a wider objective. The enzymes concerned with nucleic acid degradation in fish muscles have been found to be both numerous and active, and it is hoped that eventually a study of them will add useful information concerning the many and various chemical problems which are involved in deterioration of fish in cold storage or during processing.

A ribonuclease, free from phosphomonoesterase activity, has been obtained from muscle of lingcod and a number of its properties have been investigated. The enzyme is more stable to heat in neutral or alkaline than in acid solutions. Its optimum pH for activity is 6.5. It is strongly inhibited by fluoride, zinc sulphate, copper sulphate, monoiodoacetate and formaldehyde. No evidence for activation by metallic ions has been found. The enzyme degrades ribonucleic acid with the release of four mononucleotides which have been identified as the 3' isomers.

The ribonuclease extract also contains two enzymes that can hydrolyse bis(p-nitrophenyl)phosphate. These phosphodiesterases are separable from the ribonuclease by electrophoresis on paper in cacodylate buffer (μ 0.05, pH 7.0). They cannot degrade ribonucleic acid and their natural substrates in muscle tissues are not yet known. Unlike the ribonuclease, these enzymes are not inhibited by monoiodoacetate, but are affected similarly by other compounds tested. Enzymes capable of attacking ribonucleic acid and bis(p-nitrophenyl)phosphate have been found in muscle extracts from spring salmon, herring, lemon sole, sculpin (cabezon), dogfish and Atlantic sword fish, and thus appear to be widely distributed in marine fishes.

The phosphomonoesterase activity of lingcod muscle extract has been investigated. The extract appears to contain at least four phosphomonoesterase enzymes, only one of which is capable of hydrolysing the 3' isomers of the ribomononucleotides that are the products of the action of the ribonuclease on ribonucleic acid. This enzyme is strongly inhibited by tartrate and fluoride and has its pH optimum at 5.0. Apparently the same

enzyme can also hydrolyze the 5' isomers of the mononucleotides. The natural substrates of the other phosphomonoesterases are not yet identified.

The investigation concerning a purine nucleoside phosphorylase in lingcod muscle has been completed. This enzyme is readily prepared and has been found to be more convenient for use in preparation of the very labile substance deoxyribose 1-phosphate than the mammalian enzyme systems used elsewhere. The muscles of lingcod have also been found to possess very active phosphoglucomutase and considerably less active phosphoribomutase systems. Using a crude phosphoribomutase-containing enzyme from lingcod muscle a new phosphate ester, namely deoxyribose 1,5-diphosphate, has been isolated for the first time and described briefly. With the comparatively crude enzyme preparation used, the following reactions were observed, in all instances the final equilibrium being strongly in favour of formation of the more acid stable phosphate esters (i.e. the 6' or 5' esters): Glucose 1-phosphate $\rightleftharpoons$ glucose 6-phosphate; ribose 1-phosphate $\rightleftharpoons$ ribose 5-phosphate; deoxyribose 1-phosphate $\rightleftharpoons$ deoxyribose 5-phosphate.

In addition to the above, proof has been obtained that lingcod muscle contains very active phosphoriboisomerase and phosphoketopentoepimerase enzymes. These enzymes carry out the following reactions: ribose 5-phosphate $\rightleftharpoons$ ribulose 5-phosphate, and ribulose 5-phosphate $\rightleftharpoons$ xylulose 5-phosphate. So far the enzyme preparation used has been such that the reactions proceed rapidly through ribulose 5-phosphate to xylulose 5-phosphate, and only the xylulose 5-phosphate has been isolated. A simple method of preparing this ester in fairly pure state by preparative paper chromatography has been worked out. In connection with this work, ribulose ortho-nitrophenylhydrazone, xylulose para-bromophenylhydrazone and phosphoribonic acid have been prepared.

Antibiotics and Preservation

A careful comparative study has verified and extended previous work in which chlortetracycline (Aureomycin) was found more effective for preservation of chilled fish than oxytetracycline (Terramycin) or tetracycline. This result held for three

different preservation procedures studied, namely, (a) incorporation of 5 parts per million of the antibiotic in ice, (b) dipping fillets in solutions containing 5 parts per million, and (c) storing eviscerated fish in refrigerated salt solutions containing 1.5 parts per million. However, in the last-named procedure the antibiotic treatment was more effective in improving the "organoleptic quality" of the fish than it was in controlling the increase in bacteria. A thorough bacteriological study of fish spoilage in refrigerated sea water, with and without added antibiotics, is in progress.

It was previously reported that sorbic acid was quite effective in controlling growth of moulds on smoked fish. This year seven antifungal antibiotics have been tested as possible agents for control of mould and yeast development on smoked or other fish products. The antifungal antibiotics were added to the brine used in 0.02 per cent concentration, sorbic acid (1%) being tested for comparative purposes. While five of the antifungal antibiotics delayed mould and yeast development strongly, sorbic acid, in the higher concentration used, was more effective.

Large-scale commercial studies with chlortetracycline which have been carried out have included transport of halibut in refrigerated sea water containing the antibiotic and dipping black cod on a fishing vessel in sea water containing 50 parts per million before icing them with ordinary ice. The results of these trials have shown very significant improvement in the quality of the treated fish. Moreover, extensive microbiological assays have shown that the raw flesh of fish so treated contains either no detectable antibiotic, or amounts which would in all, or almost all cases, be non-detectable after cooking.

The studies on whale carcass preservation with antibiotics have continued. This year, on request, both oxytetracycline (Terramycin) and chlortetracycline (Aureomycin) were studied, introduction into whale carcasses being effected by placing a 50-gram (approximately 2 oz.) amount in the bombhead of the harpoon. The results were disappointing. Due to the failure of about 40% of the antibiotic charges to reach the whales, only 33 animals were treated. Bacteriological studies showed no significant improvement of these in comparison with 53 untreated whales

examined. Statistical examination of the bacteriological results obtained in the 1956 studies in which the antibiotic had been injected into the visceral cavities of the whales directly showed a highly significant improvement.

New work in progress on which there is as yet insufficient data to permit definite conclusions includes: (a) a study of the effect of the mineral elements in different types of water on distribution and stability of chlortetracycline in ice blocks; (b) effect of isoascorbic acid and various packaging methods on retarding oxidative rancidity in fish fillets, and (c) possible value of nitrites, formaldehyde and antibiotics in retarding spoilage of non-eviscerated herring intended for reduction.

Nutrition and Metabolism of Marine Bacteria

Studies on the mechanism of propionate oxidation in a marine bacterium have indicated that propionate forms propionyl adenylate and then propionyl CoA as first steps in the activation process. Oxidation of propionic and succinic acids was studied in the presence of the inhibitor fluoracetic acid. This compound inhibits citric acid oxidation, hence in its presence, citric acid will accumulate if its precursors in the metabolic sequence are supplied. From succinic acid, but not from propionic acid, considerable quantities of citric acid formed, confirming the previous conclusion that propionate is not being oxidized via succinate by these cells.

A procedure for separating and isolating tricarboxylic acid cycle intermediates produced in metabolism using celite columns has been applied in the investigation of the metabolism of propionate. When propionate $1-C^{14}$ was metabolized by resting cell suspensions of this marine bacterium, the metabolic intermediates formed were extracted and separated by celite chromatography technique. A number of radioactive peaks appeared which did not correspond to tricarboxylic acid cycle acids. None of the known intermediates in this cycle was found to have any appreciable radioactivity.

Evidence for the existence of enzymes of the tricarboxylic acid cycle in a marine bacterium was obtained. Succinic dehydrogenase, fumarase, malic dehydrogenase, the condensing enzyme and aconitase have been demonstrated. Cytochrome oxidase was

observed as well. No evidence of isocitritase was discovered. Properties of the enzymes were studied.

When the Na^+ requirement for the oxidation of exogenous substrates by whole cell suspensions of a marine bacterium was determined the di- and tri-basic acids were found to require 3 to 4 times as much Na^+ for optimum rate of oxidation as the monobasic acids and the hexose sugars. Succinic dehydrogenase, fumarase, and malic dehydrogenase enzymes in cell-free extracts of the organism not only did not require Na^+ but were more active in its absence. Malic dehydrogenase was found to require the addition of K^+ and Mg^{++} for maximum activity. The results have been interpreted to indicate that the di- and tri- basic acids must form Na^+ complexes before being transported into the cell.

Fishing Gear Investigations

The work reported last year on the testing of fishing gear materials has continued and test results have been analysed mathmematically to provide basic engineering data for fishing gear design. Much more data are still required. Information re the use of nylon in salmon gill nets, acquired over several years from the technical literature, private inquiries, and original experiment, has been published as a manual entitled, "The Selection and Care of Nylon Gill Nets for Salmon". Assistance and information on fishing gear continues to be tendered privately on request from individuals and from government and commercial organizations. In connection with the possible increased activities of the Board concerning investigation of fishing gear materials and on new fishing methods, one staff member has been absent for approximately 3 months on an extensive tour which has involved attendance at several meetings, including participation in the F.A.O. International Fishing Gear Congress held in Hamburg, Germany. Visits were made to various fishing ports and manufacturers of fishing gear, and the types of gear used in the fishing industry in the United Kingdom, Norway, Germany, and Eastern Canada were examined. Contributions are also being made toward the drafting of specifications and standard test procedures for fishing-gear materials both to the Canadian Government Specification Board and to the Food

and Agriculture Organization of the United Nations. These specifications and standards are most desirable because, at the present time, variations in test results consequent upon differing test procedures are causing confusion in the gear and fishing industries.

Biochemistry of Fish and Marine Invertebrates

Twenty-five coho salmon were captured on their way to their spawning grounds and held to ripen in tanks at the Vancouver Aquarium. Biochemical changes were followed by removing fish at intervals for analysis. Enzyme studies were conducted on principal tissues. The experiment lasted four months and during this time the fish were without food, as they would have been in their natural habitat. Changes characteristic of ripening salmon were observed. Evidence was obtained suggesting that one or more co-factors required for tissue respiration were gradually depleted over the period of the experiment. If this interpretation is correct, the phenomenon observed would be a manifestation at the cellular level of a vitamin deficiency in the fish. It was concluded that such a phenomenon could well occur during the course of upstream migration.

Uridinediphosphate galactose and the corresponding galacturonic acid and acetylglucosamine compounds have been found in fresh salmon liver. These are the only uridine compounds which occur, uridinediphosphate itself being absent. In general, the distribution of nucleotide substances in salmon liver is much simpler than that in mammalian livers. A compound which appears to be closely related to, but distinct from, uric acid is also present. An investigation concerning the excretory metabolism of fish, with special regard to trimethylamine oxide, is in progress. Levels of Na^+ and K^+ in the flesh of lemon sole were found to vary considerably with season in fish caught in the same area. That muscle tissue from different parts of the same fish may vary in Na^+ and K^+ content was noted in the case of herring.

Investigations concerning new marine sterols have progressed actively. The sterol mixture of the barnacle has been found to contain 60% cholesterol and 34% of 24-dehydrocholesterol. This is the first time this sterol has been demonstrated in

natural material. The common Pacific mussel has been found to contain a 22-dehydrosterol in the cholesterol series, in addition to the brassicasterol previously described. New techniques, such as those of gas chromatography and zone melting, are being investigated as means of isolating this sterol. Since the brine shrimp has been found to contain primarily cholesterol, it seems possible that this crustacean, from the point of view of comparative biochemistry, should be considered as primitive on the malacostracan rather than on the entomostracan stem. The in vitro conversion of cholesterol to other sterols has been achieved in a marine invertebrate.

Fish Reduction Problems

Proximate analysis of herring was carried out on fish caught at monthly intervals over a one-year period. A marked influence of gonad development on the levels of oil, water and protein was noted. In the case of oil, the lowest level was reached in March just prior to spawning. After spawning, a peak oil content (up to 24% of the body weight) was reached in June, after which a slow decline until spawning time was noted. A positive correlation was found between oil content of herring and Tester's condition factor.

The possible loss of significant amounts of protein nitrogen during the reduction of B.C. herring due to bacterial spoilage has prompted an investigation into the preservation of the whole fish using solutions of sodium nitrite, formaldehyde, and the antibiotics chlortetracycline and oxytetracycline. A preliminary test, for which only small amounts of herring were available, has produced no conclusive results. The tests will continue as soon as larger amounts of herring are available.

A study has been made to determine which of a number of different types of proteolytic enzymes available commercially would be most effective in the treatment of stickwater prior to converting it to solubles. Of the five enzymes tested, three proved to be about equally active in the process and more effective than the other two. One of the most active enzymes tested was found, by following α -amino nitrogen release during digestion, to possess endopeptidase activity almost exclusively. It was found that proteolytic enzymes could reduce the viscosity of whale solubles

effectively. The treatment can be carried out on the final concentrated product under acidic conditions at room temperature. Thus, neither special equipment nor special conditions are required in the process and at the low pH that can be employed, losses by bacterial spoilage are negligible.

For the past year an investigation has been proceeding into the stability of antioxidant-treated herring meals in storage. Neither the protein quality as determined by chick nutrition tests nor the oxidation of the oil as determined by the thio-barbituric acid test has been shown to be significantly affected by the addition of .05% of the antioxidant butylated hydroxytoluene (BHT) to the meal. In order to clear any doubts as to the method of application and effective concentration of the antioxidant, a second series of tests was initiated in January in which BHT and another antioxidant, Santoquin, were added to herring meals in concentrations from .06 to .17%. These meals are in storage at 80°F., and tests will be performed on them at four-month intervals as with the other meals.

Miscellaneous

Experiments were made in the commercial production of individual frozen oysters which brought forth one means of producing this product with existing commercial equipment. Further work was done on the mechanical brining of salt herring for the Orient. The innovations in the system which were tried in previous years were a failure but did give information on the practical limits of this method. An extension of experiments carried out in previous years indicated the feasibility of transporting crabs in chilled aerated sea water. The crabs survive the treatment well and are maintained in excellent condition.

The use of pouches made of various plastic laminates in the vacuum and controlled-atmosphere (nitrogen) packaging of fresh fish is to be investigated from the standpoint of the prevention of oxidative rancidity. For this purpose a special packaging machine has been obtained by this Station on a one year's lease. The program will commence as soon as adequate supplies of fish are available. Organoleptic and chemical tests will be used to determine the degree of oxidative rancidity in samples of packaged fish kept in refrigerated storage.

Acknowledgments

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SUMMARY NO. 1

BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION
I PHYSICAL MEASUREMENTS, PLASMA CHOLESTEROL AND ELECTROLYTE LEVELS

D.R. Idler
H. Tsuyuki
I. Bitners

Many of the physiological and biochemical changes occurring during the spawning migration of Salmonidae have been investigated. Interest has been primarily stimulated by the fact that the fish do not feed for many weeks or in some cases months. This is also a time of great activity. Tissue energy reserves must be mobilized both for the enlargement of the gonads and for the trip to the spawning grounds, which are often many hundreds of miles from the sea.

The expenditure of fat and protein reserves by the Atlantic salmon Salmo salar and the Pacific coast Oncorhynchus tshawytscha (spring salmon) has been investigated. None of these studies appear to provide data which would permit energy expenditures to be calculated either on a time or distance of travel basis. The reasons for this differ in each investigation and include: (1) non-continuous sampling during the spawning migration; (2) too few samples; (3) too few chemical analyses; (4) difficulties in converting data to a standard fish due to large variations in the dimensions of individuals. These limitations were overcome to a large degree by the studies of Pentegov et al on migrating Oncorhynchus keta (chum salmon) on the Amur river. This comprehensive study showed for the first time the energy expenditures of male and female fish per unit distance over the entire fresh water migration of approximately 700 miles. The relatively uniform size and stage of maturity of the population made it possible, within reasonable limits, to accomplish the same results as would be obtained if one could perform a complete proximate analysis on a fish, then return it to the water and recapture it further upstream. Even this magnificent effort was hampered to some degree by the variations in individual fish which apparently were sometimes not adequately compensated for by the number of samples available. Variation in flesh depot fat in limited flesh segments have been investigated for sockeye salmon (Oncorhynchus nerka) on the Columbia river. A report of limited scope is available on fat and protein analyses for sockeye on the Harrison river system.

In 1956 and again this year members of the staff of this Station collaborated with the International Pacific Salmon Fisheries Commission biologists on a program designed to study the energy expenditures of Fraser River sockeye migrants to Stuart and Chilco lakes. The Stuart Lake run has now been investigated for two consecutive years. The ability of the biologists to select a pure race of fish of nearly uniform length and weight suggested an excellent opportunity to investigate the more fundamental biochemical changes in individual organs and tissues in a manner analogous to the determination of total energy reserves in the entire fish.

This investigation was carried out on 216 fish representing both sexes of sockeye in three different phases of migration and this initial report provides necessary background information on the fish used in the phases of the project completed to date. It also demonstrates the degree of success achieved in selecting fish of uniform length and weight. There appears to be no available information in the literature for salmon concerning the electrolytes determined in the present study. Blood cholesterol has been measured on an unspecified species of sexually mature salmon taken in a river in Japan.

All fish were caught in reef nets to minimize struggle and placed in ice water to anaesthetize them prior to bleeding by cutting off the tail to sever the caudal artery. Heparin was added to the blood which was centrifuged immediately

and the plasma transported to the laboratory in dry ice. Individual fish were placed in polyethylene bags and frozen in brine at Lummi Island, and on dry ice at the more distance points. The fish were kept at -30°C until they could be processed within a few days. They were thawed overnight at 0°C by which time it was just possible to slit the belly wall and remove the frozen viscera. All tissues were kept in polyethylene bags on dry ice until the required number of organs to complete a group was obtained. Four groups of male and 4 groups of female fish were obtained from each location. The size of the groups are shown in Table I.

The internal organs of each group of fish were separated, then pooled and weighed while still frozen. Each eviscerated fish was then weighed both in water and air, as was the tail. Specific gravities were calculated from these data. Each eviscerated fish was then filleted and the entire body flesh portion kept separate from the bone, skin, head and tail.

Plasma cholesterol was determined with the Liebermann-Burchard reagent by the procedure of Sperry and Webb and sulphate sulfur was precipitated with benzidine and titrated with alkali. Well established procedures were employed to determine calcium as the oxalate, magnesium as magnesium ammonium phosphate and urea with urease.

The physical changes in the fish from Lummi Island where they were in water of low salt content to Lillooet on the Fraser River and to the spawning grounds at Forfar Creek are shown in Table II. The distances are given in Table IV. Although each fish was measured and its weight recorded, the large number of samples necessitates the reporting of averages only for each group. For this reason most of the data have been statistically analyzed.*

Changes in the tissues and organs

A detailed discussion of these changes will be given when proximate analyses are completed. For the present certain observations appear to be pertinent.

It is a well-known fact that the snout length of males increases significantly during migration whereas that of females increases but to a lesser extent. The present study confirms this fact and demonstrates the quantitative aspects for sockeye salmon on a very uniform size population. The male snout increased by 52% and the female by 25% during the migration, (Table II). The "body" length would be expected to remain constant for both sexes with possibly a very small increase during migration due to the portion of the head which was included in this measurement. The female groups (Table II) show that this constancy has been realized. The average body length of the 23 male fish taken at Lummi Island was somewhat short (49.9 cm.) but the difference was not statistically significant. This situation was anticipated and an additional 17, 35, 18 male fish were measured at the 3 consecutive points. When these

* The term "very significant" refers to probability as obtained by the "t" test of .001 or less. When p was between .05 and .001 it is described as significant. When p is greater than .05 no significance is ascribed to the data. All data are recorded as means $\pm$ standard error. Since each group of fish was not exactly the same size, slightly different values were obtained when mean values were calculated on individual fish rather than on group averages. This was particularly true for males at Lummi Island and Forfar Creek. Because of the complexity of the calculations, individual fish were analyzed statistically only for total weight, body length, flesh weight and weight of the eviscerated fish at Lummi Island. This was done to make the corrections to a standard fish as accurate as possible.

data were included, males averaged 50.2, 50.2 and 50.3 cm. at each consecutive point. All weight data for males at Lummi Island were corrected to body length 50.2 from 49.9 cm. on the previously established basis that the body weight is proportional to the length cubed when the correction is small, as it is in this instance. There was a significant weight loss in both males and females between Lummi Island and Lillooet. But in spite of the extensive depletion of fat and protein stores the males regained their original body weight while the females lost no more on the run to the spawning grounds. Tissue water is the prime factor in explaining this observation but this will be discussed when proximate analyses are completed. The losses in fat and protein with an accompanying percentage increase in body water is well established for migrating salmon and results in the very significant changes in specific gravity of both sexes (Table II). A thickening of the skin during spawning migration has been observed in spring salmon. The increase in snout length has already been mentioned. Table III shows that the tail also increased in weight. Since the fish do not feed once they begin the river migration, it is not surprising that the viscera of both sexes decreased very significantly in weight. Table III shows that this loss is primarily a reflection of a nearly complete wasting of the alimentary tract. When considering the energy expended during the migration, this factor should be taken into consideration. The heart and kidney of both sexes underwent relatively little change in weight. The spleen of both sexes first decreased, then increased in weight. The sexes started out very nearly equal in weight when the difference in body length is taken into consideration. However, a comparison of the sexes shows that the females had significantly larger livers than the males. The males had significantly larger alimentary tracts and kidneys.

Gonads

The energy which a starving fish must expend to produce milt or roe has been emphasized for Salmo salar in the rivers of Scotland. In May and June the ovaries represented only 1.2% of the total weight of the fish whereas in September and October on the upper river the ovaries were 23.2% of the fish. The testes increased from 0.15% to 3.32% of the weight of the fish. Greene's data for Columbia river O. tshawytscha shows that the ovaries represented 5% (2 fish) of the weight of the fish at the mouth of the river and 20% (4 fish) on the spawning grounds but the number of fish was limited and the fish were quite variable in size. Miescher-Rüsch emphasized that abnormal proportions were exhibited between the sexual glands and the weight of the body for extreme sizes of Atlantic salmon. He concluded that the large majority of Rhine salmon did not develop significant milt or roe while in the sea. This is not a general phenomena, however, as Paton has shown that both male and female Atlantic salmon entering rivers in Scotland showed large increases in milt and roe from May through October. For the ovaries this variation was from 1.2% in May and June to 12.4% in September and October. In the same time period the milt of incoming migrants increased from 0.15% to 2.71%.

In comparing the data for O. nerka with that outlined for other Salmonidae certain points are worthy of mention. The Atlantic salmon, S. salar, is generally a much larger fish. Thus the fish in the present study averaged about 2.5 kg. whereas the Rhine salmon examined at Basel averaged 8 kg. The O. tshawytscha on which Greene carried out his studies varied from about 7.5 to 16 kg. O. keta investigated by Pentegov averaged around 4 kg. The Atlantic salmon frequently return to the sea after spawning whereas Oncorhynchus do not. The Rhine salmon spend many months in the spawning area waiting for the gonads to ripen whereas in the present study only a few days separated arrival and spawning. However, both genera cease to take food by the time the fish reach fresh water (and probably before) and then begin an extended period of starvation at a time when the energy demands both for swimming and sexual maturation are extensive.

The study by Pentegov *et al.* on migrating chum salmon (*O. keta*) on the Amur River most closely parallels the present study. The total distance of the migration, somewhat over 700 miles, is very comparable (Table IV). The water temperatures were similar. The distance to an intermediate point on the migration is nearly identical. Chum salmon migrate somewhat slower than do sockeye. The gonads of both the male sockeye and chum salmon did not increase in weight after the first 250 miles of river migration (Tables III and IV). The gonads of both male and female chum salmon on the Amur River were heavier than those of sockeye salmon in relation to body weight on entering the river. The milt of both species appeared to decrease in relation to body weight in the later stages of migration (Table IV). For sockeye this change was not significant.

The energy expenditure to produce roe by both female sockeye and chum salmon was considerably greater than the expenditure by males to produce milt. Of particular importance was the relatively small weight of milt which must be produced by males during the starvation period in the river in comparison to the roe produced by the female (Table III).

TABLE I - Sampling of sockeye salmon.

Sex	Group	Number of fish per group		
		Lummi	Lillooet	Forfar
M	1	7	10	10
M	2	6	10	5
M	3	5	10	6
M	4	5	10	4
F	1	8	10	14
F	2	7	10	14
F	3	9	10	13
F	4	9	10	14

TABLE II - Length and weight changes of sockeye salmon during spawning migration.

Site	Sex	Groups	Total Fish gm	Total Length cm	Standard Length A	Snout Length B	"Body" Length A-B
Lummi Is.	M	4	2565±42	59.3	53.7	3.4±.11	50.2±.29
Lillooet	M	4	2371±26	59.3	54.0	3.9±.15	50.2±.00
Forfar Cr.	M	4	2583±51	61.1	55.5	5.2±.16	50.3±.28
Lummi Is.	F	4	2376±26	57.4	52.0	2.8±.04	49.2±.29
Lillooet	F	4	2156±4	57.6	52.4	3.1±.05	49.3±.40
Forfar Cr.	F	4	2168±10	58.2	52.7	3.5±.04	49.3±.26

Site	Sex	Groups	Evis. Fish gm	Head Skin Bones Tail gm	Flesh gm	Sp. gr. Evis. Fish
Lummi Is.	M	4	2322±40	881±33	1467±21	1.033±.000
Lillooet	M	4	2185±25	871±10	1302±51	1.024±.000
Forfar Cr.	M	4	2382±47	1057±10	1304±40	1.012±.000
Lummi Is.	F	4	2151±19	785±15	1335±13	1.037±.000
Lillooet	F	4	1908±41	765±20	1135±30	1.024±.000
Forfar Cr.	F	4	1775±10	834±12	934±10	1.015±.000

TABLE III - Weight and length changes in the organs of sockeye salmon during spawning migration.

Site	Sex	Groups	Heart gm	Liver gm	Spleen gm	Kidney gm
Lummi Is.	M	4	6.10 $\pm$.43	31.6 $\pm$.8	2.90 $\pm$.26	26.0 $\pm$.8
Lillooet	M	4	5.88 $\pm$.26	25.4 $\pm$.6	1.98 $\pm$.05	23.3 $\pm$.7
Forfar Cr.	M	4	5.80 $\pm$.28	37.6 $\pm$ 1	3.60 $\pm$.23	28.1 $\pm$ 1.0
Lummi Is.	F	4	5.05 $\pm$.13	40.2 $\pm$.5	2.04 $\pm$.09	21.6 $\pm$.7
Lillooet	F	4	4.95 $\pm$.21	44.6 $\pm$.9	1.50 $\pm$.08	19.9 $\pm$.2
Forfar Cr.	F	4	4.58 $\pm$.06	40.5 $\pm$ 1.6	2.17 $\pm$.18	19.7 $\pm$.2

Site	Sex	Groups	Alimentary Tract gm	Viscera gm	Gonads gm	Tail gm
Lummi Is.	M	4	90.5 $\pm$ 3.3	157 $\pm$ 5	56.5 $\pm$ 2.3	64.1 $\pm$ 2.3
Lillooet	M	4	30.1 $\pm$.4	86.7 $\pm$ 1.7	82.0 $\pm$ 2.8	58 $\pm$.5
Forfar Cr.	M	4	16.4 $\pm$ 1.0	91.5 $\pm$ 2.4	85.4 $\pm$ 2.4	82 $\pm$ 3.1
Lummi Is.	F	4	78.3 $\pm$ 1.6	147 $\pm$ 3	80.1 $\pm$.8	59 $\pm$.6
Lillooet	F	4	26.2 $\pm$.8	97.2 $\pm$ 3.6	138 $\pm$ 3.7	52 $\pm$ 1.5
Forfar Cr.	F	4	12.9 $\pm$ 2.7	79.9 $\pm$ 2.1	298 $\pm$ 2.3	77 $\pm$.9

TABLE IV - Comparison of ovaries for Stuart Lake sockeye and Amur River chum salmon.

Station	Days	Miles	H ₂ O temp. °F.	Milt %*	Roe %*
Lummi Is.	0	0	62°(est)	2.20	3.37
Lillooet	10	250	63	3.46	6.40
Sp. grounds	27	715	48	3.31	13.7
Langre Is.	0	0	63	4.11	7.02
Sophiskoya	12	253	62	4.21	10.1
Sp. grounds	33	740	48	3.08	17.4

*Expressed as percent of the body weight of the standard fish at each station.

Plasma cholesterol and electrolytes

The levels of free and esterified cholesterol found in male and female plasma are given in Table V. The "normal" total cholesterol levels at the beginning of the upriver migration were very high (570 mg.%) compared to warm-blooded animals (150-190 mg.%). However, average levels of 662 mg.% have been reported for carp blood. The decrease in total plasma cholesterol from Lummi Island to the spawning grounds (715 miles) was far greater in female (65%) than in male fish (31%). Females also lost more free cholesterol (64%) than did males (53%). Over the relatively short distance between Lummi Island and Lillooet (250 miles) the females showed no decrease in free cholesterol and a 13% decrease in total cholesterol. The males over the same distance lost 23% total cholesterol and 35% free cholesterol. It can thus be concluded that the females began to lose plasma cholesterol later in the migration than did the males but the losses in the later stages were much greater in the female. The ratio of free to total cholesterol steadily decreased as the males moved up the river. The ratio was .46 at Lummi Island, .39 at Lillooet and .34 at the spawning grounds. The females show less variation in the ratio of free to total cholesterol. The ratio was .38 at Lummi Island and Forfar Creek and .44 at Lillooet. On arrival

at the spawning grounds, which for this race occurs only a few days before spawning, both the total and free cholesterol levels were significantly higher in the male. However, for an unspecified species of sexually mature salmon (probably *O. keta*) in a Japanese river, similar levels of both free (75-80 mg.%) and total cholesterol (150 mg.%) have been reported for both sexes. The present study has shown that the plasma cholesterol levels were extremely high in sexually immature sockeye salmon and decreased with increasing sexual maturity and/or decreasing energy reserves.

TABLE V - Plasma cholesterol and electrolyte levels in migrating sockeye salmon.

Components mg.%	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Free Cholesterol	M	261±18 (23)*	170±17 (40)*	134±15 (25)*
	F	215±9 (33)*	.02** 220±24 (40)* .8**	.1** 77±6 (55)* .001**
Total Cholesterol	M	570±19	436±38	394±25
	F	572±19	.02 499±24 .05	.4 202±20 .01
Calcium	M	21.6±0.6	19.9±1.3	12.5±0.8
	F	27.3±0.4	.2 23.8±1.1 .02	.001 15.5±0.6 .001
Magnesium	M	1.58±0.16	3.71±0.25	2.15±0.11
	F	0.86±0.06	.001 2.88±0.18 .001	.01 2.18±0.12 .01
Urea N	M	6.0±0.3	4.3±0.7	4.9±0.2
	F	6.2±0.6	.05 3.7±0.5 .02	.3 4.2±0.4 .4
Inorganic Sulphate as S	M	12.5±0.2	5.5±0.3	9.6±0.3
	F	13.0±0.7	.001 3.9±0.3 .001	.001 9.8±0.2 .001

* Total number of fish divided into 4 groups.

** Significance of difference from value immediately to left.

The carbohydrate and glycogen content of salmon liver and flesh is known to be small. However, it was considered of interest to determine levels of plasma magnesium. The results (Table V) show that they were generally within the range of 1-3 mg.% reported for human serum. Carp serum is reported to contain 3.32 mg.%. The very significant increase during the migration may reflect an increased carbohydrate metabolism during this very active phase of the salmon's life, but this is purely conjecture. The recent isolation of a new sulphate-containing nucleotide from salmon liver, in this laboratory, promoted the determination of plasma sulphate. The levels (Table V) at all stages of migration were very significantly higher than those reported for human serum (0.9-1.1 mg.%) or carp serum (0.94 mg.%). The difference between the initial and final levels can probably be accounted for by blood dilution which is reported to be about 12% for *O. tshawytscha*. The significant drop during the early phase of migration and subsequent rise in the later phase is analogous to the change reported in human pregnancy. Urea nitrogen levels, 6 mg.% for

males and 6.2 mg.% for females, were lower than those normally reported for humans (10-15 mg.%) but somewhat higher than for fresh water carp (3.5 mg.%) and trout (2.6 mg.%). The changes during spawning migration can probably be largely accounted for by dilution of the blood. The calcium levels, 21.6 and 27.3 mg.%, respectively, for males and females, were much higher than the 9-11.5 mg.% reported for man or the 11.5 mg.% reported for carp serum. The calcium levels like the cholesterol levels decreased in the final stage of sexual maturity to the same magnitude as is found in humans. Parathyroid-like function has been attributed to the ultimobranchial gland of fish. However, too little is known of the functions of this gland at the present time to justify more than a suggestion that decreased activity of the gland may be associated with the continually decreasing blood calcium levels. By analogy with mammalian serum the decreased calcium levels should be accompanied by a decrease in serum protein. This will be investigated.

We wish to thank the staff of the International Pacific Salmon Fisheries Commission for their assistance and scientific advice and for making available the fish for this study. Mr. I. Bitners, a university student, temporarily employed by this Station, rendered valuable technical assistance.

SUMMARY NO. 2

BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION II CHOLESTEROL, FAT, PROTEIN AND WATER IN THE FLESH OF THE STANDARD FISH

D.R. Idler
I. Bitners

A pure race of Fraser River sockeye salmon has been sampled at the beginning, at an intermediate point, and at the end of a 715-mile spawning migration. The data show that the large number of fish taken (216) and the uniformity of the population has permitted an accurate standard fish to be calculated for each sex. In effect, the realization of a standard fish permits one to obtain the data which would be obtained if a fish could be analyzed then returned to the water and recaptured at a later time. By this technique not only percentage changes but absolute weight changes in tissue components may be determined. This report will consider the absolute weight changes in the flesh fat, protein, water and cholesterol levels in standard male and female sockeye salmon as they migrated 250 miles from Lummi Island to Lillooet and then a further 460 miles to the spawning grounds at Forfar Creek.

The sockeye salmon were caught in reef nets and transported to the laboratory at the temperature of dry ice as described in the preceding summary. The fish were thawed overnight and the frozen viscera including the kidneys were removed. The head and tail were severed and the well-scraped skin and bones were separated from the body flesh. The flesh of males and females was separated and divided into groups as previously described. The frozen flesh was put through an electric cutter and the entire sample (15 to 42 lb.) was thoroughly mixed and representative 1-lb. samples sealed in enamelled cans and either stored at -30°C. or heat processed. The heat-processed samples were employed for the analyses described in this report.

Fat was determined by a rapid method developed specifically for canned salmon and described in Summary No. 34 of this Station's Annual Report for 1956-57. Water was determined with an automatic infrared moisture balance. The fat was then taken up in acetone:ethanol-1:1 and free and esterified cholesterol determined by the Sperry-Webb procedure.

The proximate analyses for the male and female fish at each location are shown in Table I. The original moisture content and the percentage increase in moisture in the flesh was essentially the same for both sexes. The females began the spawning migration at Lummi Island with 11% more fat than the males and up to

Lillooet (250 miles) both sexes appear to have lost about the same percentage of the initial fat content resulting in a 16% greater loss for the female because of the higher fat content at Lummi Island. Both sexes began the migration with the same percentage of protein and appeared to lose the same percentage in the first 250 miles. Over the entire migration distance the females used approximately 18% more protein than the males. The percentage composition data do not show the true picture for the fat and protein consumption of the two sexes because they make no allowance for changes in the total weight of the flesh as the fish move up the river. This point is demonstrated in Table II of the preceding Summary, where it is shown that the body flesh of a standard male sockeye salmon decreases from 1467 g. at Lummi Island to 1300 g. at Lillooet and Forfar Creek. The flesh of the standard female sockeye weighed 1335 g. at Lummi Island, 1135 g. at Lillooet and only 934 g. at Forfar Creek. Table II shows the actual fat, protein and water in the flesh of an average standard male and female salmon on a weight basis at each of the three locations. Table III shows these data in a more easily interpreted form based on the changes in total weight of fat and protein per unit distance.

TABLE I - Proximate analyses and cholesterol content of the flesh of migrating sockeye salmon.

Components	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture %	M	67.2 $\pm$.11(23)*	70.7 $\pm$.23(40)*	78.2 $\pm$.23(25)*
	F	67.0 $\pm$.16(33)*	71.0 $\pm$.12(40)*	78.0-.07(55)*
Fat %	M	9.34 $\pm$.24	5.07 $\pm$.24	3.23 $\pm$.23
	F	10.55 $\pm$.12	5.59 $\pm$.19	2.70 $\pm$.11
Protein N*** %	M	3.508 $\pm$.047	3.283 $\pm$.036	2.684 $\pm$.039
	F	3.518 $\pm$.107	3.385 $\pm$.061	2.835 $\pm$.020
Free cholesterol mg./100 g.	M	25.7 $\pm$.81	30.9 $\pm$.22 <.01**	31.1 $\pm$.40 0.8**
	F	25.6 $\pm$.24	30.6 $\pm$.22 <.001**	34.7 $\pm$.77 <.001**
Total cholesterol mg./100 g.	M	29.2 $\pm$.68	34.1 $\pm$.27 <.01	36.7 $\pm$.77 >.05
	F	29.0 $\pm$.48	32.6 $\pm$.24 <.01	35.9 $\pm$.40 <.01

* Total number of fish divided into 4 groups.

** Significance of difference from value immediately to left.

*** Total flesh protein nitrogen was determined in connection with a study on the distribution of nitrogenous components of flesh, conducted by Mr. D.W. Duncan and Dr. H.L.A. Tarr.

The changes in fat for the standard fish are quite different than when considered on a percentage basis. The standard females use only 7% more fat than the standard males over the first 250 miles and they use an almost identical weight of protein. Since the initial weight at Lummi Island of the standard female fish (2376 g.) is 92.5% of the initial weight of the standard male fish (2565 g.) the female expends 15.3% more flesh fat and 6.5% more flesh protein than a male fish of the same body weight. The most significant difference in male and female fish over the 250 miles from Lummi Island to Lillooet is the change in the weight of gonads. The male milt increases by only 25.5 g., whereas the female roe increases by 57.9 g. (see preceding Summary). The female would therefore be expected to consume more body fat and protein if the energy expenditure for swimming was the same

for both sexes. The data appear to substantiate this supposition. During the final 460 miles the standard females use 57% more flesh protein and 63% more flesh fat per unit distance than does the standard male. The standard male at Lillooet weighed 2371 g. and 2583 g. at the spawning grounds, whereas the standard female weighed 2156 g. at Lillooet and 2168 g. at the spawning grounds. When the average weight of the standard fish for each sex is considered over this 460 miles the standard female expends 87.2% more flesh fat and 80.6% more flesh protein than does a standard male of the same body weight. The most significant difference in the two sexes over the final 460 miles of the spawning migration is in the weight of the gonads. The male milt increases by only 3 g., whereas the female roe increases by 160 g. An approximate calculation based on 17.6% protein and 9.2% fat for the ovaries shows that the 157 g. excess of gonad produced by the female as compared to the male represents approximately 27.6 g. of protein and 14.5 g. of fat. Table II shows that the standard female expends 15 g. more fat and 27 g. more protein than the standard male over the last 460 miles. This excellent agreement appears to offer convincing evidence that the standard female and male sockeye salmon expend nearly identical energy for swimming and the excess flesh fat and protein consumed by the female can be attributed to the larger weight of the gonads. The standard female expends 22% more flesh fat and 25% more flesh protein than does the standard male over the 715-mile migration route (Table III). The average weight of a standard male fish from Lummi Island to the spawning grounds is 2574 g. as compared to 2272 g. for the female. When this weight difference is taken into account the standard female expends 38% more flesh fat and 42% more flesh protein than a standard male of the same weight. On the same equal weight basis the standard female begins the migration at Lummi Island with 10.2% more fat and 1.2% less protein. The demands on flesh fat and protein are considerably greater for the female than for the male and only in the case of fat are somewhat greater reserves available.

For the first 250 miles the standard males expend 29% more fat than protein and the females expend 40.5% more fat. For the last 460 miles the utilization reverses and the standard male expends 100% more protein than fat while the standard female expends 92% more protein (Table III).

TABLE II - Changes in fat, protein, water and cholesterol in the flesh of migrating sockeye salmon of standard length.

Components g. in flesh	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture	M	986	921	1020
	F	894	806	813
Fat	M	137	66	42
	F	140	64	25
Protein	M	322	267	219
	F	294	240	165
Free Cholesterol	M	.377	.402	.406
	F	.342	.347	.324
Total Cholesterol	M	.428	.444	.479
	F	.387	.370	.335

TABLE III - Expenditure of flesh fat and protein by migrating sockeye salmon of standard length.

Locations	Sex	Component expended by standard fish in mg./mile	
		Fat	Protein
Lummi Island to Lillooet	M	284	220
	F	304	216
Lillooet to Forfar Creek	M	52	104
	F	85	163
Lummi Island to Forfar Cr.	M	132	144
	F	161	180

Dr. N. Paton, in referring to water changes in the flesh of migrating Salmo salar states that "it is this increase in the percentage water of the flesh which maintains the weight of the fish per fish of standard length, although the solids as a whole have diminished". This thought is prevalent in the literature in the more restricted sense that the percentage of fat plus water is a constant. There is no doubt that the percentage of water does increase as the percentage of fat decreases but the statement quoted above is not valid for the present study. The flesh of neither sex takes on water in sufficient quantity to replace depleted fat and protein reserves. Over the 715-mile route the flesh of the standard female lost 129 g. of protein, 115 g. of fat and lost (not gained) 81 g. of water (Table II). Table I shows that even on a percentage basis the fat plus water at different points for the same sex are not very constant. Between Lummi Island and Lillooet the flesh of the standard males lost 39 g. of fat, 75 g. of protein and only gained 34 g. of water, resulting in a loss in the weight of the flesh of 163 g. as compared to 401 g. for the standard female. In the present study the standard eviscerated male fish which weighs 2322 g. at Lummi Island and 2382 g. at Forfar Creek does confirm Paton's statement that the weight of the fish remains essentially constant but the constancy is due partly to the increased water in the flesh but primarily to gain in weight of the head, skin, bones and tail from 881 g. to 1057 g. The head, skin, bones and tail of the final increases from 785 g. at Lummi Island to 834 g. at Forfar Creek, but the magnitude is not sufficient to maintain the weight of the standard eviscerated female which drops from 2151 g. at Lummi Island to 1775 g. at Forfar Creek.

Both the male and female standard fish showed a loss of water for the first 250 miles of the spawning migration. The loss was 6.6% for the males and 9.8% for the females. Over the last 460 miles the males gained 10.7% water while the females lost an insignificant amount (0.87%).

Table I shows the changes in free and total cholesterol expressed in mg./100 g. of flesh. The free cholesterol per unit weight of flesh of females increased 19.5% in the first 250 miles and a further 13.5% in the last 460 miles for an overall increase of 35.6% during the migration. However, when the loss in weight of the total flesh of female standard fish is considered the total flesh, free cholesterol increases only insignificantly (1.5%) (Table II) in the first 250 miles and decreases 6.5% over the last 460 miles for an overall loss of 5.3% during the migration. The males showed an increase of 20% in free cholesterol per unit weight of flesh in the first 250 miles and no change after this point. The flesh of the standard male decreases from 1467 g. to 1302 g. over the first 250 miles and as a result the increase in free cholesterol per standard fish is only 6.6%. The weight of the male flesh does not change during the last 460 miles, neither does the percentage cholesterol.

The total cholesterol increased steadily for both sexes over the migration route when the results are considered per unit weight of flesh (Table I). However, when changes in flesh weight are taken into consideration, the standard male fish showed a gain in total cholesterol of 12% and the standard female a loss of 13% over the 715-mile migration.

The esterified cholesterol represents only 12% of the total at Lummi Island for the females and this decreased to 6.1% at Lillooet and 3.3% at Forfar Creek. The male flesh had 12% of the total cholesterol in an esterified form at Lummi Is., 9.4% at Lillooet and 15% at Forfar Creek.

The preceding Summary showed the very considerable decreases in plasma cholesterol for both sexes over the migration route. It seems surprising that while flesh reserves of fat and protein and plasma cholesterol were so extensively depleted, the cholesterol content of the flesh of both sexes actually increased per unit weight of tissue. In the case of the male even the standard fish showed an increase in flesh cholesterol, while the standard female suffered no great loss. It should be emphasized, however, that whereas the flesh of a standard male fish contained 322 g. of protein and 137 g. of fat at Lummi Island, it only contained 428 mg. of cholesterol (Table II). Thus, from an energy consideration, it makes little difference whether the fish does or does not utilize the flesh cholesterol.

The authors wish to thank the staff of the International Pacific Salmon Fisheries Commission for their assistance and scientific advice and for making available the fish for this study.

SUMMARY NO. 3

BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION
III. THE QUALITATIVE AND QUANTITATIVE DISTRIBUTION OF PHOSPHORUS
COMPOUNDS, CREATINE AND INOSITOL IN ALL MAJOR TISSUES OF THE
STANDARD FISH

Violet M. Chang
H. Tsuyuki
D.R. Idler

This project was started during the summer of 1957.

The period of the spawning migration is not only a time of starvation for salmon but also a time of great activity for both sexes. The female has the added burden of gonad production. Hence, protein, carbohydrate and fat reserves are mobilized and utilized to a very large extent to generate the energy required for this prolonged activity. For a fundamental understanding of this shift of energy from the consumed foodstuff to stored energy, a knowledge of the immediate sources of chemical energy is required. It is well known that in biological tissue, chemical energy derived from the complete combustion of protein, carbohydrates and fats is transferred through a series of electron transport enzymic mechanisms finally to the high energy phosphate bonds. This potential energy is then translated to mechanical energy necessary for motion and locomotion. The most common high energy phosphate containing natural products are creatine phosphate and the nucleotides. Adenosine polyphosphates are particularly involved in the process of muscular contraction. In vertebrates creatine phosphate serves as the main reservoir of high-energy phosphate and transfers its phosphate to ADP to form ATP and thus to maintain the concentration of this highly important compound. Nucleotides such as uridine diphosphate, adenylic acid, cytidine monophosphate and guanosine monophosphate are involved co-enzymatically in the synthesis or rebuilding of cellular material which must be continually replaced in order to maintain the life of a living organism.

At this Station we have found in salmon liver an almost complete complement of the nucleotides found in animal tissues including the glucuronic acid, galactose

and N-acetyl glucosamine derivatives of uridine diphosphate. In addition an adenylyl succinic acid derivative and its parent base, succinyl adenine have been found (Summary No. 30 of this Station's Annual Report for 1956-57).

The extensive polynucleotide/protein synthesis involved during the maturation of milt and roe necessitates either a hyperactive de novo synthesis of nucleotide from its constituents or its mobilization as the nucleotide followed by selective polymerization to form polynucleotides. If the latter is the case, as the present knowledge of polynucleotide formation indicates, then from a consideration of chemical equilibria, a high metabolic pool of nucleotides is expected. The demand on pyrophosphates both as immediate energy sources and as cellular building blocks is intimately associated with spawning migration during which time these processes must be highly active.

In 1945, Fischer hypothesized that the presence of relatively large amounts of inositol in shark fins and the absence of glycogen and other reserve carbohydrates in the liver might mean that inositol functions as a source of blood sugar by the reopening of the inositol ring. Recently, Charalampous found an enzyme system in rat kidney which catalyzed the oxidative conversion of inositol to glucuronic acid through the postulated mediation of UDP-glucose followed by UDP-glucuronic acid. Our studies on salmon liver have demonstrated the presence of UDP-glucuronic acid and UDP-galactose and by virtue of the reversible interconversion of UDP-glucose and UDP-galactose reported by Leloir it is possible that inositol may function in the manner that Fischer hypothesized.

In order to search for a solution to some of these problems as applied to spawning salmon, it was decided to study the fate of the acid and fat soluble phosphorus compounds, creatine and its phosphate and inositol. As an initial approach to this problem the acid-soluble phosphorus compounds from the various fish organs were separated into inorganic phosphorus, sugar phosphate, nucleotide phosphate, and creatine phosphate and the change which these components underwent during the spawning migration was studied. In addition the phospholipid phosphorus and inositol were also followed.

A flow sheet of the analyses performed is outlined in Figure 1.

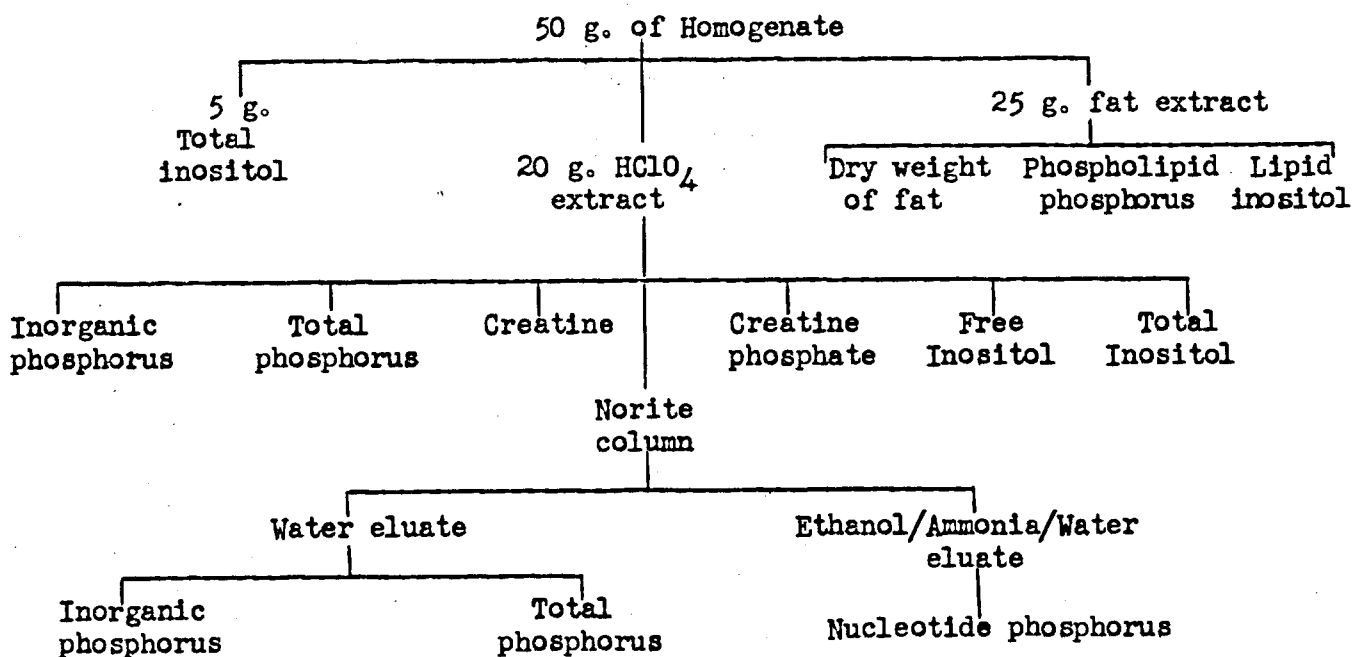


Fig. 1 - Determination of Inositol, Creatine and Acid-soluble Phosphorus Compounds in Salmon Organs.

Preparation of Tissue

The salmon were grouped and the individual organs separated and grouped as previously described (see Summary No. 1 of this report). The tissues were dipped into a large Dewar flask containing liquid nitrogen until there was no further heat exchange. They were then removed and wrapped in a piece of canvas and chilled in a box containing dry ice, where they were pounded into small pieces. These pieces were now powdered thoroughly in a large mortar and pestle (also previously chilled in a dry ice box). Sufficient liquid nitrogen was added from time to time to keep the tissues thoroughly frozen during the grinding process. The powdered tissues were then transferred quickly into chilled polyethylene bags and stored in a dry ice box until the various analyses could be performed. The preliminary powdering of the tissue before homogenizing insured uniform sampling for analytical purposes.

Preparation of Tissue Extracts

A 50 gm. sample of each powdered tissue (at $-30^{\circ}\text{C}.$) was weighed into a 100-ml. Virtis homogenizer cup. The tissues were then thawed quickly and homogenized in a Serval Omni-mixer without any addition of solvent. A 5 g. sample was weighed into a 100-ml. Kjeldahl flask for the total inositol determination. A 25 g. sample was weighed into a Soxhlet thimble and the fat extracted. The remaining 20 g. were extracted with 25 ml. of 0.6N HClO_4 , followed by another 25 ml. of 0.2 N HClO_4 at $0^{\circ}\text{C}.$ The supernatants of both extracts were combined and immediately neutralized to pH 7 with dilute KOH. Creatine, before and after acid hydrolyses of the perchloric extract, was determined by the α -naphthol procedure employing p-chloromercuribenzoate to eliminate the interference of sulfhydryl groups.

Fat Extraction.

A spatula was used to mix thoroughly 25 g. of tissue in an extraction thimble, with sufficient methanol (about 20 ml.) to break up the caked mass of tissue. It was then extracted in a Soxhlet apparatus for 5-8 hours with 100 ml. of methanol, followed by 100 ml. of CHCl_3 for the same length of time. The extracts were combined and flash evaporated. For purification, the crude fat was dissolved in 50 ml. of $\text{CHCl}_3/\text{MeOH}$ (2:1) in a 100-ml. beaker which was set inside a 1000-ml. beaker. At least 10 volumes of water was then carefully added to the large beaker to submerge the smaller one. A continuously changing surface was maintained between the organic and aqueous layers by the diffusion of the MeOH into the water. In this way water soluble contaminants are passed into the aqueous phase, leaving behind the CHCl_3 solution of fat. The CHCl_3 solution was now made up to 100 ml. and 25 ml. taken to dryness and dried to constant weight in vacuo. Phospholipid phosphorus was estimated by a total P determination on an aliquot of the remaining 75 ml. Lipid inositol was estimated microbiologically after hydrolysis of the fat extract (see total tissue inositol).

Total Tissue Inositol

To 5 g. of homogenate in a Kjeldahl flask was added 20 cc. of approximately 6N HCl. The flask was sealed and heated in an oven at 110 - $120^{\circ}\text{C}.$ for 6 hours. The flask was placed in a boiling water bath and the contents brought to near dryness by a stream of air. The residue was dissolved in a minimal volume of water and dried again as above. After this procedure was repeated a third time, most of the HCl had been removed. The residue was brought to about 20 ml. with water, the pH adjusted to 5.0 with dilute KOH and the volume brought to 25 ml. After standing overnight in the cold, the insoluble material was filtered off and aliquots of the filtrate used for the inositol determination. Recently others have found better inositol recoveries using higher concentrations of HCl for a longer time. The samples at Forfar Creek were analyzed using the more drastic conditions. Our findings, which must be confirmed and extended to other tissues, suggest that the more mild hydrolysis conditions gave better recovery (see footnote to Table II) of inositol.

Saccharomyces carlsbergensis was used for the microbiological determination of inositol. The preparation of the media was modified considerably to obtain blank readings of high transparency. The vitamin concentration of the media was doubled and the media sterilized and inoculated with washed cells (washed cells were used throughout the assay). Only a slight amount of growth was required to utilize the trace of inositol present in the media. The cells were removed by centrifugation and the pretreated media was used for the assay.

The Separation of Acid-soluble Phosphorus Compounds

1.0 g. of a 1:1 mixture of Norite A, N, F, IX (Pfanstiehl Laboratory Inc.) and Celite 535 were mixed with water and poured into a 1-cm. diameter column containing a glass wool plug at the bottom. The charcoal was washed by forcing through 20 ml. of H₂O under 2-3 lbs. pressure. One to 10 ml. quantities of the perchloric acid extract were added and the inorganic and sugar phosphates were eluted with 100 ml. of water (about 30 ml. of water was required to remove all the inorganic and sugar phosphates from the column with test samples of alimentary tract, kidney, liver, milt or roe). The nucleotides were then eluted by passing 100 ml. of EtOH/NH₂/H₂O (40:1:59) (after 40-50 ml., the u.v. absorption at 260 mμ was reduced to zero, indicating complete removal of the nucleotides).

This must be considered strictly as a progress report and the discussion must be of a very preliminary nature until more of the samples have been analyzed. Some of the variation between groups, particularly at Lummi Island, is probably caused by analytical difficulties. The phosphorus distribution in roe was an example. The data for gonads in Table I show the poor agreement between total phosphorus and the sum of inorganic, sugar and nucleotide phosphorus for all samples of roe. The total phosphorus values were found to be high, probably because finely dispersed or soluble phospholipid, which is very high in roe, was being analyzed with the acid soluble phosphorus. Lower values (in brackets Table II) were obtained after ether extraction and the sum of inorganic, sugar and nucleotide phosphorus approached the total phosphorus values. A misprint in the published procedure for the inositol microbiological assay gave some trouble with the earlier samples. Most of the analytical problems have now been resolved.

Alimentary Tract

Lipid inositol averaged only 5 to 6% of the total inositol in the alimentary tracts of both sexes at Lummi Island (Table II). The total inositol per unit weight of alimentary tract probably decreases from Lummi Island to the spawning grounds. However, the lipid inositol represents 60% of the total inositol of the males and 40% of the females at the spawning grounds. This is also reflected by the constancy of the phospholipid (Table I). The free (active in the microbiological assay) acid soluble inositol appears to decrease by about 50% from Lummi Island to the spawning grounds. It is too early to be sure but creatine levels do not appear to change too greatly for either sex per unit weight of tissue. However, the alimentary tract has been shown to decrease in weight by 82% over the 715-mile migration (Summary No. 1 of this report). Thus, for the standard fish, all components shown in Tables I and II will be utilized in amounts varying between about 80 and 90% of that present in the alimentary tract at Lummi Island. The distribution of phosphorus in the alimentary tract of males at Lummi Island is in the order of 45% inorganic, 37% phospholipid, 11% nucleotide and 7% sugar phosphate. The distribution in the alimentary tracts of females follows a similar pattern. The data is far too meagre to consider quantitatively but it is fairly apparent that at the end of the migration phospholipid phosphorus is far in excess of inorganic phosphorus.

TABLE I - Distribution of phosphorus ($\mu\text{g/g.}$ of tissue).

Tissue	Sex	Location	Acid Soluble Phosphorus				Phospholipid
			Total	Inorganic	Sugar	Nucleotide	
Alimentary Tract	M (6)*	Lummi Is.	623	337	67	135	383
	M (5)	" "	960	662	78	135	355
	M (7)	" "	677	487	105	79	472
	F (8)	" "	785	655	82	96	328
	F (7)	" "	1095	692	218	164	388
Alimentary Tract	M (10)	Lillooet	564	476	57	73	-
	M (10)	" "	734	655	94	81	-
	F (10)	" "	504	333	98	90	480
	F (10)	" "	418	317	107	70	-
Alimentary Tract	M (5)	Forfar Cr.	274	161	94	39	454
	F (13)	" "	353	218	104	36	402
* Number of individual fish which were pooled for analyses.							
Liver	M (6)	Lummi Is.	1020	684	264	-	-
	M (5)	" "	763	553	161	68	568
	M (7)	" "	747	541	177	45	806
	F (8)	" "	813	586	102	92	516
	F (7)	" "	1180	615	102	130	962
Liver	M (10)	Lillooet	831	739	111	66	-
	M (10)	" "	946	804	128	82	736
	F (10)	" "	789	615	141	96	1080
	F (10)	" "	749	624	78	90	-
Liver	M (5)	Lummi Is.	742	532	161	32	980
	F (13)	" "	872	744	122	40	1034
Kidney	M (6)	Lummi Is.	815	634	142	20	368
	M (5)	" "	562	385	171	30	468
	M (7)	" "	835	600	195	44	434
	F (8)	" "	617	560	77	33	342
	F (7)	" "	715	582	52	33	483
Kidney	M (10)	Lillooet	1071	850	200	97	-
	M (10)	" "	1240	857	222	181	285
	F (10)	" "	729	501	198	56	297
	F (10)	" "	751	562	120	52	-
Kidney	M (5)	Forfar Cr.	822	583	205	44	702
	F (13)	" "	1277	684	528	72	362
Gonads	M (6)	Lummi Is.	695	552	140	26	290
	M (5)	" "	803	662	39	48	348
	M (7)	" "	968	757	85	65	348
	F (8)	" "	1130	377	139	407	-
	Ether extracted (1134)*			(461)	(264)	(234)	361
Gonads	F (7)	Lummi Is.	1505	475	235	130	962
	M (10)	Lillooet	995	809	96	33	-
	M (10)	" "	1146	910	58	34	230
	F (10)	" "	1320	533	321	131	1464
	Ether extracted (1271)*			(519)	(461)	(102)	-
Gonads	F (10)	Lillooet	1380	586	109	170	
	Ether extracted (1278)*			(560)	(570)	(115)	
Gonads	M (5)	Forfar Cr.	775	606	208	99	374
	F (13)	" "	1166	505	316	138	1246

* See text for a discussion of ether extracted perchloric acid extracts of roe samples.

TABLE II - Distribution of Inositol and Creatine.

Tissue	Sex	Location	Inositol (µg./g. of tissue)		Creatine (µg./g. of tissue)			
			Total	Lipid	Acid Soluble		Acid Soluble	
					Free	Total	Free	Total
Alimentary Tract	M (6)	Lummi Is.	415	31	210	157	-	
	M (5)	" "	312	8	284	280	877	
	M (7)	" "	313	28	155	212	993	
	F (8)	" "	305	6	204	176	531	
	F (7)	" "	268	22	283	132	762	
Alimentary Tract	M (10)	Lillooet	345	32	194	163	724	701
	M (10)	" "	218	43	274	172	741	844
	F (10)	" "	342	77	190	102	721	743
	F (10)	" "	295	-	143	124	704	717
Alimentary	M (5)	Forfar Cr.	160	99	93	78	704	696
	F (13)	" "	206	80	103	69	749	730
Liver	M (6)	Lummi Is.	633	58	110	113	184	
	M (5)	" "	417	136	97	132	280	
	M (7)	" "	501	-	68	113	299	
	F (8)	" "	715	94	77	61	178	
	F (7)	" "	630	109	57	53	219	
Liver	M (10)	Lillooet	670	127	138	129	100	111
	M (10)	" "	607	237	178	185	102	104
	F (10)	" "	709	525	78	75	158	165
	F (10)	" "	633	-	36	58	152	150
Liver	M (5)	Forfar Cr.	650*	401	95	72	108	108
	F (13)	" "	713	488	105	96	220	215
* Value was 650 for a 6-hr. digestion with 6N HCl. It was 465 for 12-hr. digestion with 22% HCl (see text).								
Kidney	M (6)	Lummi Is.	384	23	248	196	425	
	M (5)	" "	297	53	145	233	370	
	M (7)	" "	295	-	79	238	685	
	F (8)	" "	275	42	166	136	421	
	F (7)	" "	298	38	113	96	510	
Kidney	M (10)	Lillooet	670	28	595	429	301	253
	M (10)	" "	660	-	857	648	250	271
	F (10)	" "	610	25	298	233	235	237
	F (10)	" "	598	-	305	305	253	251
Kidney	M (5)	Forfar Cr.	490	129	272	217	203	207
	F (13)	" "	458	38	367	379	410	407
Gonads	M (6)	Lummi Is.	215	55	147	105	624	
	M (5)	" "	290	48	126	153	1140	
	M (7)	" "	229	76	85	111	1100	
	F (8)	" "	442	24	57	38	142	
	F (7)	" "	480	82	38	57	134	
Gonads	M (10)	Lillooet	245	18	247	174	984	951
	M (10)	" "	290	10	257	278	1102	1090
	F (10)	" "	445	200	33	20	133	131
	F (10)	" "	490	-	31	45	131	129
Gonads	M (5)	Forfar Cr.	160	13	93	74	920	960
	F (13)	" "	398	206	4	37	114	122

Liver

The phospholipid and inorganic phosphorus represent the greater part of the phosphorus pool at Lummi Island. The phospholipid represents 45-50% and the inorganic 35-40% of the total phosphorus. The sugar and nucleotide phosphates together represent 15-20% of total phosphorus in liver. Lipid inositol appears to be a more significant component in the liver than it was in the alimentary tract. In liver, as in the alimentary tract, the lipid inositol appears to increase out of proportion to the other inositol components at the spawning grounds.

Kidney

The lipid inositol is a minor component of the total inositol of the kidney but unlike the liver the lipid inositol does not become the largest form of inositol at the spawning grounds. At Lummi Island the inorganic and phospholipid phosphorus values are about the same as for the alimentary tract (46% and 36% respectively for males). At Lummi Island the nucleotide phosphorus is very low for both sexes (Table I).

Gonads

The analytical difficulties in determining the phosphorus distribution in roe have been discussed. In the milt at Lummi Island the inorganic (59%) was the major acid-soluble form of phosphorus. The remaining phosphorus distribution was phospholipid 29%, sugar phosphate 8% and nucleotide 4%. The acid soluble inositol is consistently substantially higher in milt than in roe.

SUMMARY NO. 4

STEROID HORMONES IN MIGRATING SALMON

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H. Tsuyuki
P.J. Schmidt

This project was carried on for 2 months during the fall of 1957. It was then dropped for 4 months. The completion of the problem on salmon colour and the curtailing of the nucleotide project has permitted this project to be resumed.

The importance of steroid hormones in mammalian life processes is well established. They play an important role in regulating reproduction, ion balance, liver glycogen levels and numerous other important biochemical processes. The knowledge of hormones in fish is very scanty at the present time, although the information is extremely important to a complete understanding of sexually maturing salmon.

Our interest is particularly centred around a study of the changing hormone distribution in salmon prior to and during the spawning migration. Prior to, because of the role steroid hormones may play in "triggering" the migration, and during, because of the role the hormones play in sexual development and such processes as ion balance in mammals.

We have made several attempts to determine the levels of certain corticosteroids and 17-ketosteroids on plasma samples taken from migrating sockeye salmon. We feel that the results were extremely unreliable when accepted methods for mammalian plasma were applied to sockeye plasma. It will apparently be necessary to investigate the hormones of salmon blood in some detail before appropriate techniques can be selected. To this end, the purification of the corticosteroids, 17-ketosteroids (and estrogens, if present) of salmon blood is under investigation.

Chemical purification techniques are being combined with adsorption and paper chromatography in an attempt to obtain steroids in a sufficiently pure state for characterization purposes. It is anticipated that the majority of the hormones will not be present in levels in excess of 5 to 20 micrograms per 100 ml. of plasma. Since 100 ml. of plasma represents 4-6 adult sockeye salmon, it is anticipated that this phase of the project will take a considerable time to complete. In an attempt to expedite this program with the possible changing steroid pattern in migrating salmon, attempts to apply existing techniques, or combinations of techniques, to the determination of classes of steroids are being continued.

SUMMARY NO. 5

CHANGES IN THE PROTEIN AND NON-PROTEIN NITROGEN
FRACTIONS IN MUSCLES OF MIGRATING SOCKEYE SALMON

D.W. Duncan
H.L.A. Tarr

Since the work on this subject has recently been submitted for publication details are omitted.

The samples used were those described in Summary No. 1 of this report. The fractionation involved a subdivision of the muscle proteins into four general fractions: (1) Proteins soluble in cold, approximately neutral salt solution of comparatively high ionic strength, and insoluble in salt solutions of low ionic strength (largely actomyosin); (2) proteins soluble in cold neutral salt solutions of low ionic strength ("myogens", "myoalbumins"); (3) proteins insoluble in the above solutions (collagen and elastin, denatured myosin); (4) nitrogenous compounds not precipitated by cold 5% trichloroacetic acid solution (non-protein nitrogen).

The results may be summarized as follows:

The conditions under which the fish were obtained and the samples were prepared, which involved slow freezing and thawing and mincing the flesh in a "silent cutter" undoubtedly caused considerable denaturation of the myosin. The large number of fish samples, and the transportation distances involved, made it virtually impossible to carry out the fractionation procedure under conditions which would preclude myosin denaturation. In the procedure used denatured myosin would be found in the stroma fraction, and that this is the case is borne out by the finding that the stroma protein fraction accounted for from 14.25 to 20.55% of the total muscle nitrogen, values greatly in excess of the values (3 to 4%) which are considered normal for teleost fish. If it is assumed that true stroma proteins account for 3% of the total muscle nitrogen, and that the remaining nitrogen is denatured myosin, then the "high ionic strength" fraction would account for 54.15 to 61.6% of the total nitrogen instead of the 42.6 to 49.33% actually found. The higher values are similar to those reported for fish actomyosin in the literature.

When calculated on the basis of mg. of nitrogen per gm. of muscle (percent basis) (Table I), there is a significant decrease in the total nitrogen content of the fish as they migrate. This decrease is largely due to decreases in the nitrogen content of the high ionic strength plus stroma protein fractions. The concentration of nitrogen in the low ionic strength fraction increased significantly between Lummi Island and Lillooet, and decreased significantly between Lillooet and Forfar. The significant increase in the percentage of nitrogen in the non-protein nitrogen fraction is due to the significant decrease in the total nitrogen of the samples. The actual composition of the fraction itself in terms of mg. of nitrogen remained constant within limits of error, and this is verified in the detailed analysis of this fraction.

TABLE I - Average nitrogen content of different fraction of the muscle of migrating sockeye salmon.

Station	Sex	mg. of N per g. and % of total N on wet wt. basis						% * Recovery
			Whole Muscle	Stroma	High Ionic	Low Ionic	Non-Protein	
Lummi Island	M	mg.	35.08	6.21	15.93	7.96	4.43	98.43
		%	100	17.96	45.39	22.69	12.83	
	F	mg.	35.18	7.20	14.95	7.94	4.48	98.36
		%	100	20.55	42.60	22.60	12.61	
Lillooet	M	mg.	32.83	4.88	14.73	9.30	4.68	102.24
		%	100	14.86	44.80	28.33	14.25	
	F	mg.	33.85	5.16	14.52	9.30	4.61	99.24
		%	100	15.24	42.91	27.47	13.62	
Forfar Creek	M	mg.	26.84	4.13	13.24	6.41	4.10	103.69
		%	100	15.30	49.33	23.88	15.28	
	F	mg.	28.35	4.04	13.11	6.74	4.63	100.57
		%	100	14.25	46.24	23.75	16.33	

* Recovery of N in the four fractions as percent of the total muscle nitrogen.

The amount of nitrogen in each of the four fractions when calculated on the basis of the weight of a "standard" fish (Summary No. 1 of this report) is recorded in Table II. The values given in this table were obtained by multiplying the average nitrogen concentrations shown in Table I by the weights of standard male or female fish. The marked decrease in the nitrogen of the whole muscle is due to both the marked decrease in weights of the fish and to the lower percent nitrogen content of the muscle as migration proceeds. The sum of the nitrogen of the high ionic strength and stroma protein fractions also decreases, but the decrease is much more pronounced for females than males during the later stages of migration. Initially there is practically no change in the amount of nitrogen available to the fish in the low ionic strength fraction, but a sharp decrease occurs during the later stage of migration. The decrease in non-protein nitrogen is entirely due to the decrease in weight of the fish and not to the nitrogen concentration of the fraction.

TABLE II - Nitrogen content of muscles of migrating sockeye salmon (grams per standard fish).

Fraction	Males			Females		
	Lummi Island	Lillooet	Forfar	Lummi Island	Lillooet	Forfar
Whole Muscle	51.462	42.754	34.951	46.966	38.376	26.717
Stroma + High Ionic Strength	32.479	25.540	22.038	29.470	22.329	16.149
Low Ionic Strength	11.677	12.111	8.354	10.769	10.554	6.347
Non-protein Nitrogen	6.499	6.097	5.340	5.988	5.225	4.362

The authors are grateful to Mr. R. Moyer for making the statistical calculations.

SUMMARY NO. 6

THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE MUSCLE
OF SOCKEYE SALMON DURING SPAWNING MIGRATION

J.D. Wood

Since the results of this investigation have already been submitted for publication, they will be reported only briefly.

Previous work by D.W. Duncan and H.L.A. Tarr (Summary No. 5 of this report) indicated that there was no significant change in the amount of non-protein nitrogen (NPN) in the muscle during the migratory journey. Further investigations described here were carried out to investigate the individual compounds in the NPN fraction. The NPN constituents were found to be the same in both male and female fish and were present in approximately the same amounts in both sexes. The histidine concentration in both male and female muscle decreased to one fifth the original value during the early stages of the migratory journey and remained at that level thereafter. Some of the other constituents changed to a smaller extent, usually an increase in the later stages of the migration. This change was greater in the females than in the males. However, the increase in the concentration of these constituents in the muscle was due to a decrease in the amount of muscle per fish rather than to an increase in the amounts of the compounds themselves. During these investigations a method was developed for the analysis of the dipeptide anserine which was found in large amounts in the salmon muscle.

SUMMARY NO. 7

RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC
ACID IN MIGRATING SOCKEYE SALMON

V.M. Creelman

The samples used in these experiments are those, the preparation of which is described in Summary No. 1 of this report. These samples are being assayed for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) to see if any changes occur during migration.

Several methods of analysis for RNA and DNA were available, and the suitability of these for application to salmon muscle tissue was investigated. Briefly, the methods are as follows; RNA (purine bound ribose) being determined colorimetrically by the orcinol reaction throughout, DNA measurement will be discussed below.

Method of: (1) Ogur and Rosen (1950)

Tissue extracted in the cold with -

- (a) ethanol
- (b) ethanol and 0.1% perchloric acid
- (c) ethanol:ether
- (d) 0.2 N perchloric acid

The residue was then digested with 1.0 N perchloric acid at 40°C. for 5 hours to prepare the RNA fraction. The DNA fraction is obtained by heating the residue for 20 minutes at 70°C.

(a) Schneider 1945

Tissue extracted in the cold with -

- (a) 10% trichloroacetic acid
- (b) ethanol
- (c) ethanol:ether

The RNA and DNA are not separated in this method but are obtained in the same solution by two washings of the residue with cold 5% trichloroacetic acid and then by heating the residue at 90°C. for 15 minutes (this was varied, see below) in 5% trichloroacetic acid.

(3) Schmidt and Thannhauser (1945)

Tissue extracted in the cold with -

(a) 1.0 N perchloric acid

(b) ethanol

(c) ethanol:ether

The residue was then digested with 0.3 N KOH for 18 hours at 37°C. DNA is precipitated with 1.0 N perchloric acid; the digested RNA remains in solution. The precipitate of DNA is hydrolyzed by heating with 0.5 N perchloric acid for 20 minutes at 70°C.

Table I summarizes results of RNA measurements obtained by the three methods.

TABLE I - Levels of RNA obtained by three different extraction methods.

Method of extraction		Ribose content	RNA content as estimated from ribose*
1. Schneider		2.93 mg./100 gms.	11.2 mg./100 gms.
2. Ogur and Rosen	(A)	21.0 mg./100 gms.	80.3 mg./100 gms.
	(B)	3.9 mg./100 gms. in DNA fraction	14.9 mg./100 gms.
3. Schmidt and Thannhauser	(A)	23.6 mg./100 gms.	90.1 mg./100 gms.
	(B)	0.97 mg./100 gms. in DNA fraction	3.7 mg./100 gms.

* RNA calculated from % of purine bound ribose in salmon RNA (factor of 3.82).

The Ogur and Rosen extraction procedure resulted in ribose being found in both the RNA and DNA fractions. It was not determined if the ribose in the DNA fraction was RNA ribose or some interfering material. It was found that the levels of ribose given by the Schneider procedure were much lower than those of the Ogur and Rosen. These levels could be increased by prolonging the digestion from 15 minutes to 60 minutes but then they decreased in a further 60 minutes heating period to the level found in the 15-minute period. The highest levels found were much lower than those found by the other two methods. It appears that the low results obtained in the Schneider procedure are probably due to destruction of ribose during the heating period (this procedure is the only one of the three in which RNA is heated with acid in the presence of protein). The Schmidt and Thannhauser procedure gave high ribose levels and there were only traces of ribose found in the DNA fraction. It was found to be preferable to carry out the alkaline digestions with KOH rather than NaOH and to hydrolyse the RNA with perchloric acid rather than trichloroacetic acid. The use of KOH and perchloric acid enables a large reduction in salt content of the final solutions which is of advantage if these are to be used in paper chromatography or electrophoresis (see next page) and perchloric acid does not absorb in the ultraviolet region as does trichloroacetic acid. Further, it appeared that stirring the samples during their 18-hour digestion at 37°C. lead to more uniform results.

Duplicate muscle samples of male and female salmon from each of the three stations (see Summary No. 1 of this report) have been analysed for ribose content by means of Schmidt and Thannhauser extraction. The results are tabulated in Table II.

TABLE II - RNA in muscle of migrating salmon.

Station	Ribose ¹ content	Calculated RNA	Ribose ² content	Calculated RNA
Lummi Island	24.8 mg/100 g. 24.8 " "	94.6 mg/100 g. 94.6 " "	25.8 mg/100 g. 26.1 " "	98.5 mg/100 g. 99.6 " "
Lillooet	20.4 mg/100 g. 23.5 " "	77.9 mg/100 g. 88.8 " "	21.2 mg/100 g. 18.5 " "	81.0 mg/100 g. 70.6 " "
Stuart Lake	20.1 mg/100 g. 20.1 " "	76.7 mg/100 g. 76.7 " "	22.5 mg/100 g. 22.8 " "	86.0 mg/100 g. 87.1 " "
	¹ Males		² Females	

Analysis of other tissues for RNA is proceeding.

Work is in progress to establish a satisfactory method for DNA estimation. The indole method is very sensitive and it would be useful for the small amounts of certain tissue available. However, results with this method have been erratic, and whether this is due to some fault of the method or to variations between samples is not yet clear although the latter is perhaps unlikely in view of the RNA results. In view of the difficulties with the indole method, the diphenylamine method for DNA is also being investigated.

As it would be desirable to be able to determine not only the gross quantity of RNA and DNA in tissues, but also their composition of nucleotides, a start has been made on developing methods for this purpose.

The Schmidt and Thannhauser RNA fraction (KOH digest neutralized with perchloric acid) has been used. This fraction (following necessary concentration) was not suitable for either paper chromatography or paper electrophoretic separation of the mononucleotides due to interfering material (protein breakdown products?) in the digest. In order to separate this material from the nucleotides, the use of a charcoal column has been investigated. Nucleotides can be adsorbed on such a column and then eluted, whereas much of the interfering material is not adsorbed. This procedure provided a solution for paper electrophoresis, but recoveries of the nucleotides have not yet been satisfactory (apparently chiefly due to losses on the column). All the ribose of the Schmidt and Thannhauser RNA fraction is adsorbed onto charcoal and consequently this fraction from salmon muscle contains no free ribose or ribose phosphates as these would not be adsorbed.

SUMMARY NO. 8

CHANGE IN THE Na^+ AND K^+ LEVELS OF SOCKEYE SALMON DURING MIGRATION

R. Jonas
R.A. MacLeod

Changes in the Na^+ and K^+ levels of spring salmon during spawning migration were reported last year (Summary No. 24 of this Station's Annual Report for 1956-57). Since the International Salmon Commission and this Station's chemistry department were collaborating on a program to study energy expenditure in sockeye salmon during upstream migration, samples of this species of fish became available for analysis.

Determinations of the flesh levels of Na^+ and K^+ were made on fish taken at different points on the Fraser River from specific runs of fish.

The experimental procedures followed were the same as those reported previously.

The results obtained on the Stuart Lake runs of 1956 and 1957 and the Chilko run of 1956 are recorded in Table I.

TABLE I - Changes in Na^+ , K^+ , H_2O , fat, protein content of sockeye salmon muscle at different stages during spawning migration.

	Date	No. Fish	Area where caught	% H ₂ O	% Fat	% Protein	mg/100 gm flesh		mg/100 gm flesh	
							Na ⁺	Range	K ⁺	Range
Group I - Stuart Lake Run 1956										
1	July 4	4	San Juan	70.0	5.7	23.1	43.4	36.9-47.2	435	422-439
2	" 5	3	Lummi Is.	69.3	6.6	22.7	29.0	27.6-31.6	468	462-477
3	" 8	3	Albion	70.0	5.2	22.4	38.8	38.1-41.0	420	409-432
4	" 13	3	Hell's Gate	72.9	3.9	22.1	38.8	35.6-43.4	443	432-457
5	" 16	3	Lillooet	72.9	3.7	21.5	37.8	34.2-45.0	427	427-427
6	" 21	3	Soda Cr.	74.6	2.5	21.4	41.5	40.0-44.5	418	403-426
7	" 26	3	Fort St. James	75.7	1.6	18.4	59.7	46.0-73.5	369	353-377
8	" 31	3	Forfar Mouth	78.7	1.3	19.2	71.6	66.0-78.5	362	357-367
				(Just prior to spawning)						
Group II - Chilko River Run 1956										
1	July 30	3	Albion	69.9	5.9	22.5	31.2	26.0-38.7	425	416-443
2	Aug. 10	3	Farwell Canyon	73.7	2.9	22.8	43.7	39.3-48.8	409	398-418
3	" 20	3	Keighly Holes	72.2	3.7	23.5	51.2	48.8-52.4	401	399-403
4	Sept. 18	3	Chilko* Spawning Grounds	80.9	1.3	16.5	47.2	43.8-49.1	424	396-449
5	" 23	3	Chilko** River	87.8	1.0	10.3	36.2	26.1-45.1	297	284-309

* Alive after spawning. ** Dead after spawning.

Group III - Stuart Lake Run 1957

<u>Location</u>	<u>Miles Upstream</u>	<u>Sex</u>	<u>Na^+</u> mg/100 gm.	<u>K^+</u>
Lummi Island	At sea	Male	36.8 $\pm$ 12.1*	426 $\pm$ 24
		Female	42.5 $\pm$ 14.9	412 $\pm$ 12
Lillooet	385	Male	28.5 $\pm$ 1.5	441 $\pm$ 8.5
		Female	29.7 $\pm$ 2.4	440 $\pm$ 14.3
Forfar Creek	850	Male	69.6 $\pm$ 19.1	388 $\pm$ 14.6
(Spawning Ground)		Female	36.5 $\pm$ 2.6	427 $\pm$ 10.7

* Standard error of the mean.

Group I - Stuart Lake Run 1956. In the case of each run, a race of fish whose destination was known was followed from a point prior to its entrance into the Fraser River until it reached the spawning ground.

In the Stuart Lake run of 1956 a drop in Na^+ and a corresponding rise in K^+ occurred prior to the entrance of the fish into fresh water (see the results recorded for Lummi Island). The ion levels were restored almost to those prevailing at sea by the time the fish had reached the next check point even though the fish had by this time entered fresh water. Just prior to spawning, however, a rise in Na^+ and corresponding drop in K^+ was recorded - an observation made last year on spring salmon. Fat, water and protein concentrations in these same samples of skin-free, bone-free flesh were determined to see if changes in these could be correlated with the ion changes. No obvious relationship could be detected. It is of interest that protein changes in the flesh of sockeye salmon during migration are much less than were found to occur in spring salmon.

Group II - Chilko Run 1956. It was unfortunately not possible to obtain samples from all check points on this run. Differences between this and the Stuart Lake run are still apparent, however. Na^+ levels were lower at Albion on this run than on the Stuart Lake run. There was no sample obtained on this run exactly corresponding to the point just prior to spawning as was recorded in the Stuart Lake run. A slight rise in Na^+ to 51.2 was noted, however, in samples from Keighly Holes.

Group III - Stuart Lake Run 1957. In this run samples were obtained at fewer check points but greater numbers were obtained to ensure that the results would be of statistical significance.

The analytical results obtained on samples from Lummi Island show considerable variability. The fish from this point were first placed in water-tight plastic bags before being immersed in an ice-salt mixture for freezing. Unfortunately, several bags were damaged and for this reason there is some possibility of salt penetration into the flesh of the fish. This could account for the degree of variation recorded. Samples from all other points were frozen in dry ice so that this error could not occur again.

The values recorded on the spawning ground were obtained from fish which would have spawned about a week later. Separate analyses on males and females indicated that the rise in Na^+ which had been observed previously occurred in the males but not the females.

It was of interest to know the source of the Na^+ producing the rise in the flesh level of this ion at the spawning ground. Since the fish do not eat and fresh water contains little Na^+ , it seemed most likely that the increase in the flesh was due to a redistribution of body sodium resulting from hormone activity. Samples of fish from the 1956 Stuart run were obtained for analysis. The whole fish minus viscera and the viscera were analyzed separately (Table II).

TABLE II - Changes in Na^+ and K^+ levels on the whole fish and viscera in migrating sockeye salmon.

Stuart Run 1956	Date	#	Whole fish - Viscera				Viscera			
			mg./100 gm. sample		mg./100 gm. sample		mg./100 gm. sample		mg./100 gm. sample	
			Na^+	Av.	K^+	Av.	Na^+	Av.	K^+	Av.
San Juan	July 4	1	65.5	69.2	365	351	124.4	120.0	357	338
"	"	4	66.6		348		120.4		286	
"	"	4	75.5		340		115.3		372	
Forfar	July 31	1	110.0	102.0	290	303	87.2	109.6	299	319
Mouth	"	31	107.0		290		124.1		322	
"	"	31	89.0		329		117.6		337	

There was more sodium in the body of the whole fish minus the viscera at the spawning grounds than at sea. Since the logical source of the extra sodium would be the viscera, analyses of these were made. The average value for sodium in the viscera of fish from the spawning grounds was less than those from the sea, but the degree of variation indicates that little significance can be attached to the difference between the two average values. Much further work would be required before any explanation of the findings recorded could be offered.

SUMMARY NO. 9

BIOCHEMICAL CHANGES IN RIPENING SALMON

R.A. MacLeod
E. Onofrey
R. Jonas

As part of a general study of the biochemistry of salmon migration being conducted at this Station, the biochemistry section is undertaking an investigation of enzyme systems associated with the production of energy in the tissues. Since it would be of interest to know if the activity of these systems changes during migration and ripening of the fish, it would be desirable to study the enzymes in migrating salmon. Since the enzymes of oxidative metabolism lose their activity on freezing, it is necessary to carry out enzyme studies on tissues from freshly killed fish. For this to be done on migrating salmon under natural conditions it would be necessary to have a mobile laboratory to study samples at the site of capture. Since such facilities are not available at present, and since much preliminary work needs to be done to obtain information concerning the nature of the enzyme systems in fish associated with energy metabolism, it was decided to carry out studies on fish kept alive in tanks at the Vancouver Aquarium.

The Vancouver Aquarium had observed the previous year that pink salmon captured at their spawning grounds would spawn if transferred to tanks at the Aquarium. It was of interest to us to know if salmon captured at the entrance to the river up which they would normally travel to the spawning grounds, if placed in tanks at the Aquarium, would ripen normally and spawn.

Arrangements were made with the International Salmon Commission to obtain live sockeye salmon from the reef nets at Lummi Island. These were transported by truck in live tanks to the Vancouver Aquarium. The fish captured were part of the Stuart Lake run and under natural conditions would have travelled to the spawning grounds and spawned in about four weeks. To our great disappointment the fish contracted a bacterial infection shortly after being placed in the Aquarium and all forty died within ten days. A pure culture of an organism was isolated from the lesions which developed on these fish and was sent to Dr. Ordal, an expert on bacterial pathogens at the University of Washington, for study. Pathogenic bacteria are apparently responsible for the death of large numbers of migrating salmon, particularly when water temperatures rise above 60°C., as occurs frequently in water behind power dams. It is possible that, in the case of our fish, the shock of transporting them a long distance by truck could have lowered their resistance to infection. Less likely is the possibility that changes in water temperature were responsible for the invasion.

It was recommended that a hardier fish be used to continue our studies. Arrangements were made to obtain migrating coho salmon from the Capilano River. These were taken from a trap below the Cleveland Dam and transported to the Aquarium, a distance of only five miles. All of the fish survived until used for experimental purposes. Although the coho were vigorous, healthy fish and the experiment showed that they would ripen in the tanks, there were certain disadvantages to using these fish. In contrast to sockeye, the fish were not of uniform size and

hence it was difficult to follow progressive changes in weight of the fish with time. Furthermore, the run had not been investigated as carefully as had the sockeye runs on the Fraser. It was uncertain as to whether or not a pure race of coho had been sampled and whether or not all could have been expected to spawn at the same time. Nevertheless, the experiment was interesting in so far as it provided material for working out experimental techniques, it supplied a group of technologists with experience in handling live fish and since the fish actually ripened in the tanks, analytical data was obtained which would enable some comparison to be made of fish ripening under confined conditions with fish ripening while migrating. Finally, it has supplied some biochemical data which may help considerably to explain some of the phenomena associated with migrating salmon.

Experimental

Two tanks were used to hold the 25 fish, one of 700, the other of 500-gallons capacity. A bed of 8" gravel was placed in each tank and fresh water was introduced into the tank through holes bored in polyethylene pipe laid under the gravel. When a fish was required for analysis it was removed from the tank and transported alive to the Technological Station in a box containing water into which oxygen was bubbled. The fish was stunned by a blow on the head, weighed, its tail removed and the blood collected. The fish was then decapitated; the organs were removed, weighed and dropped into cold saline solution. Parts of selected organs were sliced for respiration studies and homogenized for the determination of glycolytic activity. The remainder of each of these organs was frozen for subsequent proximate analysis. One portion of the blood was allowed to clot and serum recovered by centrifuging; the rest was heparinized and plasma prepared. Serum and plasma were preserved by freezing until time permitted analyses for Na^+ and K^+ , glucose, protein and non-protein nitrogen and amino acids to be made. Since it was uncertain how long it would take the fish to ripen and as there were only a limited number available, the fish were analyzed one at a time at intervals of 3 or 4 days in the beginning and at longer intervals at the end of the experiment. Although considerable variation was expected from fish to fish, it was hoped that changes would be sufficiently great from the beginning to the end of the experiment to enable some degree of progression in the changes to be evident over the ripening period. Because of the limited number of fish on experiment, any which showed signs of developing superficial injuries or fungal infections were removed first from the tank for analysis. Thus, although males and females were present in almost equal numbers, these were not necessarily analyzed on alternate occasions.

Results

Weights of fish and organs at time of sacrifice. A record of the weights of some representative fish and of their various organs, expressed as percent of body weight, is presented in Table I. The last fish analyzed was killed 113 days or almost four months from the beginning of the experiment. A male and a female were left for a longer period of time in the hope that they would spawn but this did not occur in spite of the fact that the fish were transferred to a tank of spawning pink salmon in the hope that the latter might stimulate the cohoes to spawn. Over the whole period of the experiment the fish were without food. In spite of this fact they remained lively and vigorous to the end. Characteristic changes in the colour of the body surface and in the length of the snout were observed in the male fish over the period of the experiment. Reference to Table I shows that the only significant change recorded in the weights of the organs was the greatly increased percentage of the body weight that could be accounted for as eggs in the female. In fish #21 the size and number of the eggs had developed to the point where they had become 22% of the body weight. This observation and the changes noted in the appearance of the male indicated that the fish had ripened over the test period even though the final act of spawning did not take place.

TABLE I - Weights of ripening coho salmon and weights of certain organs, expressed as percent of body weight at time of sacrifice.

Fish #	1	2	3	4	5	13	16	18	21
Sex	female	female	male	female	male	female	female	male	female
Time of sacrifice*	1 day	6	9	13	15	44	57	70	93
Total wt. g.	2370	3324.5	3570	2599	2654	2055	2795	2145	2480
% heart	.143	.163	.213	.223	.193	.177	.250	.269	.218
% liver	1.53	1.41	1.32	1.58	1.32	1.39	1.85	.793	.522
% kidney	.818	.69	.715	.713	.81	.658	.905	.93	.470
% brain	.0253	.0226	.019	.0298	.032	.0312	.0275	.0312	.0331
% milt or roe	5.1	3.06	3.29	4.8	3.78	8.9	17.67	3.83	22.1
Date killed -	July	July	Aug.	Aug.	Aug.	Sept.	Sept.	Oct.	Oct.
1957	25	30	2	6	8	6	19	2	25

* In days from start of experiment (July 24, 1957).

Metabolic studies

Tissue respiration. The rate of uptake of oxygen by tissue preparations was measured using a Warburg respirometer. Slices were used in the case of liver, kidney, brain and heart muscle. In the case of skeletal muscle, bundles of fibres were separated, since suitable tissue slice preparations cannot be made. A medium recommended by Krebs for such studies was used. It contained a mixture of pyruvate, glutamate, fumarate and glucose as substrates.

The results for fish (1) and (2) at different levels of tissue tested are shown in Table II. Neither skeletal nor lateral line muscle showed appreciable O_2 uptake until considerable amounts of tissue were present in the flask. Even when enough was present to cause oxygen uptake, the activity of the tissue per unit weight increased markedly as the level of tissue increased. This suggested that some factor required for oxidative activity was diffusing from the cells and being diluted by the medium. The concentration of this in the medium would then affect the rate of oxidation by the cells. This change in Q_{O_2} with quantity of tissue tested was not so apparent with heart muscle (Fish 2) and was not evident in the case of liver, kidney and brain. There was considerable variation between Fish (1) and (2) in Q_{O_2} values for the various organs.

When Fish (3) was tested it was found that even in the case of liver, kidney and brain, an increase in Q_{O_2} with increasing levels of tissue tested was evident. This variation of Q_{O_2} with tissue level became more pronounced as the number of days the fish were on experiment increased. The phenomenon is clearly demonstrated using liver tissue from Fish #20 sacrificed 76 days after capture. (Fig. 1). The rate of oxidation of the tissue was very low when only 10 mg. was tested but increased sharply at first and then more slowly as larger amounts of tissue were used in the Warburg flask.

This change in the manner in which Q_{O_2} changed with tissue level as the length of time the fish were on experiment increased can best be ascribed to a depletion in the tissues of a factor essential for oxidative metabolism. Since the fish did not eat for the duration of the experiment, such a depletion would not be unexpected. It is not uncommon for fish to spawn and then die with apparently reasonable reserves of fat and protein still available in their body tissues. It is not unlikely that death in such cases is due to exhaustion of a co-factor essential for cellular respiration. Since such co-factors are the functional forms of vitamins in the cell, such a phenomenon would then be a manifestation at the cellular

level of a vitamin deficiency. It is planned to investigate this phenomenon further.

Another factor found to affect the Q_{O_2} value for the various tissues was the nature of the substrate present in the medium used to suspend the tissue slices. In the case of skeletal muscle and lateral line muscle, the Q_{O_2} values were increased when the four substrates in the suspending medium were removed completely. For both of these tissues the highest Q_{O_2} values occurred when fumarate was added as substrate. For kidney, liver, brain and heart the tissues were more active in the presence of the four substrates than in their absence.

TABLE II - Q_{O_2} values for various tissues from coho salmon.

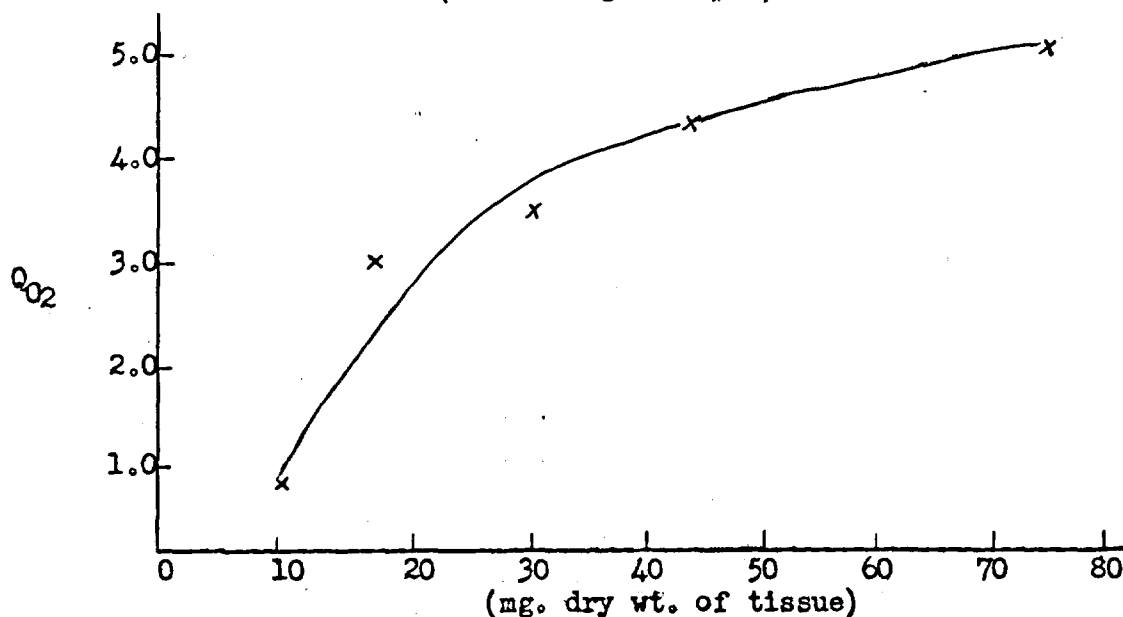
Tissue	Dry wt. tested	$Q_{O_2}^*$	Dry wt. tested	$Q_{O_2}^*$
	<u>Fish (1)**</u>		<u>Fish (2)**</u>	
Skeletal muscle	11.1	-†	35.5	.084
" "	14.9	-†	77.7	.63
Lateral line muscle	22.5	-†	50.3	.62
" " "	45.0	-†	104.9	1.22
Heart muscle	-	-	18.2	5.22
" "	-	-	31.9	5.58
Liver	18.5	6.5	11.9	3.1
" "	31.7	6.45	21.8	2.95
Kidney	13.9	2.66	11.6	6.5
" "	27.4	2.85	26.5	5.3
Brain	13.1	7.95	14.0	5.14
" "	21.7	8.85	30.2	6.5

* Q_{O_2} = μ l O_2 taken up/mg. dry wt./hr. Atmosphere - O_2 , temp. 25°C.

** Fish (1) sacrificed 1 day, and Fish (2) 6 days after the beginning of the experiment.

† No significant oxygen uptake.

Fig. 1 - Change in Q_{O_2} of liver tissue with amount of tissue tested. (Data using Fish #20)



Glycolytic studies

Conditions used in these studies were patterned after those found to be most satisfactory for animal tissues. Homogenates were prepared at a concentration of 10% in .25 M sucrose. The medium used in the main compartment of the Warburg flask contained inorganic ions and co-factors known to be active in glycolysis. Glucose or other substrates to be tested were introduced from the side-arm. The reaction was carried out in an atmosphere of 95% N₂, 5% CO₂ at a temperature of 25°. Glycolytic activity was measured manometrically by following the rate of release of CO₂ by the acids formed in metabolism. Lactic acid production was determined by direct analysis at the end of the experiment. In Table III representative results obtained in glycolytic studies are shown. When glucose was introduced from the side arm, Experiment A, heart muscle homogenate was the only one which showed a significant capacity to attack glucose. In the case of skeletal muscle, addition of glucose produced some suppression of endogenous CO₂ release. In all cases, a fairly high level of endogenous CO₂ release was observed. This was found to be due largely to the presence of hexose di-phosphate (HDP) in the reaction mixture as shown in the next experiment. In Experiment B, neither HDP nor glucose was included in the suspending medium. HDP was introduced into the flask from the side arm after equilibration. The results show that in some cases all, and in other cases most, of the endogenous activity noted in Experiment A could be ascribed to HDP in the reaction system.

TABLE III - Glycolytic activity of fish tissue homogenates. Effect of glucose and hexose di-phosphate on CO₂ production.

Tissue	Experiment A		Experiment B	
	No glucose	+ glucose	No HDP	+ HDP
	Q* CO ₂ values			
Skeletal muscle	53.7	17.1	12	78
Lateral line muscle	127	130	0	125
Heart muscle	46	341	13	107
Liver	65	64	31	23
Kidney	78	81	7	64
Brain	131	144	51	221

* μ l CO₂ released per mg. N per hour. In Expt. A, HDP (hexose di-phosphate) was present in the main compartment and glucose was introduced from the side arm during equilibration. Fish #1 was used as a source of all tissue homogenates in Expt. A except Brain which was from Fish #4. In Expt. B Fish #15 was the source of homogenates for all tissues except Heart which was from Fish #17.

Lack of capacity of a homogenate to attack glucose does not necessarily mean that glycolysis does not occur in the corresponding intact tissue. It could mean that enzymes capable of breaking down ATP and hence of interfering with glucose phosphorylation are released during the homogenizing procedure. Various efforts were made to obtain preparations capable of attacking glucose but these were not successful. No activity was shown toward fructose, galactose or ribose.

As the results in Table III show, heart muscle homogenates were very active in breaking down glucose to lactic acid. Q_{CO₂} values, particularly with fish used early in the experiment were very high. A tendency for Q_{CO₂} values to drop off toward the middle and end of the experiment could possibly be due to depletion of a co-factor necessary in glycolysis.

Much data on blood composition changes, proximate analyses of tissues and lactic acid production during glycolysis have yet to be completed. When all the information is available, it should be possible to understand better at least some of the phenomena associated with salmon migration.

The authors wish to express their appreciation to the following persons: to Dr. Clemens for his advice, encouragement and assistance in arranging for live fish to be obtained and for a place to keep them; to Mr. Murray Newman and his staff at the Vancouver Aquarium for much kind advice and assistance in caring for the fish; to Mr. Rod. Hourston, Chief Biologist, Department of Fisheries, for arranging for us to obtain migrating coho salmon, and to Mr. Royal and the staff of the Salmon Commission for making it possible to obtain sockeye salmon. We also wish to acknowledge the advice and encouragement of Dr. Hoare with whom we had many helpful discussions.

SUMMARY NO. 10

PREDICTING THE COLOUR OF CANNED SOCKEYE SALMON FROM THE COLOUR OF THE RAW FLESH

P.J. Schmidt
D.R. Idler
I.I. Bitners

This project was described in last year's Annual Report (Summary No. 33 of this Station's Annual Report for 1956-57). At that time the colour measurements of the raw sockeye samples from 416 individual fish and 259 canned samples had been completed. Only preliminary observations and calculations were presented. Since then, colour measurements and oil content determinations have been made on the remaining canned samples (over 200) and all the data subjected to statistical analysis. Since the nature of the problem and the procedures used were described in last year's report, they will not be discussed here.

Comparison of 3 colour-measuring instruments.

Seven samples of both the raw and processed fish were subjected to colour measurement using the Beckman DU spectrophotometer, the Lovibond-Schofield Tintometer and the Gardner Color-Difference Meter. These measurements were converted to I.C.I. dominant wave-length and % excitation purity and are recorded in Table I. The 3 instruments did not give identical results. Such discrepancies have been observed by other investigators working with different products. Agreement between the spectrophotometer and Color-Difference Meter was better than between the spectrophotometer and Lovibond-Schofield Tintometer. Because of the long period of time required for the spectrophotometric measurements, considerable darkening of the sample surface occurred before the measurements were completed and may account for some of the discrepancies. Agreement between instruments was better for processed samples than for raw samples. This is not surprising because the reproducibility of measurements with a given instrument was found to be much better for the processed samples, and may be attributed to the fact that the raw samples were not so uniform in colour, had an irregular surface texture and a slight translucency. The Color-Difference Meter has several advantages over the other two instruments for these measurements. Because of the nature of the raw samples, more accurate results can be obtained with an instrument viewing a larger sample area as does the Color-Difference Meter. In addition, this instrument is much more convenient and more rapid than either of the other two.

Colour measurements of raw and processed sockeye salmon.

Typical reflectance spectra of a sample before and after processing are shown in Figure 1. Processing increased the brightness throughout the entire visible region, particularly above 560 mμ.

TABLE I - Comparison of instruments for colour measurement.

<u>Raw Sockeye Salmon</u>									
Spectrophotometer				Colour Difference Meter			Lovibond Schofield		
Sample No.	Y,%	Dominant wave-length mμ	Purity %	Y,%	Dominant wave-length mμ	Purity %	Y,%	Dominant wave-length mμ	Purity %
1	8.39	603.6	56.0	9.0	602.3	71.2	11.5	604.7	70.5
2	9.97	598.2	58.3	12.2	598.9	66.5	11.8	603.0	66.0
3	10.6	599.0	57.5	11.5	600.0	69.7	14.5	599.7	70.5
4	10.0	598.7	57.5	11.5	600.1	68.0	12.3	601.7	70.4
5	12.1	595.6	54.0	14.0	596.8	64.2	16.6	599.4	59.1
6	9.96	600.0	58.0	12.1	600.1	68.0	12.9	600.5	73.2
7	10.7	600.0	63.3	13.2	600.2	68.4	14.5	602.0	67.4
<u>Processed Sockeye Salmon</u>									
1	30.3	590.0	49.4	34.9	588.9	50.3	48.0	590.1	48.3
2	35.9	588.6	43.6	38.6	587.4	43.6	41.7	589.7	44.3
3	35.2	589.8	48.3	39.6	589.1	49.3	40.7	590.4	47.3
4	33.6	589.3	50.0	37.8	589.0	48.1	40.7	590.8	46.2
5	36.2	587.6	43.0	40.8	586.3	41.5	43.7	589.2	38.7
6	32.2	589.5	50.2	36.5	588.5	47.9	33.1	590.0	45.6
7	30.6	590.0	48.0	33.9	488.8	48.2	33.9	591.2	44.6

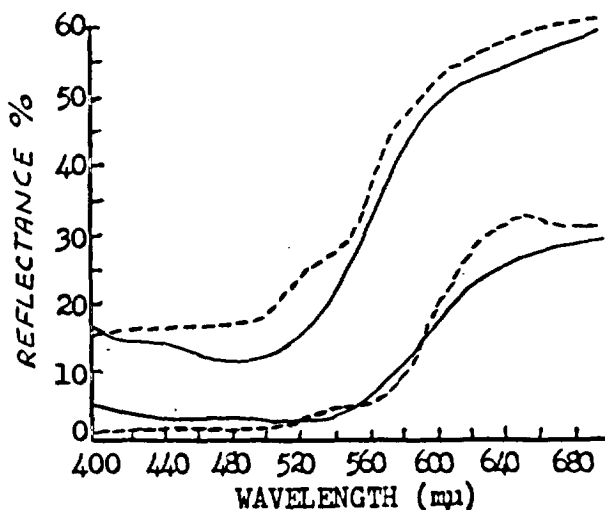


Fig. 1. Reflectance curves for (1) reference tile for canned salmon measurements; (2) canned salmon; (3) reference tile for raw salmon measurements; (4) raw salmon.

The Rd, a and b (Gardner Color-Difference Meter measurements) readings for 60 samples were selected to contain approximately the whole range of colours, were converted to I.C.I. trichromatic values and then to dominant wavelength and % excitation purity. Extensive changes occurred in each of the 3 colour dimensions during the cooking process (Table II). The samples became much lighter while the hue was shifted from red to orange-red and orange and the purity decreased.

TABLE II - Range of colours from converted Colour Difference Meter measurements of 60 samples of sockeye salmon before and after processing.

Colour attribute	Range	
	Raw	Processed
Lightness, %	9.00 - 21.1	28.6 - 52.5
Dominant Wavelength, mμ	591.0 - 602.3	582.3 - 592.2
Purity, %	41.7 - 73.3	29.1 - 60.0

Colour measurements of canned salmon.

The a readings ranged from 7.1 to 29.8 and had an average value of 19.9 while the b readings varied from 19.5 to 26.9. There was excellent correlation between the a/b ratio and the dominant wavelength. However, there was very good agreement between the dominant wavelength and the a value alone, the linear correlation coefficient being 0.991 for the 60 samples previously mentioned. In addition, the a value was found to correlate well with purity and lightness, the correlation coefficients being 0.943 and -0.834 respectively. This meant that with an increase in a value, there was an increase in dominant wavelength, purity and darkness. During the course of these measurements it became evident that the a readings were a good measure of visual colour preference. A panel of qualified representatives from the salmon canning industry and from the Canadian Government Canned Fish Inspection Laboratory were consulted to make visual colour judgments of approximately 100 samples of canned sockeye salmon. Their evaluations showed that the higher the a value, the more preferable was the colour of the product. In collaboration with this panel it was concluded that there should be 3 commercial colour grades of sockeye salmon with specifications defined in terms of a values as follows: grade 1, the best grade, should have a values greater than 20; grade 2 should have a values not greater than 20 and not less than 14.5, while grade 3 should have a values less than 14.5. On the basis of these specifications, Table III lists the % of fish that were grade 1, 2 and 3 when canned from each of the 8 areas where the salmon were caught. These data show that in general the sockeye salmon from the more southerly areas had a better colour. Only the Rivers Inlet area contributed any appreciable number of grade 3 canned salmon. Particular difficulty in colour grading sockeye salmon from this area had been experienced by the industry.

TABLE III - Percentage of sockeye salmon from different areas in each colour grade.

Area of capture	No. of fish	% of fish in each grade when canned.		
		Grade 1	Grade 2	Grade 3
West coast of				
Vancouver Island	40	70.0	30.0	0
Fraser River	79	73.4	25.3	1.3
Rivers Inlet	95	22.1	53.7	24.2
Juan de Fuca	78	44.9	55.1	0
Johnstone Strait	57	61.4	36.9	1.7
Bella Coola	37	54.1	43.2	2.7
Pender Harbour	10	80.0	20.0	0
Fitzhugh Sound	20	35.0	65.0	0
All areas	416	51.0	42.8	6.2

Prediction of a value of canned salmon.

The problem of grading was thus reduced to predicting the a value of the canned product from the colour of the raw fish. The a readings of the raw samples had an average value of 34.7 and ranged from 16.8 to 43.3, while the b readings ranged from 14.9 to 25.8 with most of the readings falling between 18 and 23. As with the canned samples, the a/b ratio was found to be directly proportional to the dominant wavelength. Linear correlation coefficients between the a values of the canned samples and the a, a/b and Rd values of the raw samples were as follows: $\underline{a} = 0.637$; $\underline{a/b} = 0.787$; $\underline{Rd} = -0.170$. Regression equations for predicting the a value of the canned salmon from either the a or a/b value of the raw fish were as follows: $\underline{a_c} = 0.550 \underline{a_r} + 0.786$ and $\underline{a_c} = 18.479 (\underline{a/b})_r - 10.801$, where the sub-letters c and r refer to canned and raw sample readings respectively. The standard errors of estimate were 2.72 a units for the first equation and 2.18 for the second. From the visual colour evaluations made by the panel members mentioned earlier, the permissible grade tolerances for canned salmon were estimated to be ± 1 a unit. This meant that a values as low as 19 for grade 1 and as high as 15.5 for grade 3 were permissible, while the a values for grade 2 could extend from 13.5 to 21.0. On the basis of the above specifications, the a value separating grade 1 and 2 canned salmon was 20, while that separating grade 2 and 3 was 14.5. From the second regression equation, the a/b ratios for the raw fish corresponding to these a values were calculated to be 1.67 and 1.37, as shown graphically in Fig. 2. The grade specifications in terms of a/b ratios for raw fish can be stated thus: grade 1 - a/b value must exceed 1.67; grade 2 - a/b value must exceed 1.37 but must be less than 1.67; grade 3 - a/b value must be less than 1.37. When the 416 samples were graded on their a/b ratios it was found that 37 samples or 8.9% fell in the incorrect grade on the basis of the a value specifications and the limits outlined earlier for the canned fish. Of the incorrectly graded samples 15 of them with an average a value of 17.5 were placed in grade 1 instead of grade 2, 18 samples with an average a value of 21.9 were placed in grade 2 instead of grade 1, 4 samples with an average a value of 12.9 were in grade 2 instead of grade 3, and only 1 sample was incorrectly placed in grade 3. The samples that were incorrectly graded were not from any particular area nor of any particular period of the season. The average a value of the canned samples placed in each of the 3 grades was as follows: grade 1 - 21.0; grade 2 - 19.0; grade 3 - 11.2. The % of fish in grades 1, 2 and 3 now was 56.9, 39.7 and 3.4 respectively. When a similar grading procedure was applied using the a values of the raw fish rather than the a/b ratios, 12.5% of the samples were incorrectly graded. This method failed particularly in correctly placing the grade 3 samples. These results indicated that the colour grade of canned sockeye salmon could best be predicted from the a/b ratio of the raw fish and that the prediction within specified limits was 91% accurate.

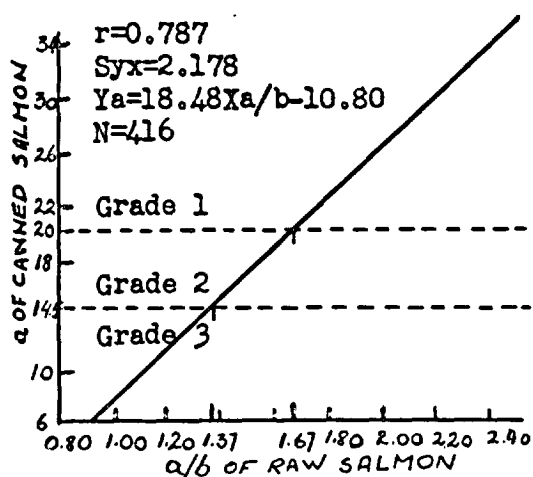


Fig. 2. Regression line for estimating a readings of canned sockeye salmon.

If visual grading, based on these specifications, were desirable, at least 2 material reference standards would be required, one having an a/b ratio of 1.67 and the other having a ratio of 1.37. The a values of 20.0 and 14.5 for canned fish were applied to the first regression equation to give the best a values for such reference standards, namely 34.9 and 24.9. To retain the correct a/b ratios, the b values were calculated to be 20.9 and 18.2 respectively. The average R_d values for 76 raw samples whose a/b ratios varied from 1.65 to 1.69 was 12.9 while that for 23 samples with a/b ratios ranging from 1.30 to 1.45 was 14.1. Thus the complete specifications for such reference standards are: (a) $R_d = 12.9$, $a = 34.9$, $b = 20.9$; (b) $R_d = 14.1$, $a = 24.9$, $b = 18.2$. Visual grading, utilizing standards with such specifications, is currently under investigation and is described in Summary No. 12 of this report.

Relationship of oil content to colour.

The oil content of each of the 416 cans of processed sockeye salmon was determined. The data in Table IV show that in general the better colour grades of sockeye salmon had a slightly higher oil content. However, on an area basis this was not always true. For example, Juan de Fuca samples had a higher average oil content (14.9%), but a lower average a value than the samples from 4 other areas. For a given area the grade 1 samples had a higher average oil content than the grade 2 samples, except for the Rivers Inlet and Pender Harbour areas.

TABLE IV - Average a readings and average oil contents of sockeye salmon of 3 different colour grades from 8 different areas.

Area	No. of fish	Average oil content			Average a reading	Average oil content
		Grade 1	Grade 2	Grade 3		
West Coast of Vancouver Is.	40	10.5	9.39	None	21.1	10.1
Fraser River	79	12.3	11.4	9.39	21.4	12.0
Rivers Inlet	95	9.58	9.67	9.48	17.0	9.58
Juan de Fuca	78	15.5	14.4	None	20.4	14.9
Johnstone Strait	57	12.4	12.3	11.5	21.2	12.4
Bella Coola	37	9.86	8.97	2.19	19.2	9.28
Pender Harbour	10	9.39	10.8	None	21.5	9.67
Fitzhugh Sound	20	9.00	7.18	None	19.6	7.82
All areas	416	11.9	11.1	9.28	19.9	11.3

Effect of increased temperature and time of processing on colour.

Samples from 10 sockeye salmon with an average a/b ratio of 1.74 when raw, were processed in the usual manner at 116°C. for 20 minutes after which the average a value of the canned samples was 22.6. From the same fish another 10 samples with an a/b ratio of 1.76 were processed at 121°C. for 90 minutes, resulting in an average a value of 21.1 for the canned products. A third group of 10 samples from the same fish with an average a/b ratio of 1.79 was processed at 116°C. for 2 hours, the resulting canned samples having an average a value of 21.0. These data show that a 5°C. increase in the temperature of processing or a 30-minute increase in the cooking time resulted in an increased colour loss.

Effect of post-mortem storage conditions on colour.

Samples were taken from fresh sockeye salmon for immediate colour measurements while the remainder of the fish was stored in either crushed ice or refrigerated sea water at -1.1°C. for colour measurement later. Another batch of fish was divided crosswise into 2 equal parts to permit simultaneous storage in ice and refrigerated

sea water. The results, shown in Table V, indicate that the colour of canned salmon was not adversely affected by the post-mortem storage of the raw fish in either ice or refrigerated sea water.

TABLE V - The effect of post-mortem storage in ice and refrigerated sea water on the colour of canned salmon.

<u>Post-mortem</u> storage	No. of fish	Average <u>a/b</u> ratio of raw samples	Average <u>a</u> value of canned samples
None	10	1.66	21.5
10 days in ice	10	1.74	21.4
None	4	1.67	20.8
12 days in seawater at -1.1°C.	4	1.73	21.0
6 days in ice	11	1.69	20.4
6 days in seawater at -1.1°C.	11	1.72	19.1

The only phase of this project that is still being investigated is the development of material standards for visual grading. Colour grading by means of an automatic photoelectric method measuring a/b ratios of the raw fish would of course be more satisfactory. However, it is felt that the commercial application of such a method should be left to the industry themselves.

SUMMARY NO. 11

PREDICTING THE COLOUR OF CANNED SALMON FLESH FROM THE OIL
CONTENT AND THE COLOUR OF THE OIL FROM THE RAW FLESH

P.J. Schmidt
D.R. Idler

During the course of the investigation of the fading of salmon on canning (see Summary No. 10 of this report), transmission measurements were made on oils extracted from both the raw samples and their corresponding canned samples from 30 different sockeye salmon. This was done to enable more precise assessment of the colour changes that occur on canning.

Extraction of oils.

Two adjacent steaks were cut from each fish. One steak was canned, using normal procedure. Oil samples were then extracted from each of the steaks. To 40 grams of skin and bone free flesh was added 100 ml. of acetone and a little BHA (an antioxidant) in a Waring Blendor jar. Nitrogen was blown into the solvent for a few minutes, the jar closed and the mixture then homogenized for 30 seconds. The solution was then filtered on a Buchner funnel and the residue replaced in the Blendor jar with 100 ml. of methylene chloride, mixed again for 30 seconds, and filtered on the Buchner funnel. The residue on the funnel was washed with 100 ml. of methylene chloride. The filtrates were combined and then cooled in a dry ice-acetone bath. The water extracted from the flesh and present in the filtrate was frozen in this way and the ice was filtered off on a Buchner funnel. The solvent was then evaporated in vacuo at a temperature of 30°C., using a "flash evaporator" with a dry-ice acetone bath for condensing purposes. The final traces of solvent were removed using a high vacuum pump, in place of the water suction pump.

Transmission measurements.

Transmission readings using a Beckman DU spectrophotometer were taken at 10 mμ intervals over the visual region (400-700 mμ). The normal cuvettes with a 1 cm. light path could not be used because of the low transmission values of the oils. Cuvettes with an 0.5 mm. light path were used. I.C.I. co-ordinates were calculated using the summation method of Judd. These measurements would have been more accurate if cuvettes with an even smaller light path had been available. The colour of the oils was so intense that the transmission readings were negligible in the region from 450 to 550 mμ for most samples. In addition, to obtain good agreement between oils from duplicate samples of flesh, great care must be exercised during the extraction to prevent destruction of the pigments. Also, the removal of the last traces of solvent from the oil must be ensured.

Results.

Of the I.C.I. coordinates, the x value was considered to be most significant, as it is a measure of redness. The correlation between x value of the oil from the raw fish (x_r) and the x value of the oil from the canned fish (x_c) was not as good as might be expected, the correlation coefficient being 0.687. The regression equation for predicting x_c from x_r was calculated to be as follows:

$$x_c = 0.99 x_r - 0.067$$

For the 30 fish involved, x_c varied from 0.41 to 0.58, while x_r varied from 0.54 to 0.63.

Calculations were made relating the x value of the oil from the raw fish and the oil content of the fish with the a value of canned flesh (a_c), which is a measure of the colour quality of canned fish (see Summary No. 10 of this report). The final colour of the canned product should be proportional to the amount of oil present and to the redness of that oil. In other words, it should be possible to predict a_c from x_r and oil content. The average values a_c , x_r and oil content for the 30 fish were $a_c = 20.8$, $x_r = .594$ and oil content = 13.4. From the data for the 30 samples, the contribution to the a value of the canned product per unit of oil content and per unit of x_r were calculated and the following formula derived:

$$a_c = \% \text{ oil } (0.85) + 63.0 (x_r) - 28.0$$

The standard error of estimate for this equation is 1.80 a units, which is actually better than the 2.18 standard error of estimate for the equation for predicting a_c from a/b values of the raw flesh. However, the latter equation was derived from data involving 416 individual fish while the above equation is based on data for only 30 fish.

The following table gives data for 10 different fish, selected to indicate the type and range of values:

Predicted and actual a values of canned sockeye salmon.				
Sample	$x_r(1)$	% oil	Predicted $a_c(2)$	Actual a_c
1	0.62	13.2	22.3	21.7
2	0.54	17.3	20.7	19.6
3	0.60	13.6	21.4	23.3
4	0.60	15.8	23.2	24.6
5	0.60	8.1	16.7	14.4
6	0.63	10.9	21.0	21.9
7	0.58	14.5	20.9	21.6
8	0.60	9.2	17.6	16.7
9	0.56	16.4	21.2	19.0
10	0.62	10.7	20.2	19.7

(1) x_r = I.C.I. x value for oil from raw flesh.
 (2) a_c = a value of canned flesh as determined by a Gardner Colour-Difference Meter.

SUMMARY NO. 12

MATERIAL STANDARDS FOR VISUAL COLOUR GRADING OF SOCKEYE SALMON

P.J. Schmidt

On completion of the work described in Summary No. 10 of this report, this project was initiated to develop visual standards which could be used commercially to colour grade raw sockeye salmon prior to canning. The previous work showed that the specifications for two such standards for separating sockeye salmon into 3 grades were as follows:

(1)	Rd = 12.9	a = 34.9	b = 20.9
(2)	Rd = 14.1	a = 24.9	b = 18.0

Steel plates painted with automobile paint and baked were prepared with these specifications. However, this was found to be unsuitable. Because the plates lacked the translucency and the non-uniform surface of salmon flesh, satisfactory colour comparisons by eye could not be made. An attempt was then made to produce polyester resin plates. White, red and yellow pigments were mixed with the clear resin in various proportions. However, great difficulty was experienced in attaining the correct colour specifications and at the same time produce plates with translucency similar to that of raw sockeye flesh. To duplicate the surface texture of the flesh, different moulds were cast from the cut surface of a salmon steak. Plaster of paris and lead were found to be unsuitable, while Wood's metal proved to be of some value for this purpose. Occasionally the Wood's metal mould was destroyed by the heat generated during the setting of the resin. Although several hundred replicas have been made, none have been entirely satisfactory. However, it is felt that suitable plates can be made by further experimentation. This will be attempted shortly so that a visual colour grading system can be tested during the forthcoming salmon season.

SUMMARY NO. 13

MARINE STEROLS: 24-DEHYDROCHOLESTEROL FROM BARNACLES

U.H.M. Fagerlund
D.R. Idler

An investigation of the sterols from a barnacle (Balanus glandula) was started last year (Summary No. 31 of this Station's Annual Report for 1956-57). It has now

been possible to separate chromatographically the azoyl ester mixture prepared from the crude sterols into three zones. The sterol of the fastest moving zone (Zone 3) was identified as cholesterol and accounted for 59.8% of the total sterols. The middle zone, which represented 6% of the total sterols, has not yet been characterized. The slowest moving zone (Zone 1, 34.2%) when hydrolyzed was found to be contaminated with a small amount of a $\Delta^{5,7}$ -sterol, which was removed with maleic anhydride. The purified sterol and its derivatives had properties different from those of any known sterol. Hydrogenation of the sterol with Adams' catalyst in glacial acetic acid produced cholestanol with the uptake of 2 moles of hydrogen and demonstrated that the original sterol was a readily reducible cholestadienol. The modified Liebermann-Burchard reaction showed that the cholestadienol had a double bond in the 5 position and that the second double bond made no significant contribution to the reaction, thus eliminating most nuclear double bonds from consideration. Ozonolysis resulted in the isolation of acetone as its 2,4-dinitrophenyl hydrazone in good (51%) yield and completed the identification of the sterol as 24-dehydrocholesterol. The synthesis of this sterol via the Wittig reaction from 3β -acetoxy-5-choleinaldehyde and triphenylphosphine isopropylidene, reported in Summary No. 32 of this Station's Annual Report for 1956-57, has been repeated with a larger amount of material, and the properties of the synthetic sterol and its derivatives after extensive purification were identical with those of the sterol from barnacle.

With respect to the comparative biochemistry of marine invertebrates, the question of the significance of 24-dehydrocholesterol in barnacle must await a comprehensive study of the sterols of other crustaceans. However, by analogy with Mollusca the results of this report already appear of interest. In Mollusca, as we ascend the evolutionary scale, we find a high percentage of Δ^7 -sterols in chiton, large quantities of 24-methylencholesterol in Pelecypoda, and almost entirely cholesterol in Gastropoda and Cephalopoda. Descending the evolutionary scale in Crustacea we find that cholesterol is the principal if not the only sterol (other than small amounts of provitamin D) in Malacostraca (crabs, shrimps, etc.); Cirripedia (barnacle) has been shown here to contain cholesterol as a major component but also 24-dehydrocholesterol in large amounts. It remains to see whether cholesterol either continues to decrease or is absent in Ostracoda, Copepoda and Branchiopoda. The day may not be too far distant when the sterols of a marine invertebrate will be predictable from its position in the evolutionary scale. From the biochemical viewpoint there is already evidence that unsaturation of the cholesterol sidechain may be an important factor in cholesterol metabolism. Further studies on lower forms of marine life may well provide a means of isolating possible intermediates.

A sterol, "desmosterol", isolated as a minor component (2%) of the sterols of chick embryo, has been reported to be 24-dehydrocholesterol. A sterol claimed to have properties identical with those of the sterol described in this summary, has also, coincidental with our report at the ACS meeting in 1957, been reported as a minor component of rat skin and stated to be 24-dehydrocholesterol. These reports can not be evaluated until the data have been published.

We recently reported the synthesis of 25-dehydrocholesterol and showed that it is not identical with two products prepared by dehydrohalogenation and assigned this structure. We had available for comparison an acetate of one of the earlier preparations which showed no apparent evidence of deterioration. Part of the infrared spectrum of this compound was published by us and it showed no significant absorption at 890 cm^{-1} characteristic of a terminal methylene group. The only conclusion that could be reached at that time was that the earlier preparations were probably 24-dehydrocholesterol. However, the properties of the synthetic product reported here show that this is not the case. One of the earlier investigators (Dr. W.G. Dauben) has synthesized a new batch of the sterol in question and kindly supplied us with a sample so that we might resolve the problem. In a personal communication, Dr. Dauben reached the conclusion that his earlier preparation must have

been a mixture of 25-dehydrocholesterol and 24-dehydrocholesterol. The infrared spectrum of the new preparation has significant absorption at 890 cm^{-1} . We have recorded spectra of synthetic 24-dehydrocholesterol, 25-dehydrocholesterol prepared via the Wittig reaction and the new product prepared by dehydrohalogenation and found the latter to be a mixture composed of 40% 25-dehydrocholesterol and 60% 24-dehydrocholesterol. It must be concluded that both the earlier preparations were originally predominately 25-dehydrocholesterol and must have undergone extensive changes on storage. However, it is also probable that the composition of such a sterol mixture would vary with the reaction conditions and the number of recrystallizations.

In a preliminary experiment to obtain the sterols from barnacles, 22.5 lb. of ground-up, fresh barnacles were extracted by shaking with three batches of acetone. The first extract was heated to boiling before shaking. The mass was finally washed with ether and the combined extracts were evaporated to dryness. Moisture (38.1%) was determined on an aliquot of wet barnacles, and the oil content (0.83%) was determined on a sample of the dried material. The nonsaponifiable portion (4.28 g.) was chromatographed on a silicic-acid: Celite column, 7.5 cm. x 10 cm., and the sterols (1.77 g.) eluted with methylene chloride, after washing the chromatogram thoroughly with Skelly Solve C and Skelly Solve C-benzene 1:1. To obtain larger quantities of sterol, 233 lb. of fresh barnacles were extracted three times with hot acetone in a pilot plant. The oil (300 g.) gave 93 g. of nonsaponifiables and 12.6 g. of crude sterols when chromatographed as desired.

The azoyl esters of the crude sterol mixture were chromatographed on silicic-acid: Celite, using 5.5:1 Skelly Solve C-benzene developer as previously described. Three zones developed, all well separated from each other.

Zone 1 azoyl ester was crystallized from benzene-ethanol and hydrolyzed. Calculated from the ultraviolet spectrum the sterol contained 0.85% of provitamin D. The acetate was treated with maleic anhydride in the usual manner to remove provitamin D. The residue from the maleic anhydride treatment was acetylated and the acetate, crystallized from aq. ethanol, melted at 101.5° , $[\alpha]_D^{24} - 39.2$.

Anal. Calcd. for $\text{C}_{29}\text{H}_{46}\text{O}_2$: C, 81.63; H, 10.87. Found: C, 81.59; H, 10.75.

The acetate was hydrolyzed and the sterol crystallized from aq. methanol, m.p. 117° , $[\alpha]_D^{22} - 38.7^\circ$.

Anal. Calcd. for $\text{C}_{27}\text{H}_{44}\text{O}$: C, 84.31; H, 11.53. Found: C, 84.35; H, 11.44.

The benzoate was crystallized from acetone, m.p. 129° , $[\alpha]_D^{22} - 15.4^\circ$.

Anal. Calcd. for $\text{C}_{34}\text{H}_{48}\text{O}_2$: C, 83.55; H, 9.90. Found: C, 83.86; H, 9.58.

Molecular rotational differences: 24-Dehydrocholesterol: $\Delta^{\text{Ac-18}}$; $\Delta^{\text{Bz}} + 74$; Δ^5 -sterols, $\Delta^{\text{Ac-35}} \pm 16$; $\Delta^{\text{Bz}} + 16$.

The acetate (16.3 mg.) was reduced in glacial acetic acid with platinum oxide catalyst. The hydrogen uptake was 1.78 ml. The theoretical amount for two double bonds is 1.71 ml.

The reduced acetate was crystallized from aq. methanol, m.p. 111° , $[\alpha]_D^{22} + 13.6^\circ$, no m.p. depression with cholestanyl acetate, m.p. 111° .

Anal. Calcd. for $\text{C}_{29}\text{H}_{50}\text{O}_2$: C, 80.87; H, 11.70. Found: 80.91; H, 11.79.

The sterol was crystallized from aq. methanol, m.p. 141° , $[\alpha]_D^{22} + 23.6^\circ$, no m.p. depression with cholestanol, m.p. 142° .

Anal. Calcd. for $C_{27}H_{48}O$: C, 83.43; H, 12.45. Found: C, 83.35; H, 12.57.

Ozonolysis. Zone 1 sterol (530 mg.) was suspended in 10 ml. acetone-free acetic anhydride - acetic acid (4:1) and cooled in an icebath. Ozone was passed through the solution until all of the solid had disappeared. Water and zinc dust were then added and the mixture was heated in a waterbath in order to decompose the ozonide. The volatile fraction was steam distilled into a water trap and the contents of this trap in turn steam distilled into a 2,4-dinitrophenylhydrazine solution. A 2,4-dinitrophenylhydrazone precipitated immediately. The crude product (175 mg.) was recrystallized twice from aqueous ethanol and melted at 128° , mixed m.p. 128° with the 2,4-dinitrophenylhydrazone of acetone (m.p. 128°).

Anal. Calcd. for $C_9H_{10}N_4O_4$: C, 45.38; H, 4.23. Found: C, 45.34; H, 4.38.

The 2,4-dinitrophenylhydrazone derivatives of the ozonolysis product and that of acetone were chromatographed in isopropanol-water (1:1) on Whatman No. 1 filter paper treated with 5% olive oil in chloroform. The descending chromatograms were run for $2\frac{1}{2}$ days. The derivatives were also run both ascending and descending in methanol-isooctane (1:1) on untreated paper. The two 2,4-dinitrophenylhydrazones had identical R.f. values differing from those of formaldehyde, acetaldehyde and isobutyraldehyde.

Zone 3 azoyl ester was crystallized from benzene-ethanol and hydrolyzed to the sterol which was crystallized twice from aq. methanol, m.p. 148° , $[\alpha]_D^{22} - 40.2^{\circ}$, mixed m.p. 148° with cholesterol.

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found: C, 83.82; H, 11.92.

The acetate crystallized from aq. ethanol, m.p. 116° , $[\alpha]_D^{22} - 47.0^{\circ}$, mixed m.p. 116° with cholesteryl acetate.

Anal. Calcd. for $C_{29}H_{48}O_2$: C, 81.25; H, 11.29. Found: C, 81.34; H, 11.23.

The benzoate was crystallized from acetone, m.p. 145° , $[\alpha]_D^{22} - 14.2^{\circ}$, mixed m.p. 145° with cholesteryl benzoate.

The infrared spectrum and the modified Liebermann-Burchard reaction were identical to those of authentic cholesterol.

SUMMARY NO. 14

MARINE STEROLS: THE STEROLS OF STARFISH

U.H.M. Fagerlund
D.R. Idler

In a typical extraction of the sterols from starfish, 25 lb. of starfish were cut up so that the internal organs could be removed from the shells. Moisture (79.2%) was determined on an aliquot of the wet organs which weighed 5 lb. These were extracted four times with hot acetone. The oil weighed 51.2 g. (2.26% of the wet organs). Phospholipids weighed 16.1 g., and the nonsaponifiable portion 11.7 g. (22.8% of the oil). Chromatography on alumina gave 3.8 g. of sterols (32.5% of the nonsaponifiable). The calciferous shells (20 lb.) were finally chopped in a suitable cutting machine and the oil extracted with acetone as above. The yield of sterol was 500 mg.

The azoyl esters, when chromatographed on silicic acid: Celite (1:1 by volume) separated into two major zones. Zone 1 (ca. 25%) and Zone 2 (ca. 70%) were both fast acting in the Liebermann-Burchard reaction. A minor zone preceded Zone 2 but it has

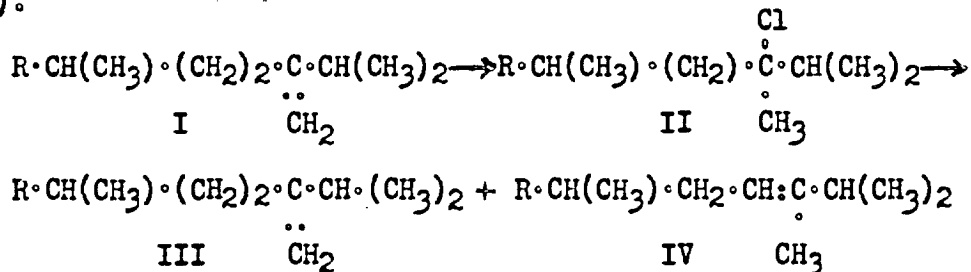
not been investigated. A second minor sterol ran slower than Zone 1 and judging from its chromatographic behaviour it is probably a polyfunctional alcohol or ketone.

The sterol from Zone 1 has one of its double-bonds in the 7(8) position as evidenced by the Liebermann-Burchard reaction. The infrared spectrum has prominent absorption bands at 1637 cm^{-1} and 885 cm^{-1} associated with a terminal methylene group. When hydrogenated with PtO_2 catalyst in acetic acid it takes up only one mole of H_2 which is consistent since a 7(8) double-bond resists hydrogenation under these conditions.

The acetate of Zone 1 was treated with HCl in chloroform. Dehydrohalogenation was then accomplished by refluxing in acetic anhydride. The product was ozonized in a mixture of acetic anhydride and acetic acid. Water was added and the mixture steam distilled. Initially a trap containing a solution of 2,4-dinitrophenyl hydrazine (DNPH) was used, which after a few minutes was replaced with a dimedone solution. The DNPH precipitate was chromatographed on alumina and separated into two zones which on paper chromatograms developed into three spots which run with the same R.f. values as formaldehyde, acetone (Zone 1) and methylisopropyl ketone (Zone 2). The dimedone derivative was identified as that of formaldehyde.

The remaining dimedone solution was steam distilled into a 2,4-DNPH solution. The precipitate was identified by paper chromatography as the DNPH's of methylisopropyl ketone and acetone.

The evidence points to the presence of a sidechain configuration as in I. During the HCl -treatment Cl adds to C_{24} (II). The subsequent dehydrohalogenation proceeds predominately in two ways to produce III (45%) and IV (45%) which on ozonolysis give the volatile compounds that were found. The small amount of acetone presumably is formed by dehydrohalogenation also producing a small amount of the 24-dehydro isomer (10%).



SUMMARY NO. 15

MARINE STEROLS: EVIDENCE FOR THE PRESENCE OF A
 Δ^{22} -CHOLESTENOL IN MUSSELS

U.H.M. Fagerlund
D.R. Idler

The presence of $\Delta^{5,7,22}$ -cholestatrienol in a mussel Mytilus edulis has been reported by J. Van der Vliet. During an investigation of the mussel Modiolus demissus we have found evidence for the presence of a Δ^{22} -cholestenol, which is not a provitamin D.

A crude mussel sterol preparation was obtained from E.I. duPont de Nemours & Co. Provitamin D was removed with maleic anhydride in the usual manner. The sterol mixture was then crystallized once from aqueous ethanol and ozonized. The ozonides were decomposed with Zn and the volatile decomposition products steam distilled into a 2,4-dinitrophenyl hydrazone (DNPH) solution. The hydrazones were chromatographed on a silicic-acid:Celite column with a petroleum-ether:benzene mixture (1.5:1). Three zones developed. The middle zone, which accounted for 20-30% of the mixture was crystallized twice from aqueous methanol, m.p. 118°C ., mixed m.p. 120° with

isovaleraldehyde DNPH (m.p. 120°), mixed m.p. 90° with methyl isopropyl ketone DNPH (m.p. 122°).

The middle zone was chromatographed on paper as described above (see Summary No. 13 of this report). It moved with the same R.f. value as isovaleraldehyde DNPH.

Anal. Calcd. for $C_{11}H_{14}N_4O_4$: C, 49.6; H, 5.3. Found: C, 49.7; H, 5.3.

SUMMARY NO. 16

MARINE STEROLS: CHOLESTEROL CONVERSION BY STARFISH IN VITRO

U.H.M. Fagerlund
D.R. Idler

This project was started in order to determine to what degree the nature of the sterols of marine invertebrates are related to the type of sterols supplied with the food.

In each experiment a mussel was injected with cholesterol 4-C¹⁴ in a few drops of salmon oil. A starfish previously fed solely on mussels was kept in a small tank of sea water. The mussel was placed in contact with the oral cavity of the starfish which in most experiments began opening the shell almost immediately. The starfish was kept alive up to 9 days after the feeding and was then cut up and extracted with acetone. The sterol azoylesters were developed on a column into three zones (see Summary No. 14 of this report). A large portion of the activity was found as unchanged cholesterol (Zone 3). The activity of Zone 1 was lower than that of Zone 2.

Some activity was also found in various fractions of the saponified oil. The results are still too preliminary to justify a detailed presentation of the data. The evidence strongly indicates that the starfish is able to convert ingested cholesterol to the sterols which it normally deposits in its tissues. In the majority of the experiments conducted thus far the starfish after nine days had still not transformed a large portion of the ingested cholesterol. In one experiment the conversion of cholesterol was excellent and the conditions used in this experiment will be individually examined in an effort to produce consistently high yields of the starfish sterols. Further studies may, apart from the original goal, reveal some of the pathways of sterol transformation in this marine invertebrate.

SUMMARY NO. 17

PHOSPHOROUS COMPOUNDS IN SALMON LIVER

H. Tsuyuki
Violet M. Chang
D.R. Idler

Previous reports (Summaries No. 3 and 30 of this Station's Annual Report for 1956-57, respectively) on the acid-soluble nucleotides of salmon liver have dealt briefly with some of the methods involved in their isolation and characterization and the nature of a new peptide containing nucleotide, 6-succinyl adenosine-5'-phosphosulfate (peptide), was described in detail. Tentative characterizations of the other nucleotide fractions in liver were also included in these previous reports.

All of the major components in each fraction have now been characterized and a more detailed picture of the acid-soluble nucleotides of salmon liver, together with the methods used for their separation and identification, is reported.

The following abbreviations are used in this report: UMP uridine-5'-phosphate, UDP uridine-5'-diphosphate, UTP uridine-5'-triphosphate, AMP adenosine-5'-phosphate,

ADP adenosine-5'-diphosphate, ATP adenosine-5'-triphosphate, IMP inosine-5'-phosphate, IDP inosine-5'-diphosphate, ITP inosine-5'-triphosphate, GMP guanosine-5'-phosphate, GDP guanosine-5'-diphosphate, GTP guanosine-5'-triphosphate, DPN diphosphopyridine nucleotide, UDPAG uridine-5'-diphosphate acetyl glucosamine, UDPGAL uridine-5'-diphosphate galactose, UDPGA uridine-5'-diphosphate glucuronic acid, UDPX uridine-5'-diphosphate derivative where X is a variable, P_t total phosphorus, P_i acid-labile phosphorus.

Preparation of acid-soluble fractions.

The livers were excised from live spring salmon at sea and immediately frozen in dry ice. The powdered tissue was homogenized in a precooled Waring blender with cold 0.6 N perchloric acid (1.51 ml. per g. of tissue) and centrifuged. The residue was extracted a second time with the same volume of cold 0.2 N perchloric acid. The supernatants were combined and neutralized with 5 N potassium hydroxide to pH 7.0. All operations were carried out at 0°C. The slightly soluble potassium perchlorate (.75 g. per 100 ml. at 0°C.) began to precipitate immediately, and after standing a few hours the supernatant was decanted, lyophilized to one-quarter its volume, and a further crop of potassium perchlorate removed to reduce the salt concentration. The sample was chromatographed on Dowex-1 anion exchange resin employing gradient elution as described by Hurlbert and co-workers. The liver nucleotides were first separated into fractions A to J with 4 N formic acid. Each of these fractions was then further purified on Dowex-1, using an ammonium formate-formic acid solvent system.

Low temperature chromatography.

When the nucleotide pattern from a column operated at room temperature according to Hurlbert and co-workers was compared to that obtained at 0°C., the differences observed warranted the use of refrigeration throughout the chromatographic procedures. A Gilson fraction collector pan was insulated to minimize heat exchange and the refrigerant was circulated through the pan and jacketed column at a rate sufficient to maintain the temperature of each at 0°C.

Analysis and rechromatography.

The nucleotide fractions were initially located by reading their optical densities at 260 mμ in a Beckman spectrophotometer. Subsequently, the nucleotides were located automatically by means of an ultraviolet recorder attached to a Gilson fraction collector. In this system a combination of filters permitted ultraviolet light in the region of 257 mμ to pass through a quartz cell through which the eluent passed. Thus only material absorbing in the 257 mμ region is recorded. When the results from several column runs obtained by the automatic ultraviolet recording device were compared to the readings from the Beckman DU, it was found that with the exception of a few very small components, all major and minor peaks were detected.

Appropriate fractions of a peak were pooled and the solvents were removed by freeze-drying and the nucleotides analyzed or again chromatographed. The ammonium formate was removed by several methods: (a) by freeze-drying for several days; (b) by ion exchange (IR-120) followed by freeze-drying, and (c) by freeze-drying followed by high-vacuum (10^{-7} mm. of Hg.) sublimation for a few hours. The samples were then dissolved in water and aliquots taken for the various analyses.

Analytical Methods

Ultraviolet light. The maximum and minimum ultraviolet absorption of the nucleotides in acid (pH 1-2) was read from a Beckman model DK spectrophotometer recording. The nucleotide concentrations in acid were calculated from the appropriate extinction values compiled by the California Foundation for Biochemical

Research. Adenine succinic acid was determined by absorption at 266 mu, using $E=16.9 \times 10^3$ at pH 1.0.

Phosphate. Inorganic phosphate was determined by the method of Lowry and Lopez. All recorded values for total and labile phosphate have been corrected for inorganic phosphorus. For labile phosphate the sample was hydrolyzed directly in 18 x 150 mm. calibrated colorimeter tubes, containing 4.0 ml. of 1 N sulphuric acid, for 15 minutes in a boiling water bath. The colour was developed by the Fiske-Subbarow method and read after 15 minutes in an Evelyn colorimeter with a 660 mu. filter. For total phosphorus, the samples were brought to dryness in colorimeter tubes and digested with 1.0 ml. of 4 N perchloric acid for at least one hour at 160°C. The tubes were then partially cooled, one drop of 30% hydrogen peroxide added and the contents digested for an additional 40 minutes. The colour was developed as for labile phosphate.

Sugars. Ribose was determined according to Meijbaum. The ribose values of pyrimidine ribosides were used qualitatively. Uronic acid was determined by the Dische method, reducing sugars by the Folin and Malmros method, and N-acetyl glucosamine by the method of Reissig and co-workers. In the Reissig assay, the large difference in the extinction values enabled qualitative distinction between N-acetyl glucosamine and N-acetyl galactosamine.

Paper chromatography.

Whatman No. 1 and occasionally No. 3 papers were used in ascending development. Where it was necessary to elute the nucleotides for further analyses, washed papers were used because of ultraviolet absorbing contaminants in the paper. The papers were washed with a 1:1 mixture of ethanol and 1.2 N acetic acid, rinsed thoroughly with water, followed by ethanol according to the procedure of Kay and co-workers. The solvent systems used are indicated in the tables. The free bases and their derivatives were located by use of a mineral light lamp, the hexoses and uronic acid by aniline phthalate, hexosamines and acetyl hexosamines by the modified Elson-Morgan reagent and phosphorus by the Hanes and Isherwood method. Both the purine and pyrimidine nucleotides were hydrolysed to their free bases by heating for 2 hours at 175°C. with 90% formic acid.

Results

Fractions A to J inclusive have invariably been present in every column that has been analyzed. However, the relative amounts of each fraction showed considerable variation between individual salmon and between sexes. Each of the fractions has been analyzed and the principal components characterized.

The components of fractions A, B, C, D, H and J are summarized in Table I, together with the analytical data for their identification. Fraction A was separated into three components (A_1 , A_2 and A_3) by column chromatography using ammonium formate as the developer. When each of these was subjected to further separation on paper, A_1 proved to be a single component, DPN, showing a typical cyanide reaction. A_2 was composed of two fractions A_{2a} and A_{2b} , which were AMP and succinyl adenine respectively. These two components behave chromatographically alike in many systems but could be separated in n-butanol-acetic acid. A_3 was also separated into two components on paper. A_{3a} exhibited the ultraviolet absorption of uric acid. However, uricase did not reduce the absorption at 283 mu. and it was distinguishable from uric acid by paper chromatography using n-propanol-water (60:40). A_{3b} has not yet been identified.

Peak B proved to be a single component, GMP.

TABLE I - Analyses of fractions A, B, C, D, H and J.

Frac- tion	Compo- nents	Chromatographic properties similar to*	Spectra in acid		Per mole			Base*
			max.	min.	P _t	P _l	Ribose	
A	A ₁	DPN ^{1,7}	256	236	1.82	0	-	Adenine ^{4,6}
	A _{2a}	AMP ^{1,7}	257	230	.88	0	0.91	Adenine ^{4,6}
	A _{2b}	Succinyl adenine ^{1,7}	270	231	0	-	0	
	A _{3a} ⁷		283		0			
	A _{3b} ⁷		257					
B	B _a	GMP ^{1,7}	257	228	1.04	0	1.20	Guanine ^{4,6}
C	C ₁	Hypoxanthine ^{1,4,6}	248	215	-	-	-	Hypoxanthine ^{1,4,6}
	C ₂	UMP ^{1,7,9}	261	230	.93	0	+	Uracil ^{4,6}
	C ₃	IMP ^{1,7,9}	248	224	1.04	0	1.0	Hypoxanthine ^{1,4,6}
	C ₄							Uracil and hypoxanthine
	C ₆		256	231				Adenine ^{4,6}
D	D	ADP ^{1,3}	257	231	2.0	0.92	1.0	Adenine ^{1,4,6}
H	H	GDP ^{1,3}	257	230	2.0	1.06	-	Guanine ^{4,5}
J	J	ATP ^{1,3}	257	236	3.1	2.2	-	Adenine ^{4,6}

* Superscripts refer to solvent systems used as follows:

(1) Isobutyric acid/conc. ammonium hydroxide/water, 66/1/33; (2) ethanol/1 M ammonium acetate (pH 3.8), 7.5/3; (3) 0.1 M phosphate pH 6.8/ammonium/sulphate/n-propanol, 100/60/6; (4) aqueous ammonia, pH 10.0; (5) n-butanol/water/formic acid, 77/13/10; (6) water-saturated n-butanol/ammonia, 95/5; (7) n-butanol/acetic acid/water, 2/1/1; (8) isobutyric acid/conc. ammonium hydroxide/water, 57/4/39; (9) ethanol/1 M ammonium acetate pH 4.5, 65/35; (10) ethyl acetate/pyridine/ammonium hydroxide/water, 40/20/12/12, paper treated with boric acid; (11) n-butanol/acetic acid/water (boric acid saturated), 4/1/5; (12) n-butanol/pyridine/water, 45/25/40.

After a second column purification fraction C was separated into six components. However, during the initial stages of this investigation, when the ion-exchange procedure was carried out at room temperature, it is significant that only two components (UMP and IMP) were obtained. Presumably the less stable remaining components were destroyed during column chromatography. The first component, C₁, proved to be hypoxanthine. When the known hypoxanthine derivatives IMP, IDP and ITP were treated with 4 N formic acid for three days at room temperature (conditions considerably more drastic than the chromatographic procedures) there was no evidence of free hypoxanthine. At present, there is no explanation for the presence of hypoxanthine in this fraction except as a product of the partial decomposition of one of the C fractions C₄, C₅ or C₆. Components C₂ and C₃ were shown to be UMP and IMP respectively. C₄ consists of

the bases uracil and hypoxanthine. The identification of C5 and C6 is continuing.

Fraction D consisted solely of ADP, H of GDP and J of ATP (Table I).

Fractions F, G and I are all derived from UDP and have been identified as UDPAG, UDPGAL and UDPGA, respectively. Whereas, UDPAG has been isolated and characterized from yeast, animal liver, hen oviduct and mung bean seedlings, UDPGAL from mung bean seedlings, and UDPGA in rabbit liver and mung bean seedlings; their presence in marine forms of life has not yet been reported. The analytical data on which the identification of F, G and I is based appear in Table II. The characterization of UDPAG is based on a molar ratio of Base:P_t:P_l: reducing value: N-acetyl glucosamine of 1:2:1:1:1. N-acetyl glucosamine was identified by paper chromatography and by colorimetric assay and differs from N-acetyl galactosamine by having an extinction value about three times greater at 585 mμ.

TABLE II - Properties of UDPX compounds in salmon liver.

Frac- tion*	Chromato- graphic properties similar to**	Base**	Per mole			Reduc- ing value	Uronic acid	N-acetyl glucosa- mine	X Component**
			P _t	P _l	Ribose				
F	UDPAG ^{1,2,3}	Uracil ^{4,6}	1.88	.92	+	1.08		1.05	N-acetyl ^{2,10} glucosamine
G	UDPGAL ^{1,2,3}	Uracil ^{4,5}	2.05	.92	+	1.0			Galactose ^{11,12}
I	UDPGA	Uracil ^{4,5}	1.97	1.01	+	+	.86		Glucuronic ^{7,9} acid

* All three fractions showed a uridine-type spectra.

** Superscripts refer to solvent systems in Table I.

Fraction G was identified as UDPGAL by virtue of an equimolar amount of UDP and reducing sugar. The sugar was shown to be galactose on several paper chromatograms. Fraction I was likewise shown to be UDPGA by a UDP to uronic acid ratio of approximately unity. Glucuronic acid was distinguished from galacturonic acid by paper chromatography and by the ease with which the former compound lactonizes.

Fraction E has been described fully in the previous year's report and will not be discussed here.

The acid-soluble nucleotide distribution in fish liver and other tissues are considerably different. Whereas in rat liver and in wheat plants the mono-, di-, and tri-phosphates of cytidine are present, these are absent in the fish liver as are GTP, UDP and UTP. The nucleotide picture appears to tend towards simplicity compared to these other two tissues. The absence of UDP which should appear in the region of fractions F, G and I is significant. This nucleotide was not detected though it has been searched for specifically. It would appear then that unless low temperature column chromatography is employed, UDP appears as a decomposition product of relatively labile UDPX compounds. The role of UDPX compounds is interesting to consider. Fischer hypothesized that the presence of relatively large amounts of inositol in shark fins and the absence of glycogen and other reserve carbohydrates in the liver might mean that inositol may function as a source of blood sugar by the reopening of the inositol ring. Recently, in his biochemical studies on inositol. Charalampous discovered an enzyme system from rat kidney which catalyzed the oxidative conversion of inositol to glucuronic acid through the postulated mediation of UDP glucose followed by UDP glucuronic acid. An analysis of a number of fish livers showed that we

have in this tissue a set of conditions very suggestive of the role of inositol as a carbohydrate storage product. Inositol analyses from a number of tissues from salmon including the liver indicate the presence of this compound at relatively high levels. The presence of reducing sugars in fish appears to be a post-mortem phenomena rather than an actual presence of these compounds in fresh fish. UDPGAL and UDPGA were found to be present in significant concentrations in the liver and the absence of UDP glucose is noteworthy. The reversible conversion of UDP glucose to UDPGAL has been demonstrated by Leloir in S. fragilis. UDPGAL AND UDPGA, therefore, might be implicated in the conversion of inositol to glucuronic acid in addition to other co-enzymic roles in which these nucleotides are involved.

SUMMARY NO. 18

AN AUTOMATIC MULTI-STAGE SEMI-MICRO ZONE-MELTING APPARATUS

A.P. Ronald
F.C. Freeman
D.R. Idler

The technique of zone melting, which is well known in metallurgy has recently been successfully applied to the purification of organic compounds. The limited success of chromatographic techniques in the sterol field suggested that if a suitable apparatus were available, zone melting might find application to the problems in our laboratory.

This method for the purification of organic compounds requires that a narrow, molten band be allowed to travel slowly down a vertical column of the solid substance, which is contained in a suitable glass tube, resulting ultimately in the separation of the impurities which are concentrated at the bottom of the column. Due to the similarity of some impurities to the compounds undergoing purification in our laboratory, preliminary trials indicated that separations could not be expected except after many cycles. With this in mind it was decided that an automatic apparatus capable of simultaneous purification of at least four columns of material should be constructed.

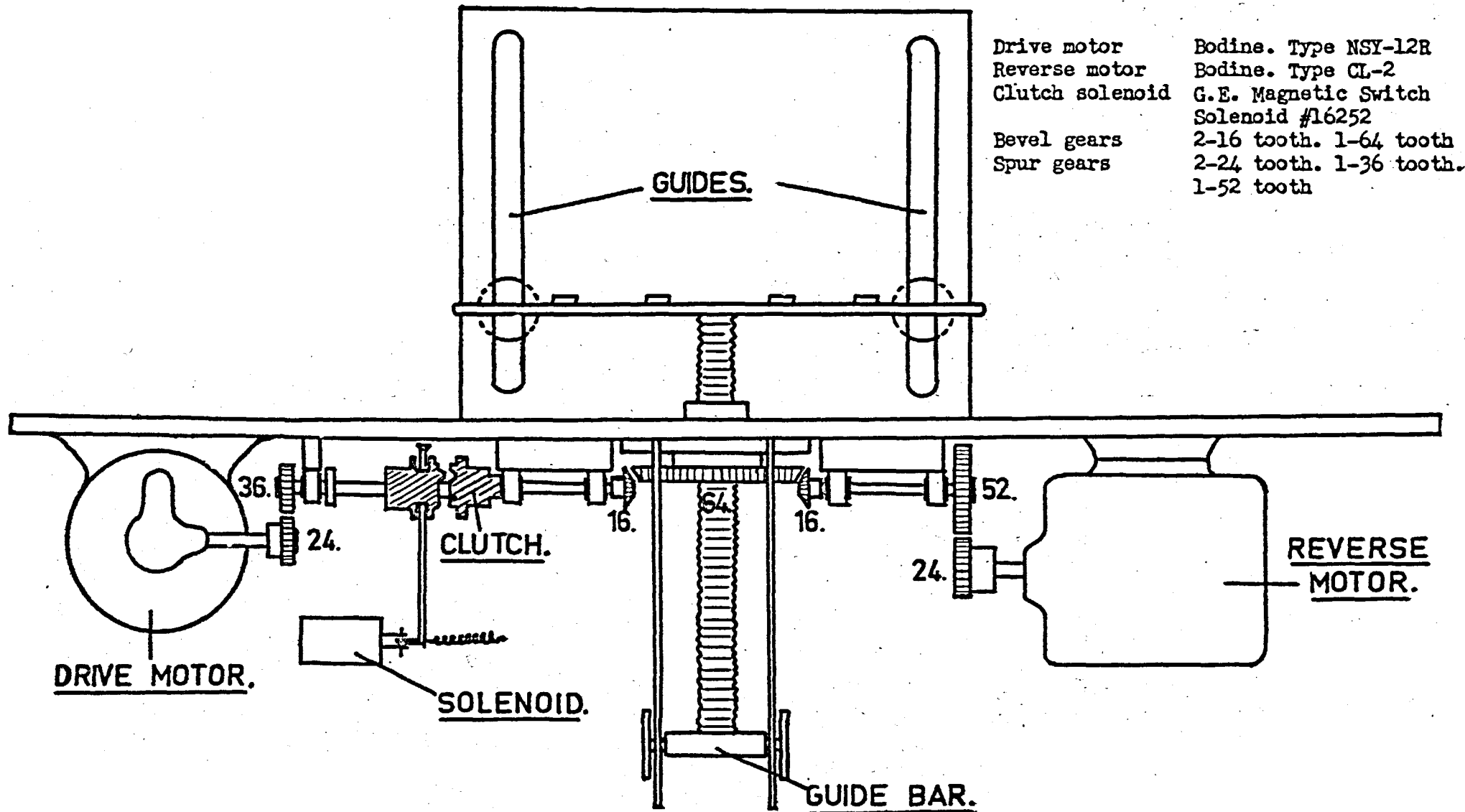
Examination of the literature on this technique showed that the only type of semi-micro apparatus available was not designed for automatic recycling. A design for an automatic macro model exists but the disengagement of the drive motor by the system of pawl and ratchet was not clearly understood by us, and it did not seem a suitable apparatus for adaptation to micro scale.

Principle of operation

The apparatus (Figure 1) consists of a drive motor which raises a platform at the rate of 1.25 inches per hour. The platform supports four tubes which contain the material to be purified. As the platform rises, the tubes pass through a heater which creates a well-defined molten zone of material. The molten zone then passes slowly down the column until the bottom is reached. The platform then reverses in direction and travels quickly to its starting position and again commences its slow ascent.

The slow drive speed required for raising the platform is attained by means of a synchronous motor with a drive shaft speed of 120 revolutions per hour. This is further reduced by external gears to 20 revolutions per hour. The last reduction is by a bevel gear which has an internally threaded hub (Figure 2), through which passes a 3/8-inch diameter rod with 16 threads to the inch. The rod is fixed by means of a guide bar attached at right angles to the rod and sliding in a slot so that it cannot rotate. The bevel gear on completing 16 revolutions raises the platform 1 inch. The gear turns 20 times in 1 hour, thus the platform travels at the rate of 1.25 inches per hour.

Fig. 1 General layout of mechanical components.



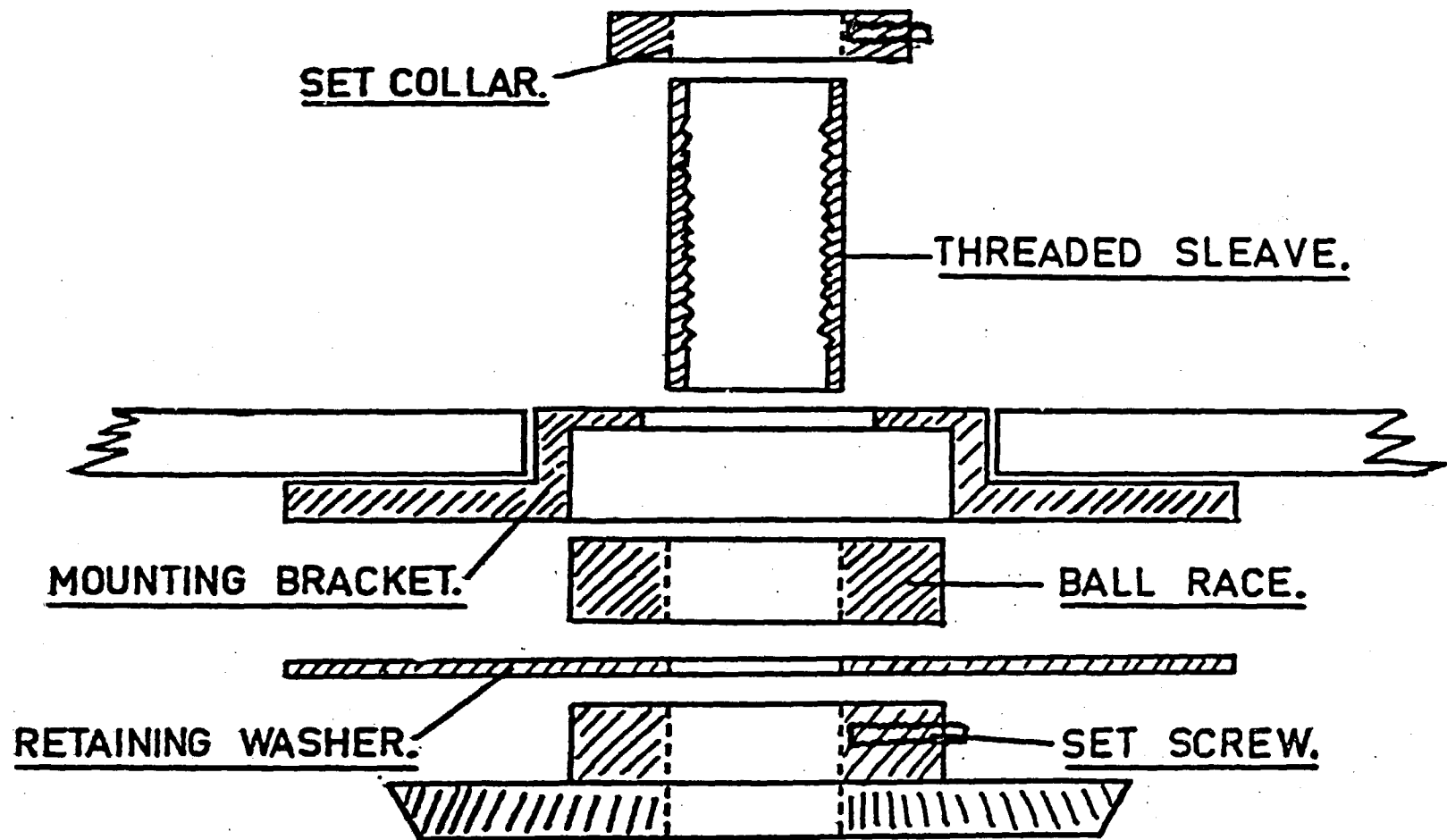


Fig. 2 Exploded view of bevel gear assembly.

The automatic recycle is accomplished in the following manner. On reaching a predetermined height the guide bar, attached to the bottom of the threaded rod, lifts a lever which operates a micro switch. The power is then cut off from the drive motor which is then disengaged from the system through the action of a solenoid operated dog-clutch. The reverse motor then cuts in, returning the platform to its original position, where on striking another micro switch it is stopped. After about 15 seconds the clutch disengages and the drive motor starts. The delay before the commencement of the recycle is effected by the incorporation of a delay mechanism in the circuit, which was installed to avoid damage to the gear system.

Operation of apparatus

Reference should be made to Figure 3 and Table I in the following more detailed description of the operation of the apparatus. The electronic delay circuit has been so designed that when it is switched on, 15 seconds elapse before the relay coil (#1) is energised. Energising of the coil cuts off, through the action of relay (#1A), the filament voltage to tube (VI) and at the same time the plate voltage is made to tube (V2). In this manner the relay is held on until the circuit to the coil is broken, at which point even immediate application of current to the circuit would not energise the coil until 15 seconds had elapsed, which is the time required for the filament of tube (VI) to heat up and allow sufficient current to flow to energise the coil. Relay (#1B) controls the action of the clutch solenoid and the drive motor. When coil (#1) is not energised the clutch solenoid is in operation. This disengages the drive motor from the system and also closes the micro switch (M3). This switch is so positioned that the circuit to the reverse motor is not complete unless the clutch is fully disengaged. However, even with the clutch disengaged the reverse motor will not come into action until its particular circuit has been completed. This can only occur in the following manner. Micro switch (M1) is closed either by the action of the bar attached to the threaded rod or by manual manipulation. Coil (#2) is then energised and relay (#2) closes and breaks the circuit to the delay unit. When the circuit to the delay unit is broken, relay (#2A) makes a circuit to its own coil; this is necessary as micro switch (M1) is opened as soon as the reverse motor comes into operation. Relay (#2B) makes a circuit to micro switch (M3). When the filament of tube (V2) has cooled sufficiently to cut off the power supply to coil (#1), relay (#1) opens, the drive motor stops, the clutch disengages and completes the circuit through micro switch (M3) to the reverse motor. The rod then travels down until the bar strikes micro switch (M2) opening the self-holding circuit to coil (#2). Relay (#2) opens, the reverse motor stops, the circuit to the delay unit is made and after 15 seconds coil (#1) is again energised. The clutch disengages, the drive motor starts and a new cycle commences.

Construction of apparatus

The mechanical components were assembled on an aluminum plate 10 x 24 x 3/8-inches. The bevel gear assembly was first manufactured and assembled and then mounted in the centre of the plate. The reverse motor gear shaft was then assembled, the 16-tooth bevel gear was fastened to one end of a 2-inch length of $\frac{1}{4}$ -inch stainless steel shaft, by means of a pin driven through the hub of the gear and through the shaft. The 52-tooth spur gear was fastened to the other end by drilling a $\frac{1}{4}$ -inch hole in the gear and fitting it to the shaft and a small hole was drilled parallel to the shaft so that it cut a keyway from the shaft and the gear. The hole was then tapped and a screw inserted which held the shaft and the gear firmly together. The gear assembly was mounted on $\frac{1}{4}$ -inch brass pillow blocks which were fastened to an aluminum mounting block of such a thickness that the bevel gears meshed correctly. A similar procedure was followed for the mounting of the slow speed drive motor shaft. A hub was brazed to each spur gear which was to be mounted on a motor shaft and a hole drilled and tapped on each hub and a set screw inserted. The gear was held in position on the drive shaft of the motor by means of the set screw. This allows for easy

mounting, simple adjustment, quick removal and also for disengagement of any motor while tests are being carried out on the correct positioning of the electrical components of the system. If the gear system is not mounted properly and any tightness occurs, difficulty will be experienced in disengaging the clutch. To avoid this, careful alignment is necessary and the clutch teeth should be angled as much as possible. The micro switches (M1, M2) can be mounted in many ways. One convenient mount is on the side of a right-angled bracket which is in turn mounted on the outside of the guide bar support. A slot is cut in the side of the bracket to allow for easy positioning of the micro switches. The mechanism of the micro switch is actuated through coming in contact with that part of the guide bar which protrudes through the guide bar support.

The four-element heater unit is constructed from single loops of #26 bare nickel-chromium wire attached to copper rods approximately 5 inches in length and 1/8-inch in diameter. The rods are held in place by a bakelite strip which provides both insulation and a means of adjusting the height of the heater. Each heater is controlled by a rheostat, wired in the manner of a voltage divider (Fig. 4). The type of circuit used was found to give the best individual control for each heater. With all the heaters working at maximum temperature the load is about 13 amps. However, the 6-volt centre tapped 10 amp. Hammond transformer used in our apparatus worked quite satisfactorily and there was no indication of heating due to overloading.

TABLE I - Values of component parts of electrical circuit.

	<u>Resistors</u>
R1	100-ohm 50-watt
R2, R3, R4, R5	2-ohm 50-watt rheostats
R6, R7, R8, R9	Loop of #26 bare nickel chromium wire
	<u>Condensers</u>
C1	50-mf. electrolytic
	<u>Tubes</u>
V1, V2	117Z6GT. Converted to an ordinary triode, thus doubling its current carrying capacity.
	<u>Relays</u>
1.	P & B Relay - KA 11D. 110-volt. D.C. D.P.D.T.
2.	Guardian Relay - Series 200. 115-volt. A.C. D.P.D.T.
	<u>Switches</u>
M1, M2, M3	Unimax. Type 2 HBW-1, Micro switch
On-off	10-amp. D.P.S.T. toggle switch
	<u>Transformers</u>
T1	Hammond. Type 1129R60CY 6-volt. 10-amp. centre tapped.
	<u>Fuses</u>
	2 2-amp. Type 3-AG.
	<u>Indicator Lights</u>
N1, N2	Neon bulb. Type GE - NE 51. Resistor type mountings should be used with this bulb.

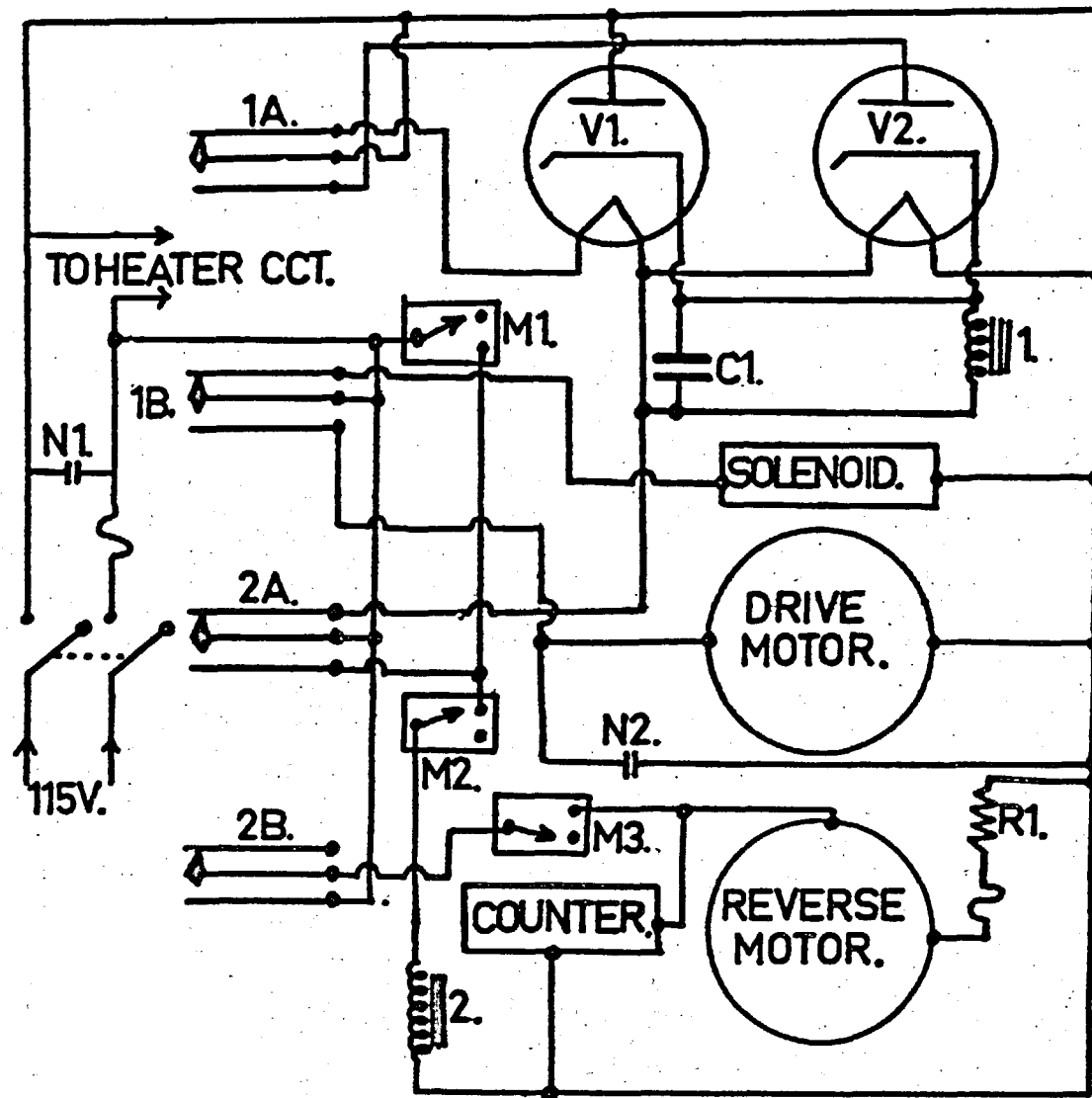


Fig. 3 Circuit diagram of apparatus.

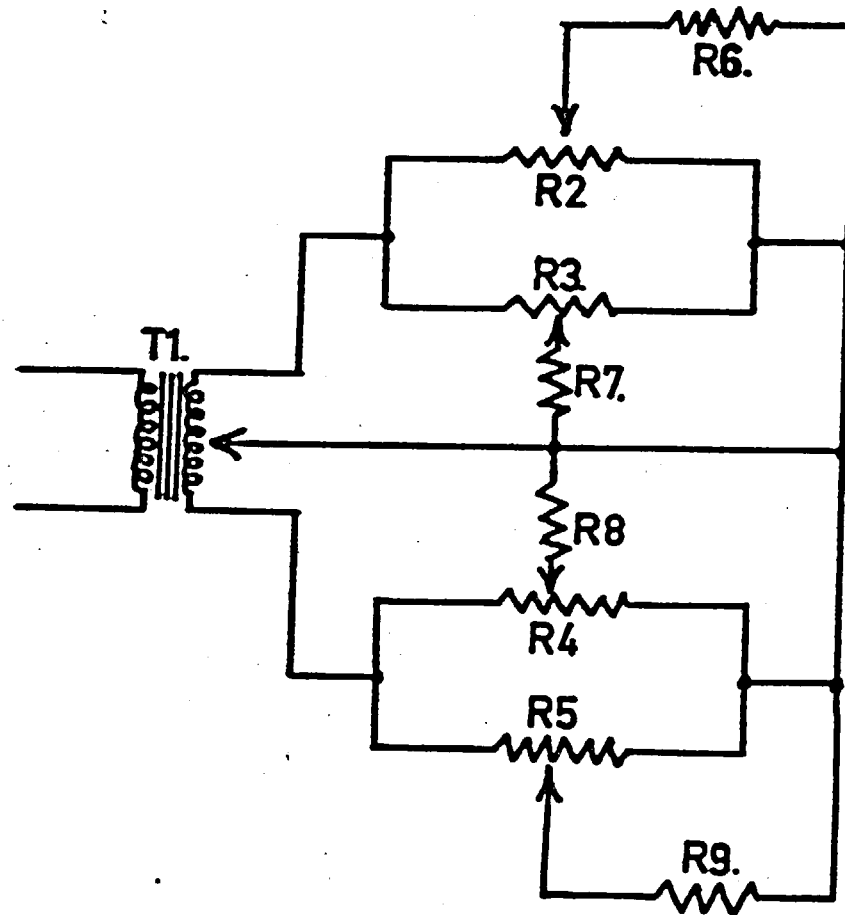


Fig. 4. Heater circuit.

SUMMARY NO. 19

THE PRESERVATION OF WHOLE HERRING PRIOR TO REDUCTION

N.A.W. LePage
B.A. Southcott

The possibility that a considerable percentage of the British Columbia herring catch destined for reduction is wasted, due to the formation of volatile products by bacterial action, has prompted this investigation. There is a special likelihood of bacterial spoilage occurring at the high ambient temperatures prevailing during the summer, and when large gluts of herring make immediate reduction impossible.

The Norwegians have had considerable success in preserving their catches of herring, using formaldehyde and sodium nitrite, though the latter is apparently more commonly used. For the purpose of this investigation it was decided to use these two chemicals and the antibiotics oxytetracycline and chlortetracycline (OTC and CTC).

In order to achieve the best results a preservative should be applied as soon after netting the herring as possible, preferably at the time the fish are loaded into the hold of the seiner. An alternative to this procedure would be the application of the preservative after the fish have been landed and are stored in fish bins awaiting reduction.

Originally it was hoped that idle fish bins at a local fish plant could be used in storing large quantities of treated fish. Unfortunately, a current strike of the British Columbia herring fishermen prevented this. However, a small amount of herring were obtained, using a gill net and by "jigging". While strictly fresh these fish were sprayed with small amounts of concentrated solutions of the preservatives and were stored in plastic containers at an average temperature of 42°F. for 1 week. (Up to 40% formaldehyde and sodium nitrite solutions calculated at .05% of the weight of the fish, and up to 1000 parts per million OTC and CTC solutions calculated at 1 ppm of the weight of the fish were used. Mixtures of the preservatives were used as well.)

Samples of the whole herrings were taken daily, blended in a Waring Blendor, and total bacterial counts made. Although the treated fish seemed to develop off odours less quickly than the control, the bacterial counts were quite scattered and inconclusive. This was probably due to the very small number of fish used in the experiment. As soon as larger quantities of herring become available, this investigation will be resumed.

SUMMARY NO. 20

THE STABILITY OF ANTIOXIDANT-TREATED HERRING MEALS IN STORAGE

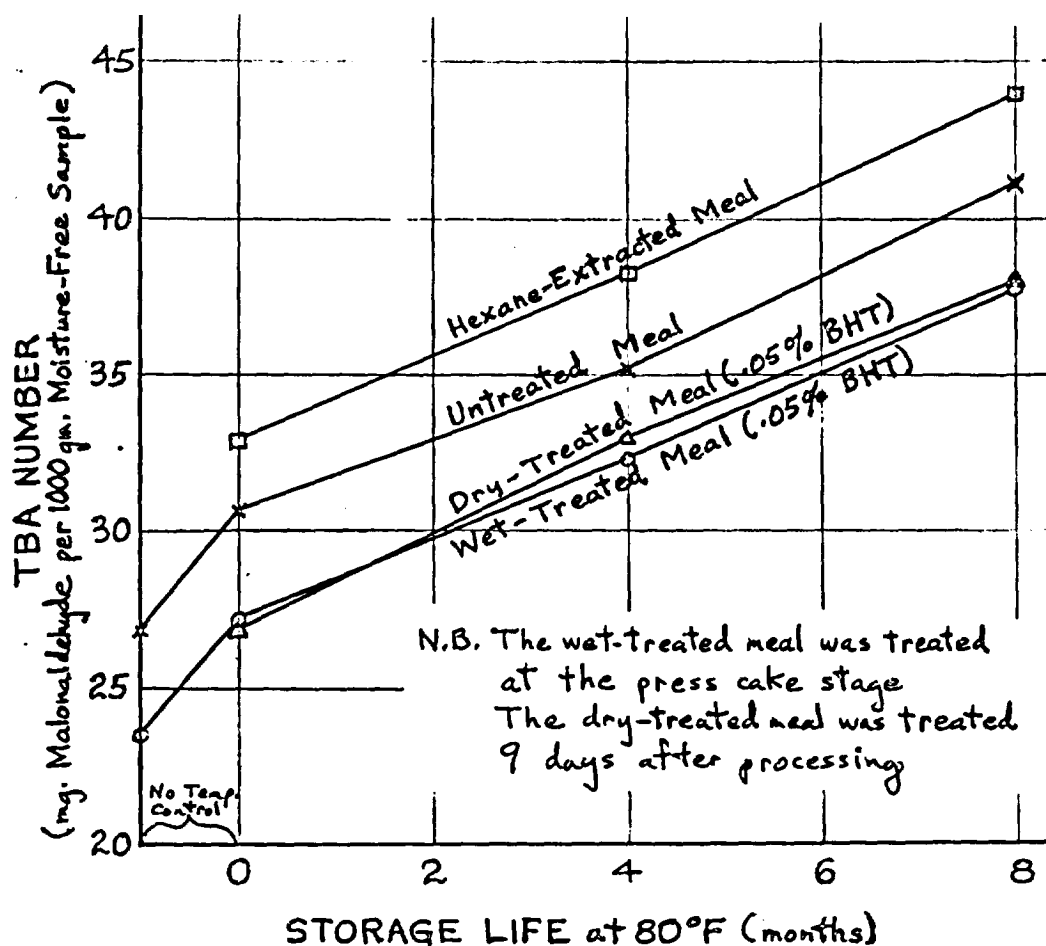
I. THIOBARBITURIC ACID TESTS FOR OIL OXIDATION

N.A.W. LePage

The results of 2-thiobarbituric acid (TBA) tests made on four herring meal samples (Summary No. 44 of this Station's Annual Report for 1956-57) are presented graphically in Fig. 1.

All four meals show a similar rate of increase of the TBA number (milligrams of malonaldehyde per 1000 grams of sample) after eight months in storage at 80°F. The fact that the oil in the two BHT-treated meals seems to have oxidized as rapidly as that in the untreated control, suggests the possibility that (1) the wet-treated meal did not receive sufficient BHT (0.05%) to allow for steam distillation in the drying operation, and (2) the distribution of BHT in the dry-treated meal was not sufficiently uniform to give protection. Apparently, in the case of the hexane-extracted meal, the constituent in the oil giving rise to the TBA reaction was not removed by the extraction.

Fig. 1 Thiobarbituric acid tests for oil oxidation.



Because of the uncertainties in the application of the antioxidant, a further series of herring meals have been treated using BHT both in a finely powdered form (200 mesh) and dissolved in herring oil, and Santoquin (Santoquin has been shown to be a more powerful antioxidant than BHT, but as yet has not been approved by the Food and Drug Authorities.) dissolved in herring oil. The powdered BHT was added at approximately .17% of the finished meal to two duplicate lots, using a metering device closely resembling a garden spreader. The BHT in oil was sprayed into two lots of meal at approximately .11% and .06%. The Santoquin-fish oil solution was sprayed into one lot of meal in approximately .06% concentration. Two control meals were obtained: one with approximately .6% of herring oil added and one without additional oil. The antioxidants were added immediately after the drying operation and prior to grinding of the meals. These meals are in storage at 80°F. and will be tested using TBA and chick nutrition tests.

The writer gratefully acknowledges the assistance of Mr. David Barbour of the British Columbia Packers Ltd., who provided the spraying apparatus and the approximate analyses of the meals, and of Mr. Robert Wilson of North Shore Packing Co., Ltd. and Mr. Michael Kreuger of Prince Rupert Fishermen's Co-operative Association, who made available the facilities of their plants for the purposes of this investigation.

THE STABILITY OF ANTIOXIDANT-TREATED HERRING MEALS
IN STORAGE. II. CHICK NUTRITION TESTS

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(Dept. Poultry Science, U.B.C.)
N.A.W. LePage

Concurrent with the TBA tests of Part I, chick nutrition tests were performed at four-month intervals using the four herring meals. The composition of these meals is set out in Table I.

TABLE I - Proximate analyses of herring meals.

	Meal 1 Wet-treated (.5% oil + .05% BHT)	Meal 2 Dry-treated (.5% oil + .05% BHT)	Meal 3 Hexane-extracted Control	Meal 4 Untreated Control (.5% oil)
Moisture %	7.9	8.9	8.0	8.8
Fat %	8.0	8.8	0.3	8.2
Protein %	75.0	73.9	81.0	74.1
Ash %	10.6	10.3	11.3	10.5

Each of these meals was tested for its protein nutritive value as the sole source of supplementary protein in two diets, one containing added folic acid and the other deficient in folic acid (unless this was supplied by the herring meal). The protein content of each diet was 21%. Tables II and III show the average weights of the chicks at intervals during the storage period for each meal.

TABLE II - Growth of chicks fed herring-meal-supplemented-diet (with added folic acid).

Herring meal supplement	Average weights at 24* or 26 days (grams)		
	Storage interval		
	Start*	4 months	8 months
1	338	362	320
2	317	362	332
3	319	358	317
4	316	371	307

N.B. All meals stored at 80°F. 2 weeks after processing.

TABLE III- Growth of chicks fed herring-meal-supplemented-diet (no added folic acid).

Herring meal supplement	Average weights at 24* or 26 days (grams)		
	Storage interval		
	Start*	4 months	8 months
1	266	279	219
2	262	302	222
3	266	304	268
4	267	287	223

N.B. All meals stored at 80°F. 2 weeks after processing.

From the data of Tables II and III it would appear that antioxidant treatment of the herring meals had no significant effect on their protein quality. The hexane-extracted meal after 8 months storage seems to have retained more folic acid than the other meals. However, no definite conclusions can be reached as to the effect of BHT on the protein quality of herring meal until the completion of the second set of trials outlined in Part I.

The TBA and chick nutrition tests at the end of twelve months of storage have not yet been completed.

SUMMARY NO. 21

EFFECT OF ENZYME TREATMENT ON THE VISCOSITY OF WHALE SOLUBLES

J.R. McBride
D.R. Idler
R.A. MacLeod

Recently, the whaling industry has expressed some concern over the high viscosity of much of the whale solubles being produced.

In view of the fact that this problem of viscosity in whale solubles appeared to be quite similar to the problem of viscosity in herring solubles, it was of interest to know if proteolytic enzyme treatment would have the same capacity to reduce viscosity in whale solubles as it had been found to have in the production of herring solubles. In the case of whale solubles, it was decided to apply the enzyme hydrolysis procedure to the final product rather than to the primary stickwater as had been done for herring solubles. This change in procedure was necessary because of the limited holding facilities for whale stickwater during the peak periods of production at the whaling station.

As the final product is acidic, (pH about 3.5), commercially available proteolytic enzymes which display activity in an acid medium were chosen for study. Three commercial proteolytic enzymes, Papain, Bromelin, and Rhozyme B-6, were selected. As Rhozyme B-6 has a pH optimum of about 6.5, it would not be expected to have activity at the low pH of the whale solubles unless it proved to be active over a wide pH range. As the enzyme is already used for producing herring solubles, it was considered of interest to determine its effect on whale solubles. Papain, another of the enzymes tested, is used extensively as a meat tenderizer.

In the first experiment, Table I, the enzyme hydrolysis was carried out at the pH to which the whale solubles is normally lowered to prevent bacterial spoilage. In the second, Table II, the enzyme hydrolysis was carried out at pH 5.0. During the first hundred minutes of each experiment the samples were mixed by stirring. This agitation produced a lowering of the viscosity in the control sample (without added enzyme). The lowering in the control, however, was not permanent since, as soon as the stirring ceased, the viscosity values rose to their previous levels. The results in the presence of Rhozyme B-6 paralleled the control when the pH of the solubles was 3.5, Table I, indicating that this enzyme was not effective at the low pH. Both papain and bromelin, however, produced a very satisfactory lowering of the viscosity of the solubles and the viscosity value remained low even after the product had stood without stirring for 48 hours. Even when the products of enzyme digestion were cooled to 10°C., they remained fluid. The viscosity values at this temperature accentuated the greater effectiveness of the bromelin in lowering viscosity.

At pH 5.0, Table II, Rhozyme B-6 showed some activity though it was still not as effective as papain or bromelin in the process. This result with whale solubles differs from findings with herring solubles, since in the case of the latter product, all three enzymes were found to be similarly effective in reducing viscosity at this pH.

TABLE I - Changes in the viscosity of whale solubles treated with commercial proteolytic enzymes at pH 3.5.

Time	Control	Rhozyme B-6	Papain	Takamine Bromelin
Stormer Values*				
0	64.6	64.6	64.3	64.4
20 min.	52.2	52.1	44.5	41.7
40 "	48.6	51.2	40.2	35.8
60 "	49.4	48.8	40.2	35.6
80 "	48.8	48.9	39.6	31.9
100 "	48.8	48.9	39.9	31.9
160 "	55.1	55.3	38.4	30.6
24 hr.	63.0	64.2	29.7	25.9
48 "	64.4	65.8	28.2	25.9
10°C.	**	**	64.3	43.7

* Each value represents the number of seconds required for a paddle in the sample to make 100 revolutions when a force of 200 grams is applied.

** Gel too heavy for test.

Each enzyme was added at a level of 0.5% of the total solids in the solubles sample used (solids content 48%). Samples stirred continuously for the first 100 minutes. pH of solubles 3.5, temperature 25°C.

TABLE II - Changes in the viscosity of whale solubles treated with commercial proteolytic enzymes at pH 5.0.

Time	Control	Rhozyme B-6	Papain	Takamine Bromelin
Stormer Values*				
0	38.7	38.6	38.6	38.7
20 min.	34.4	33.8	29.1	28.7
40 "	34.3	33.8	27.5	24.2
60 "	34.5	33.4	26.7	23.2
80 "	34.4	33.4	26.9	22.6
100 "	34.5	33.4	26.7	22.5
160 "	35.2	33.4	25.4	21.4
24 hr.	42.4	32.5	24.4	22.8
48 "	42.6	31.1	24.0	22.8
10°C.	**	156.1	35.0	34.3

* See Table I.

** Gel too heavy for test.

Conditions as in Table I, except that pH of solubles was 5.0.

The results of this study indicate that enzyme treatment can be used to reduce the viscosity of whale solubles. The treatment can be carried out on the final concentrated product under acidic conditions at room temperature. Thus, neither special equipment nor special conditions are required in the process and at the low pH that can be employed, losses by bacterial spoilage are negligible.

SUMMARY NO. 22

RELATIVE ACTIVITY OF VARIOUS COMMERCIAL PROTEOLYTIC
ENZYMES IN THE PRODUCTION OF HERRING SOLUBLES

J.R. McBride
R.A. MacLeod
D.R. Idler

As noted in previous Annual Reports of this Station (1955-56-57), the use of acid to precipitate proteins in herring stickwater is limited in its scope for the production of herring solubles. The appearance of an unknown gel factor, not precipitated by acidulation, in the herring stickwater during the months of October to April inclusive, results in a solubles of high viscosity. In addition to this undesirable physical characteristic, considerable difficulty is experienced in the breaking of the oil-water emulsion caused by the gel factor. Since herring solubles are sold on the basis of protein content, any oil present in the solubles is an economic loss to the producer.

However, the recent use of commercial proteolytic enzymes in the solubilizing or partial hydrolysis of insoluble protein in the food industry has made available an alternate process for the production of herring solubles.

Although Rhozyme B-6, a commercial proteolytic enzyme, has recently been used successfully in B.C. as a means of reducing the viscosity of solubles, no systematic study has been carried out to determine which of a number of different types of proteolytic enzyme available commercially would be most effective in this process.

In this study several different types of commercially available proteolytic enzymes have been evaluated on the basis of their capacity to reduce the viscosity of herring solubles.

In order to insure a common substrate for all enzyme experiments, a large reserve of primary herring stickwater was divided into two parts. One part was used for enzyme studies without further treatment, the second part was centrifuged in a Servall centrifuge at 12,000 r.p.m. for 20 minutes to remove the remaining oil and insoluble solids. Thus, two herring stickwater substrates were available, "primary" stickwater and "pure" stickwater. The primary stickwater was used to duplicate to some extent actual commercial conditions since it contained more nearly the same level of total solids as commercial stickwater. The "pure" stickwater was also used since suspended solids which have an effect on viscosity had been removed and the effect of the enzyme would be expected to be due almost entirely to its effect on the gelling agent. Aureomycin was added to both samples of stickwater as a preservative at 2 parts per million (p.p.m.), based on the total solids content of the stickwater.

Each enzyme was tested over a series of concentrations ranging from 0.5% to .0005% of the total solids present in the stickwater. A control sample was carried out with each separate enzyme studied. Enzyme activity was measured by converting the treated stickwater to solubles and determining the viscosity of the resulting product. Viscosity, as measured by the pour residue test, was determined at the end of a 24-hour setting period at 10°C. and 25°C.

The rate of liberation of α -amino nitrogen with time of treatment was also followed. Free α -amino N was determined by Sahyun's modified Sorensen formal titration method on aliquots removed at different times from the digestion mixture.

The total solids content of the herring stickwater and solubles was determined by means of a Cenco moisture balance.

At the completion of each enzyme digestion, the herring stickwater substrate was boiled for five minutes at a pH of 3.0, cooled, and the pH readjusted to pH 5.0.

The stickwater was then concentrated under vacuum in a glass distillation system to approximately 45% solids.

Table I shows the results obtained by the use of five commercial proteolytic enzymes on primary stickwater. All five enzymes are most effective in lowering the viscosity in the two-hour period when tested at the .5% level. This is the level recommended for the use of their enzyme by the manufacturers of Rhozyme B-6.

TABLE I - The effect of several commercial proteolytic enzymes on the gel factor in primary stickwater.

Enzyme	Level of Enzyme*	Free α -amino Nitrogen released (mg/ml)			Total solids gms. %	Viscosity**	
		Time				% retained	
		$\frac{1}{2}$ hr.	1 hr.	2 hr.		24 hr/25°C	24 hr/10°C
Rhozyme B-6	Control	0.000	0.000	0.000	42.6	27.49	100
"	0.5%	0.0470	0.0470	0.0504	42.2	3.95	7.70
"	0.05%	0.0332	0.0470	0.0470	42.1	13.29	23.01
"	0.005%	0.0168	0.0168	0.0214	42.0	100.00	26.29
"	0.0005%	0.0000	0.0000	0.0000	42.3	100.00	28.95
Merck Ficin	Control	0.0028	0.0004	0.0004	41.0	17.08	100
"	0.5%	0.0924	0.1064	0.1288	42.0	23.11	27.52
"	0.05%	0.0462	0.0462	0.0574	41.5	28.84	37.22
"	0.005%	0.0168	0.0168	0.0392	40.8	46.80	100
"	0.0005%	0.0000	0.0000	0.0224	40.9	35.71	100
Takamine Bromelain	Control	0.0024	0.0024	0.0024	42.0	20.05	100
"	0.5%	0.1400	0.1428	0.1484	42.0	4.16	11.99
"	0.05%	0.0700	0.0808	0.0812	45.0	25.65	37.39
"	0.005%	0.0364	0.0616	0.0627	42.0	20.88	25.35
"	0.0005%	0.0280	0.0252	0.0252	41.2	23.61	40.81
Takamine Protease	Control	0.0000	0.0000	0.0000	42.0	16.8	100
"	0.5%	0.1456	0.1652	0.2184	42.1	5.7	14.8
"	0.05%	0.1040	0.1320	0.1544	42.0	3.4	17.0
"	0.005%	0.0743	0.0967	0.1191	42.6	15.4	25.1
"	0.0005%	0.0644	0.0812	0.1036	41.5	36.6	100
Papain	Control	0.0000	0.0000	0.0084	42.0	24.89	100
"	0.5%	0.0010	0.0212	0.0500	41.8	20.89	26.05
"	0.05%	0.0000	0.0084	0.0120	41.6	26.03	39.71
"	0.005%	0.0000	0.0000	0.0084	41.7	20.50	100
"	0.0005%	0.0000	0.0000	0.0000	41.6	23.48	100

* The percentages quoted are percentages of the total solids present in the sample of stickwater used.

** Viscosity of solubles prepared from stickwater after 2 hrs. incubation at the following pH and temperature: Rhozyme B-6^{ph} 6.0, 60°C; Ficin pH 5.0, 40°C; Bromelain pH 5.0, 60°C; Protease pH 8.0, 40°C. and Papain pH 5.0, 37°C.

Of the five enzymes tested, Rhozyme B-6 proved to be the most effective, though bromelain and Takamine protease were almost as good. Both ficin and papain were somewhat less active. Determinations of α -amino nitrogen were run to see if the extent of reduction in viscosity of solubles could be correlated with the amount of α -amino nitrogen released by the enzyme. It will be seen, however, that in the case of Rhozyme B-6 at the lower levels of enzyme tested, where little or no release of α -amino nitrogen could be detected, significant decreases in viscosity occurred. This is probably due to the fact that a limited breakdown of the protein

chains to peptides resulted in a large decrease in viscosity, though few α -amino groups were actually released. On the other hand, in the case of ficin, which was only of limited effectiveness in reducing viscosity, a significant release of α -amino nitrogen was detectable at the lowest level of enzyme tested. This would suggest that ficin, in contrast to the Rhozyme preparation, is relatively more active in splitting off end group amino acids and this could occur without materially reducing the length of the protein chains. Thus, a large release of α -amino groups could occur without causing a significant decrease in viscosity of the sample.

The following table, Table II, shows the results obtained on the use of the five commercial proteolytic enzymes on "pure" stickwater, that is, stickwater from which suspended solids have been removed by high speed centrifugation. Again, all the enzymes were active at the 0.5% level and all showed considerable activity even at the .0005% level. This demonstrates the lower gel capacity of the clarified stickwater and indicates that suspended solids contribute significantly to the viscosity of solubles prepared from primary stickwater - an observation which has also been made by other investigators. Again, Rhozyme B-6, on a weight basis appears to be somewhat more active than the other enzymes tested, though there was less difference between the activity of the enzymes when clarified stickwater was the substrate than when "primary stickwater" was used.

There is an important difficulty associated with the determination of the relative activity of commercial enzyme preparations. All such preparations are subject to batch to batch variation. New production methods could also improve the activity of a particular manufacturer's enzyme preparation. Results such as those obtained here would be valueless for comparative purposes unless the activity of these and other batches used could be referred to a readily available reference standard. We have chosen hemoglobin substrate powder obtainable from the Worthington Biochemical Corporation as reference standard. The activity of the commercial enzymes used in this study toward this substrate, based on their capacity to release α -amino nitrogen, is shown in Table III. The conditions under which the test was run are also included with the table. Since two batches of the same commercial enzyme would be more likely to have the same activity toward stickwater if their activity toward the reference standard was the same, it would be advisable to first determine the activity of a batch of enzyme toward the reference standard before deciding on its suitability for use in the preparation of solubles.

The writers are indebted to Rhom and Haas Company for a sample of Rhozyme B-6, to Takamine Laboratory Inc. for samples of Bromelin and Protease, and to Merck and Company, Inc. for a sample of Ficin.

The Papain used was purchased from Nutritional Biochemicals Corporation.

TABLE II - The effect of several commercial proteolytic enzymes on the gel factor in "pure" stickwater.

Enzyme	Level of enzyme	Free α -amino Nitrogen released (mg/ml)			Viscosity*		
		Time			Total solids gms. %	% Retained	
		$\frac{1}{2}$ hr.	1 hr.	2 hr.		24 hr/25°C	24 hr/10°C
Rhozyme B-6	Control	0.0040	0.0090	0.0090	44.4	1.81	100.00
"	0.5%	0.0230	0.0510	0.1080	46.2	0.64	0.93
"	0.05%	0.0280	0.0510	0.0510	47.5	0.69	0.88
"	0.005%	0.0160	0.0160	0.0160	44.9	0.82	0.96
"	0.0005%	0.0160	0.0160	0.0160	47.5	2.99	13.05
Merck Ficin	Control	0.0000	0.0000	0.0020	48.8	8.09	77.21
"	0.5%	0.0012	0.0472	0.0896	47.9	0.87	0.95
"	0.05%	0.0140	0.0224	0.0308	46.0	3.58	8.32
"	0.005%	0.0000	0.0028	0.0252	48.8	1.68	12.05
"	0.0005%	0.0000	0.0018	0.0270	49.3	1.59	11.87
Takamine Bromelain	Control	0.0028	0.0028	0.0028	52.2	3.84	100.00
"	0.5%	0.0488	0.0784	0.0784	55.0	3.07	6.11
"	0.05%	0.0000	0.0056	0.0224	51.4	3.85	9.52
"	0.005%	0.0028	0.0028	0.0128	48.7	1.36	2.77
"	0.0005%	0.0000	0.0100	0.0100	48.2	2.09	3.15
Takamine Protease	Control	0.0000	0.0000	0.0000	49.0	2.5	100.00
"	0.5%	0.0980	0.1148	0.1400	47.5	0.55	0.65
"	0.05%	0.0084	0.0168	0.0476	48.1	0.58	1.20
"	0.005%	0.0000	0.0122	0.0250	47.3	1.20	3.76
"	0.0005%	0.0000	0.0194	0.0194	46.7	1.08	3.59
Papain	Control	0.0000	0.0028	0.0028	47.2	1.32	100.00
"	0.5%	0.0392	0.0924	0.1064	52.2	1.65	7.08
"	0.05%	0.0840	0.0840	0.0868	45.5	2.00	4.58
"	0.005%	0.0784	0.0784	0.0814	47.2	3.12	9.78
"	0.0005%	0.0006	0.0014	0.0044	46.2	2.93	9.36
-	Control	0.0000	0.0000	0.0006	50.5	4.33	100.00
Rhozyme B-6	0.0005	0.0020	0.0026	0.0128	46.0	1.98	8.64
Merck Ficin	"	0.0084	0.0128	0.0296	45.5	3.96	10.91
Takamine Bromelain	"	0.0228	0.0256	0.0396	52.0	4.91	8.9
Takamine Protease	"	0.0000	0.0084	0.0168	47.8	2.04	6.80
Papain	"	0.0000	0.0062	0.0086	51.0	5.09	44.72

* Viscosity of solubles prepared from stickwater after 2 hrs. incubation at the following pH and temperature: Rhozyme B-6 pH 6.0, 60°C; Ficin pH 5.0, 40°C; Bromelain pH 5.0, 60°C; Protease pH 8.0, 40°C, and Papain pH 5.0, 37°C.

TABLE III - Activity of commercial proteolytic enzymes toward Worthington hemoglobin substrate powder.

Enzyme tested	Conditions* pH Temp.		Activity (mg. g-amino nitrogen released per mg. of enzyme per hour)		
			1st hr.	2nd hr.	Avg. for 2 hrs.
Rhozyme B-6	6.0	60°C	.777	.119	.448
Bromelin	5.0	60°C	.705	.072	.388
Papain	5.0	37°C	.143	.013	.078
Protease	8.0	40°C	.408	.422	.415
Ficin	5.0	40°C	.971	.368	.669

* Test system: Hemoglobin substrate solution was prepared by making a 2% solution of hemoglobin powder in water. Undissolved matter was removed by centrifugation. Total solids content of clear supernatant was 1.71 gm/100 ml in this case. For each enzyme activity study, 400 ml of substrate solution was adjusted with NaOH or HCl to the required pH and warmed to the desired temperature in a water bath. A 10 ml aliquot of substrate solution was removed to determine g-amino nitrogen level at 0 time. An amount of enzyme equivalent to 0.5% of the total solids present in the substrate solution (.034 gm. in this case) was suspended in 10 ml of H₂O and the suspension added to the solution with stirring. Aliquots of 10 ml were removed at 30-minute intervals for 2 hours for the determination of g-amino nitrogen.

SUMMARY NO. 23

PROXIMATE ANALYSIS OF HERRING AT MONTHLY INTERVALS

J.R. McBride
D.R. Idler
R.A. MacLeod

The investigation reported in Summary No. 26 of this Station's Annual Report for 1956-57 has been continued to enable proximate analyses of herring to be made at monthly intervals for a consecutive 12-month period. The methods of analysis and preparation of herring for analysis were identical to that given in the previous report.

All herring used for analysis were obtained from the lower British Columbia coast except for the months of December and January when, due to a local strike, herring were procured from the Prince Rupert area. The results obtained from this investigation are given in Table I.

Examination of the results given in Table I indicate a marked influence of gonad development and maturation on the levels of oil, water and protein in herring. The most significant changes were noted immediately following the spawning period.

During March, the period immediately preceding spawning, the lowest oil level and the highest water level were recorded. However, upon spawning in April, a significant increase in the oil level and drop in the water level were noted. Also, an apparent drop in the protein content took place immediately following spawning. The oil content continued to increase rapidly until the month of June, when a maximum oil level of 24.35% was attained. Although a slight drop in the oil content was noted in July, a relatively high oil content was maintained from July through to the end of

December. From January until the time of spawning in March, a gradual but continual decrease in the oil content occurred. There appeared to be a somewhat higher oil level in female than in male herring.

TABLE I - The oil, water, ash and protein content of whole herring.

Month	Sex	Gonads % total body weight	% water	% fat	% ash	% protein ¹	Total: % ash + % water + % oil + % protein
Jan. 1957	Male	15.30	70.10	9.47	2.47	17.59	99.63
"	Female	10.01	70.37	9.50	2.36	17.38	99.61
Feb. 1957	Male	16.40	72.09	7.76	2.35	17.84	100.04
"	Female	16.20	72.00	8.04	2.28	17.00	99.32
Mar. 1957	Male	20.80	76.43	4.90	2.43	16.79	100.55
"	Female	19.20	76.70	5.40	2.78	16.68	101.56
Apr. 1957	Undetermined*	-	71.90	10.91	2.30	14.81	99.92
May 1957	"	-	74.77	8.09	2.41	15.13	100.40
Jun. 1957	"	-	57.69	24.35	2.65	14.25	98.94
Jul. 1957	"	-	66.49	16.53	2.36	15.00	100.38
Aug. 1957	"	-	65.31	18.06	2.29	14.31	99.97
Sep. 1957	Male	3.68	64.20	17.61	2.34	15.44	99.69
"	Female	2.40	63.40	18.01	2.37	15.31	99.09
Oct. 1957	Male	5.45	65.00	16.20	2.46	15.94	99.60
"	Female	2.91	64.75	16.70	2.37	15.94	99.76
Nov. 1957	Male	10.61	66.80	14.40	2.41	16.90	100.51
"	Female	4.02	68.30	13.05	2.45	16.61	100.41
Dec. 1957	Male	12.80	64.60	16.55	2.31	17.62	101.08
"	Female	7.29	64.00	16.60	2.47	17.00	100.07
Jan. 1958	Male	18.10	69.10	11.66	2.28	17.69	100.73
"	Female	14.70	68.60	11.72	2.39	17.75	100.54

* No evidence of milt or roe.

¹ Nitrogen x 6.25.

As noted in the previous investigation, and confirmed in the present study, an inverse ratio existed between the level of oil and the level of water.

The range in the percentages of protein recorded in Table I is from 14.25% in June to 17.84% in February. The reason for such differences can only be speculated upon with the data at hand. There is a close parallel between gonad development and increase in the percent protein of the herring in this study. Other investigators have noted similar variations in the protein levels of herring but have suggested that several factors besides gonad development, such as race and spawning and feeding cycles, might also play an important role in these variations.

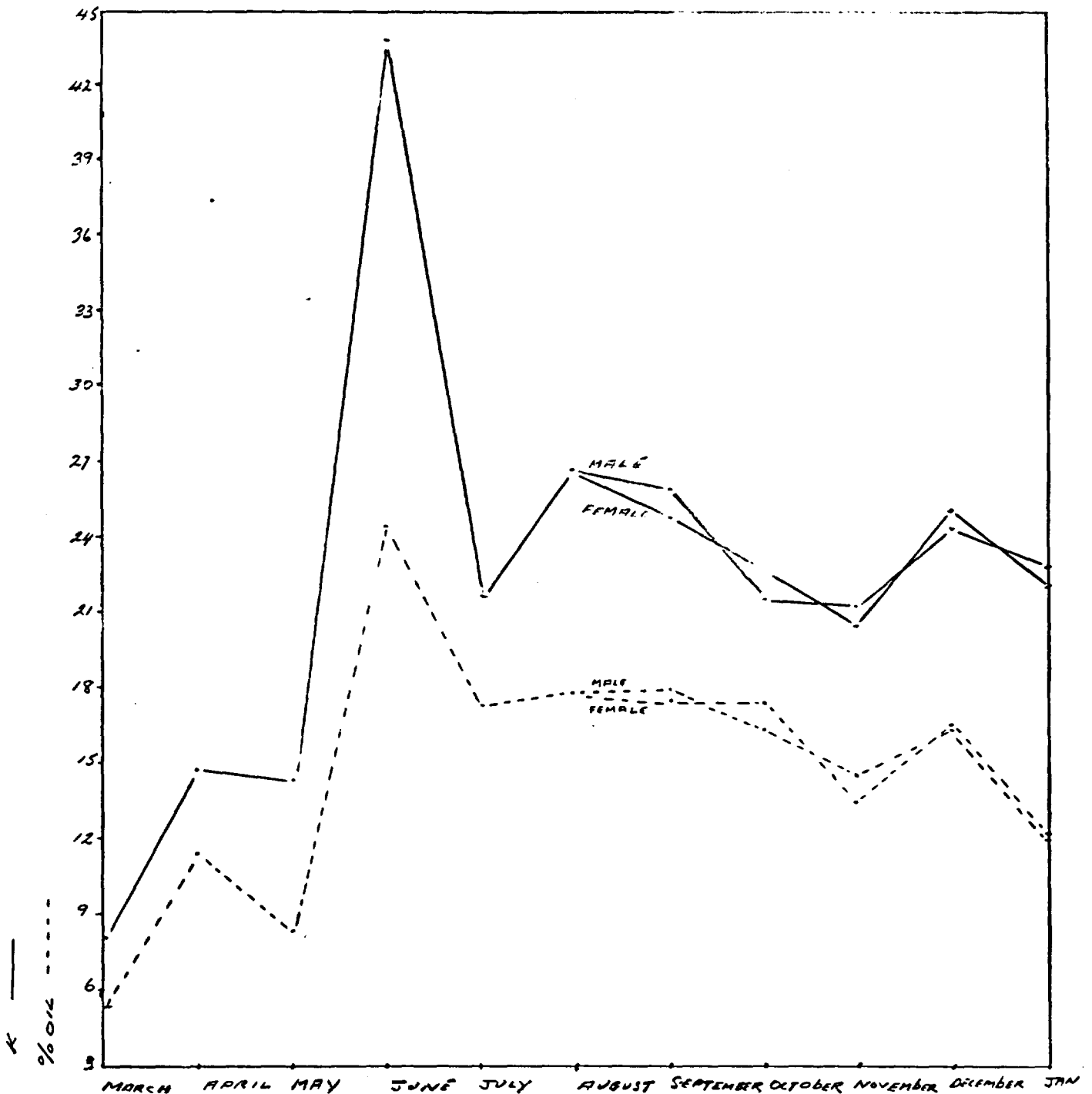
The percentages of ash, although erratic in two instances, i.e., in March and June, appear to present a relatively constant level for the whole year. Furthermore, no apparent differences exist in the level of ash between male and female herring.

In the 1956-57 Annual Report of this Station, a preliminary study was reported on the correlation between the condition factor of herring as measured by $K = \frac{W}{L^3} \times 100$ and the level of oil in herring. It was noted that in this investigation no positive correlation existed between this condition factor and the level of oil. However, a second condition factor, Tester's condition factor, based on the specific gravity of herring, has indicated a positive correlation with the level of oil in herring. The

condition factor (K) was measured in the equation $K = \frac{W_a}{W_w}$, where W_a is the weight of the fish in air and W_w is the weight of the fish in water at 13°C.

It is apparent from the results depicted in Fig. 1 that there is a positive correlation between fat present and Tester's condition factor in herring. The values obtained suggest that Tester's condition factor varies with season and reflects changes in the percentage oil content of herring.

Fig. 1 Variation in the percentage oil and Tester's condition factor (K) in herring sampled at monthly intervals over the year 1957-58.



SUMMARY NO. 24

THE MAILLARD REACTION: ENZYMES CONCERNED WITH DEGRADATION OF FISH MUSCLE NUCLEIC ACIDS AND INTERCONVERSION OF SUGAR PHOSPHATE ESTERS

FISH MUSCLE RIBONUCLEASE

N. Tomlinson

The investigation of fish muscle ribonuclease (RNase) (Summary No. 13A of this Station's Annual Report for 1956-57) has been continued.

Assay of enzyme activities

RNase activity has normally been assayed by the method previously described (Summary No. 13A of this Station's Annual Report for 1956-57), but in the measurement of this activity in extracts following electrophoresis on paper, because of the low activities to be measured, longer incubation periods were necessary (6 to 9 hours). Such results are reported simply as optical density of uranylacetate-trichloroacetic acid solutions (prepared as in the usual assay) at 260 m μ . All light absorption measurements were made with a Beckman model DU spectrophotometer, 1 cm. cells being used throughout.

Phosphodiesterase activity was measured with bis(p-nitrophenyl)phosphate as substrate. The digestion mixture consisted of sodium succinate buffer pH 6.0, 0.025M, bis(p-nitrophenyl)phosphate (sodium salt), 0.0018M, and enzyme. The incubation temperature was 25°C. Aliquots (0.33 ml.) of the reaction mixture were removed immediately after addition of the enzyme and after a suitable period of incubation (normally 2 hours). These were at once added to 3 ml. of 0.1 N NaOH. The optical densities of the resulting solutions were measured at 400 m μ and the p-nitrophenol released determined by reference to a suitable calibration curve. Reaction blanks with enzyme omitted were used.

Phosphomonoesterase activity was measured either by determination of orthophosphate released from RNA as substrate (reaction mixture deproteinized with an equal volume of ice-cold 10% TCA) or by measurement of p-nitrophenol released from di-sodium p-nitrophenyl phosphate (Sigma Chemical) as substrate. The reaction mixture with the latter compound as substrate consisted of sodium succinate buffer pH 4.5, 0.025M, substrate, 0.018M, and enzyme. The incubation temperature and time were 25°C. and $\frac{1}{2}$ -hour, respectively. Measurements were performed as for the phosphodiesterase determinations described above.

When enzymic reactions were allowed to proceed for longer periods than a few hours, chlortetracycline.HCl (5 p.p.m.) was added to the reaction mixture to prevent bacterial growth. At the end of the experiment these reaction mixtures were examined microscopically for bacteria by means of direct smears, results being discarded if any evidence of bacterial growth was found.

Zone electrophoresis of proteins and nucleotides and paper chromatography of nucleotides

Electrophoresis of proteins was carried out using Whatman No. 3 paper at 1°C. and a field strength of 25 volts/cm. for $3\frac{1}{2}$ hours. The use of field strengths lower than 25 volts/cm. was found to be unsatisfactory, as the much longer time required resulted in poor separation of the proteins due to diffusion. The buffer solution found most suitable, of several tried for this purpose, was sodium cacodylate, μ 0.05, pH 7.0. Protein on papers was located, following electrophoresis, by staining with bromophenol blue. The bound stain was estimated by cutting segments 2 mm. wide from the paper, eluting them 30 minutes in 3 ml. of 0.01 N NaOH and then measuring the optical density of the eluates at 575 m μ .

For assay of enzymes separated by paper electrophoresis, segments cut from the papers without drying were added directly to suitable buffer-substrate solutions. Blanks in such assays consisted of paper segments cut from protein-free areas of the paper, then treated in the same manner as the enzyme-containing segments.

Electrophoresis of nucleotides was conducted under conditions similar to those used in the electrophoresis of proteins but with the following buffer solutions: (I) ammonium formate, μ 0.1, pH 3.5; (II) sodium phosphate, μ 0.1, pH 5.8; and (III) sodium phosphate, μ 0.1, pH 6.2.

Paper chromatography of nucleotides was carried out on Whatman No. 1 or No. 3 paper with the following solvent systems: (A) isobutyric acid-0.5 N ammonium hydroxide (10/6, v/v), pH 3.7; (B) 0.1 M sodium phosphate pH 6.8 - ammonium sulphate-isopropanol (100/60/2, ml./g./ml.); (C) 1 M ammonium acetate pH 9.0 - ethanol (30/70, v/v) saturated with sodium tetraborate.

Nucleotides were located visually on paper with the aid of a Mineralight lamp. The nucleotides were estimated by elution in 4 ml. of 0.01 M HCl followed by measurement of the optical density of the eluate at the appropriate wavelength in the ultraviolet region. Reference blanks were prepared from pieces of paper of the same sizes as those of the pieces containing the nucleotides, but cut from nucleotide-free portions of the paper at the same distances from the origin.

Further purification of lingcod RNase

The stability of lingcod RNase to heat at several pH's was investigated. The enzyme was found to be much more sensitive to heat at pH 2.5 or 3.5 than at pH 6.6 or 9.0. At these pH's 99%, 85%, 21% and 50%, respectively, of the total activity was destroyed in 10 min. at 58°C. However, while traces of the phosphomonoesterase activity also present in the muscle extracts were still detectable following heating at the lower pH's, none was detectable following heating at pH 9.0 with either RNA or p-nitrophenyl phosphate as substrate. As it was desirable to obtain an enzyme extract free from phosphomonoesterase activity in order that the products of RNase action could be investigated, heating at pH 9.0 was made a part of the purification procedure. Table I summarizes the results of the modified procedure, the earlier steps of which have been described in the preceding report. It will be noted that the extract possessed phosphodiesterase activity, as measured with bis(p-nitrophenyl)phosphate as substrate, in addition to RNase. Bis(p-nitrophenyl)phosphate was introduced as a substrate at this time in an attempt to ascertain if the RNase activity might be due to the action of a relatively non-specific phosphodiesterase, or if such phosphodiesterase(s) might be present.

The data indicate that the RNase and phosphodiesterase activities are not due to the same enzyme, as the losses in the two activities and also the changes in their specific activities during the pH 9.0 heating step are not equal. It should be noted that the loss in phosphodiesterase activity indicated is a maximum, as a portion of the loss must be attributed to the removal of the phosphomonoesterase which, when present, increases the apparent activity of the phosphodiesterase under the test conditions.

An extract, freed from phosphomonoesterase activity as described above, was subjected to electrophoresis on paper with the results recorded in Fig. 1. The position of the RNase activity did not correspond to the position of the two major phosphodiesterase activities, and the position of none of these three activities corresponded exactly with either of the two major protein peaks. A portion of each phosphodiesterase-containing area was obtained free from RNase activity, consequently it appears that neither of the diesterases can degrade RNA. On the other hand, the RNase may have low activity against bis(p-nitrophenyl)phosphate, as eluting the peak of the RNase activity (1 cm. segment) from the paper and subjecting it to electrophoresis once more did not

greatly reduce the phosphodiesterase activity found at the position of the RNase peak. However, the phosphodiesterase activity found in this position is of such a low order of magnitude that the accuracy of the measurement is perhaps open to question.

TABLE I - Partial purification of ribonuclease from muscle of lingcod (*Ophiodon elongatus*).

Extract after	Total activity ¹	Ribonuclease		Phosphodiesterase		Phosphomonoesterase	
		Specific activity ²	% recovery	Specific activity ³	% recovery	Specific activity ⁴	% recovery
Clarification	2952	1.64	100				
Several steps ⁵	1260	11.5	43	0.19	100	7.5	100
pH 9.0, 58°C. for 10 min.	638	7.0	22	0.14	62	0	0

¹ Units from 800 g. lingcod muscle.

² Units/mg. protein N.

³ μM p-nitrophenol released/2 hr./mg. protein N.

⁴ μM p-nitrophenol released/hr./mg. protein N.

⁵ $(\text{NH}_4)_2\text{SO}_4$ precipitation, lyophilization and isoelectric precipitation.

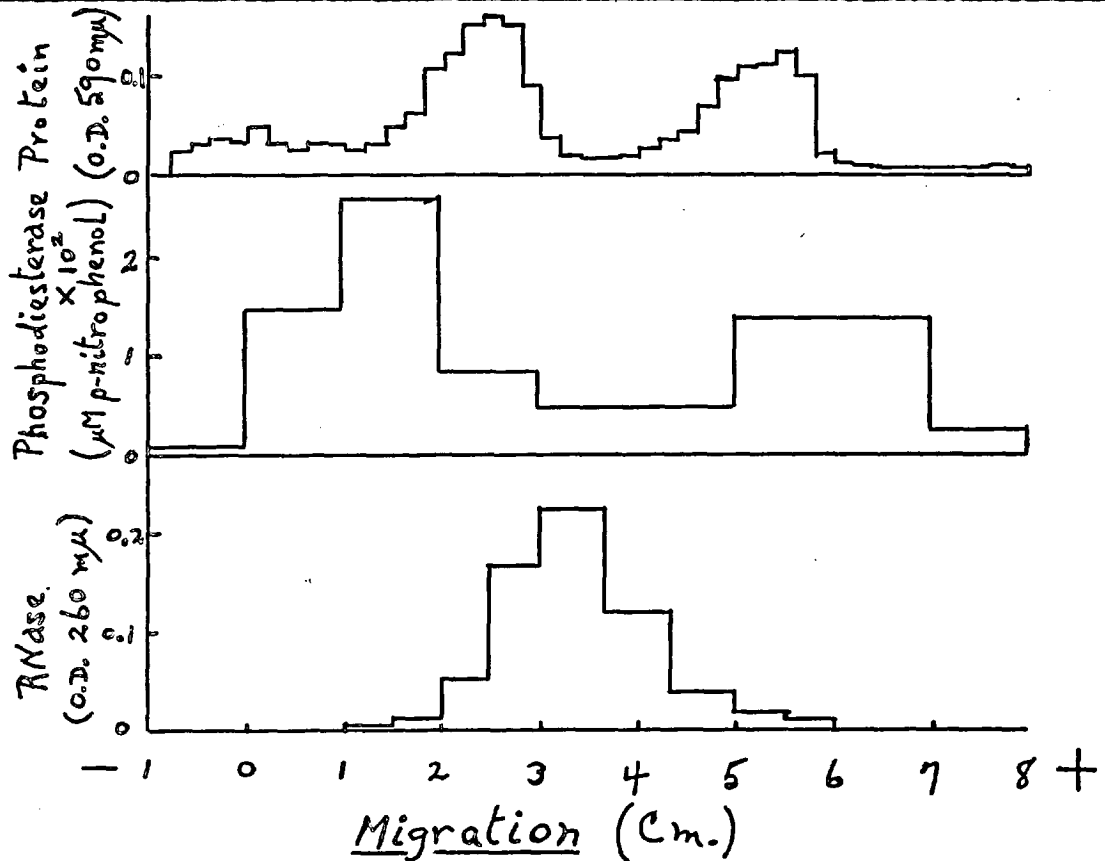


Fig. 1. Electrophoresis of lingcod muscle extract on paper. 9.6 μg . protein N applied/cm. length of starting line. Total protein N in strip for protein-bound dye determination, 14 μg ., for phosphodiesterase, 115 μg ., and for ribonuclease, 48 μg . p-nitrophenol released by phosphodiesterase control 0.0825 μM . Increase in O.D. at 260 m μ by RNase control, 0.533.

The influence of pH and possible inhibitors and activators on lingcod RNase and phosphodiesterase activities.

The influence of pH on the RNase and phosphodiesterase activities of a purified lingcod preparation was investigated and each was found to exhibit a maximum activity near pH 6.5 (Fig. 2). Only one activity peak was found in the curve for phosphodiesterase, although two enzymes were present.

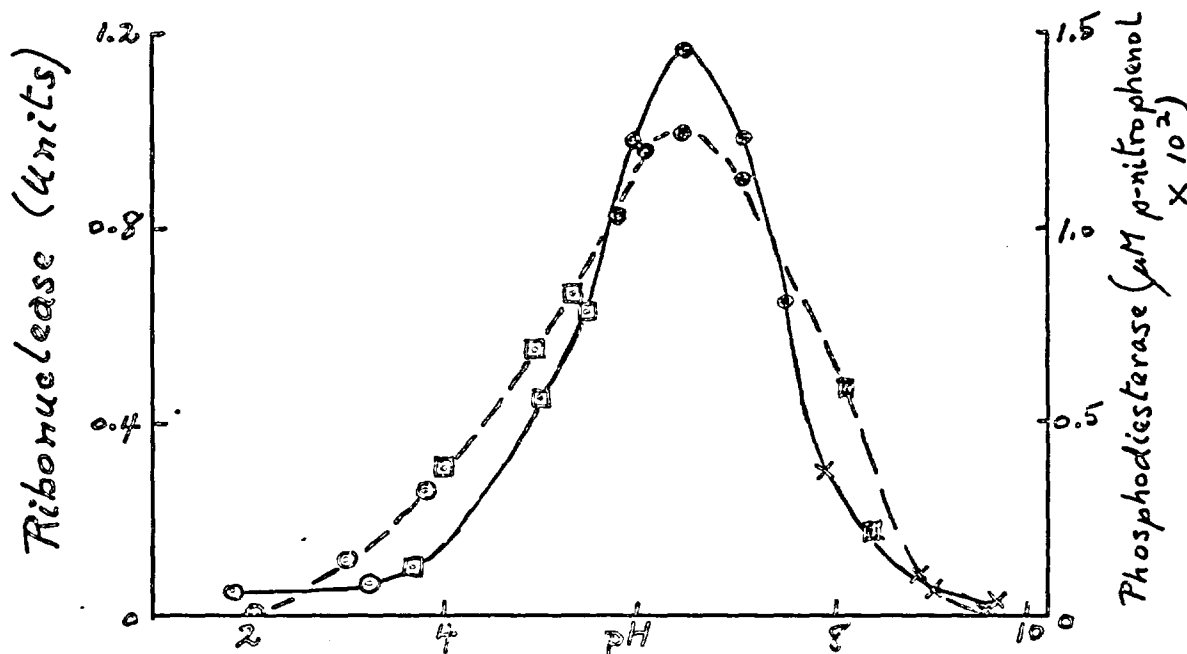


Fig. 2. Influence of pH on the RNase and phosphodiesterase activity of lingcod muscle extract. RNase ———. Phosphodiesterase ———. Buffers (0.025M): Glycine.HCl O—O, sodium acetate □—□, sodium cacodylate ●—●, sodium diethylbarbiturate ■—■, sodium borate x—x. pH's indicated are those of the complete reaction mixture.

The influence of a number of possible inhibitors and activators on the RNase and phosphodiesterase activities was examined. Results are tabulated in Table II. Zn^{++} , Cu^{++} and fluoride (the latter at high concentration) strongly inhibited both activities, while monoiodoacetate and formaldehyde inhibited RNase, but had little or no effect on phosphodiesterase. No appreciable activation of either activity was observed with any of the compounds tested.

Products of the action of lingcod RNase on RNA

The final products of the breakdown of RNA by the phosphomonoesterase-free lingcod extract were investigated by incubating RNA with the extract for 48 hours, then separating the products of the reaction by ionophoresis in buffer I. From their migration on ionophoresis and from their ultraviolet absorption spectra in neutral, acid and alkaline solution, adenylic, cytidylic, guanylic and uridylic acids were tentatively identified as products of the reaction. By chromatography in solvent C, the possibility that any of the four were 5' isomers was eliminated. The four 5' mononucleotides have R_f values of 0.02 or less in this solvent, while the 2' and 3' isomers of adenylic, cytidylic, guanylic and uridylic acids have R_f values of 0.1, 0.11, 0.065 and 0.13, respectively. The four mononucleotides from the digestion mixture behaved as did known mixtures of the 2' and 3' isomers in this solvent. Adenylic and guanylic acids from the reaction mixture were finally identified as the 3' isomers by co-chromatography on paper with known compounds in solvent B.

TABLE II - The influence of possible activators and inhibitors on RNase and phosphodiesterase activities of lingcod muscle extract.

Addition ¹	Concentration M	% inhibition (-) or activation (+)	
		RNase	Phosphodiesterase
None		0	0
NaCl	0.01	+7	+4
"	0.05	0	0
"	0.1	-8	0
Na ₂ SO ₄	0.005	+4	0
NaF	0.0005	-4	0
"	0.005	-33	-21
"	0.1	-89	-98
ZnSO ₄	0.005	-59	-69
CuSO ₄	0.005	-93	-93
MgSO ₄	0.005	-3	+7
CaCl ₂	0.005	0	+3
² EDTA	0.025	-23	-3
(EDTA+	0.025)		
(ZnSO ₄	0.005)	-32	-12
DL-tartrate	0.005	-3	+3
Monoiodoacetate	0.01	-98	0
"	0.005	-91	0
"	0.0005	-55	0
Formaldehyde	0.5%	-88	-21

¹ Enzyme incubated in buffered solution with addition indicated for 15 minutes before the substrate was added.

² Di-sodium ethylenediamine tetraacetate.

Cytidylic and uridylic acids were identified as the 3' isomers by ionophoresis. Buffer III was used with cytidylic acid and buffer II with uridylic acid. These buffers may be used to distinguish between the three isomers of these nucleotides. Under the conditions described above, in 3 hours and 50 minutes in buffer III, the 5'-, 2'-, and 3'-monophosphates of cytidine migrated 21, 22.5 and 23.7 cm., respectively, and in the same time in buffer II, the 5'-, 2'-, and 3'-monophosphates of uridine migrated 19.5, 22 and 23 cm. Examination of the reaction mixture by two dimensional paper chromatography with solvent A as the first, and solvent B as the second solvent, revealed four ultraviolet absorbing spots corresponding to the positions of the four mononucleotides identified above, and a small amount of ultraviolet absorbing material that streaked a short distance in solvent A, but that did not move appreciably in solvent B. Spectrophotometric estimation of the mononucleotides separated from the reaction mixture by ionophoresis resulted in 83% of the RNA phosphorus added to the digestion mixture being accounted for in the four mononucleotides (RNA phosphorus added 3.92 μ M. Recovered in adenylic acid 0.85 μ M, cytidylic acid 0.61 μ M, guanylic acid 1.08 μ M and uridylic acid 0.71 μ M). The possibility that small amounts of other mononucleotides may have been formed cannot be ruled out, but none was detected by the methods used. A similar procedure to the above was carried out, but with an enzyme solution purified by repeated electrophoresis on paper as described above. All four 3'-mononucleotides were again identified from the digestion mixture, but only 25% of the RNA phosphorus was accounted for by these compounds. Examination by two dimensional paper chromatography, as described above, revealed the presence of the four mononucleotides, together with a relatively large amount of material streaked a short distance from the origin in solvent A, but not moving appreciably in solvent B. Thus the enzyme purified in this manner releases all four 3'-mononucleotides from RNA, but the extent to which it can degrade RNA is not clear, for sufficient purified enzyme has not yet been avail-

able to carry out what could be considered as exhaustive digestion of the substrate. The electrophoretic method of purification is laborious and yielded only very small amounts of the purified material. Further examination of the details of the initial stages of the breakdown of RNA by this enzyme must await the development of a purification method that will provide a much higher yield.

The ability of the lingcod extract (freed from phosphomonoesterase) to degrade RNA prepared from lingcod muscle (Summary No. 12 of this Station's Annual Report for 1956-57) was investigated. The material was degraded, but at a rate only about 15 to 20% of that observed with yeast RNA. As a matter of interest, the rates of digestion of these two substrates by crystalline pancreatic ribonuclease was also determined. Again, the yeast RNA was the more rapidly digested of the two.

Occurrence of RNase and phosphodiesterase activities in muscle of other marine fish

Extracts were prepared from the muscle tissue of several marine fish. These were carried through the ammonium sulphate precipitation, dialysis and lyophilization steps of the purification procedure used with lingcod, and were then examined for RNase and phosphodiesterase activities. The results are presented in Table III.

TABLE III - RNase and phosphodiesterase activities in muscle extracts of some marine fish.

Fish	Total protein N ¹	RNase Units/mg. protein N	Phosphodiesterase µM p-nitrophenol/mg. protein N released (x10 ³)
	mg.		
Dog-fish (<u>Squalus</u> <u>suckleyi</u>)	117	0.5	75
Herring (<u>Clupea</u> <u>pallasii</u>)	27	1.7	120
Lemon sole (<u>Parophrys</u> <u>vetulus</u>)	67	0.8	9
Sculpin (<u>Cabezon -</u> <u>Leptocottus</u> <u>armatus</u>)	46	3.0	90
Spring salmon (<u>Oncorhynchus</u> <u>tshawytscha</u>)	138	0.17	2
Swordfish (<u>Xiphias</u> <u>gladius</u>)	88	0.5	93

¹ In preparation from 200 g. wet weight muscle tissue.

Evidence was obtained that both activities were present in the extracts of all fish examined. Apart from the fact that the enzymic activities are present in these fish, too much importance should not be attached to the quantitative aspects of the data, for the assays were arbitrarily conducted under conditions optimum for lingcod

enzymes, and these conditions may not be optimum for enzymes from other fish. Also, variations up to about 2-fold have been observed between the activities of preparations from different lingcod, the reasons for which are not known.

SUMMARY NO. 25

THE PHOSPHOMONOESTERASE ACTIVITY OF FISH MUSCLE EXTRACTS

N. Tomlinson

During a study of ribonuclease in lingcod muscle extracts, the presence of phosphomonoesterase(s) was also demonstrated (Summary No. 13 of this Station's Annual Report for 1956-57). Phosphomonoesterases probably play an important part in the degradation of many compounds in fish muscle (e.g. nucleotides, sugar phosphates) and for this reason a study of these enzymes has been undertaken in conjunction with the study of ribonuclease.

Materials and methods

The method of preparation of the enzyme extract was the same as that described previously (Summary No. 13 of this Station's Annual Report for 1956-57) for ribonuclease, i.e., extraction at pH 6.2, ammonium sulfate precipitation, dialysis, and lyophilization. Other steps will be described below with the results.

Two substrates were used in measurement of phosphomonoesterase activity. The first, di-sodium-p-nitrophenyl-phosphate (NPP), was selected for use as it could be expected to be a satisfactory substrate for several different phosphomonoesterases that might be present in the extracts. The second, adenosine-3'-phosphate (AMP), as it would serve as substrate for those particular phosphomonoesterases (nucleotidases) capable of attacking the 3'-nucleotides now known to be the products of the action of the ribonuclease of lingcod on RNA (Summary No. 26 of this report).

Enzyme assay systems with either substrate contained 0.025 M buffer, of composition and pH as indicated in figures and tables. Substrate concentrations were 0.018M for NPP and 0.0013M for AMP. Enzymatic hydrolysis of NPP was measured by determination of p-nitrophenol (NP) released (see Summary No. 26 of this report), and hydrolysis of AMP by colorimetric measurement of orthophosphate (Pi).

The pH values given in figures and tables are those of the complete reaction mixture.

Results

(a) With NPP as substrate -

Figure 1 shows the influence of pH on the phosphomonoesterase activity of an extract prepared from lingcod muscle as described above (referred to hereafter as the "crude" extract) and of the same extract following heating at 25°C. for 1 hour at pH 4.5 ("pH 4.5 purified" extract). The shape of the two curves suggests the presence of at least two phosphomonoesterases in the "crude" extract, one relatively stable at pH 4.5, the other labile. A further 15 minutes heating at 30°C. and pH 4.5 did not alter the shape of the curve given by the "pH 4.5 purified" extract, indicating that the activity of this extract observed at pH 5.0 was not due to a residue of the enzyme labile at pH 4.5.

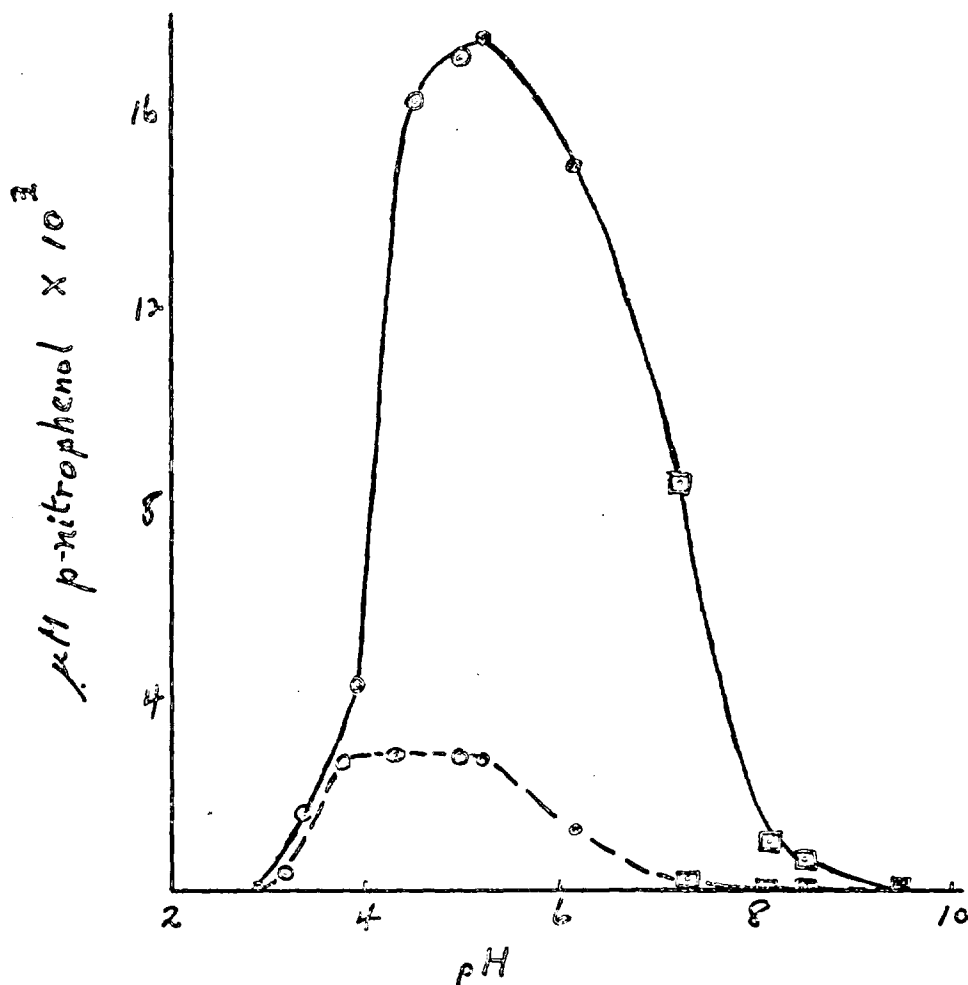


Fig. 1 Influence of pH on phosphomonoesterase activity of "crude" —●—, and "pH 4.5 purified" - - -○- extracts of lingcod muscle. Buffers. Glycine. HCl ○—○, sodium succinate ●—●, tris. HCl □—□, and sodium borate ■—■. Substrate p-nitrophenyl phosphate.

Figure 2 shows the influence of pH on the activity of the "crude" extract measured with or without a 15-minute preincubation of the enzyme at the pH of measurement prior to addition of the substrate.

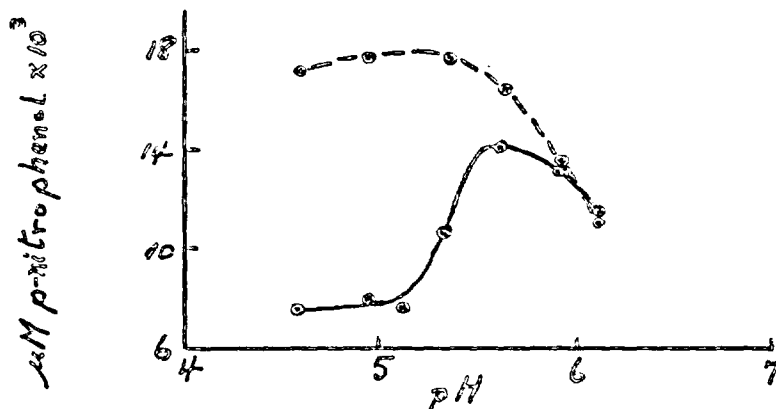


Fig. 2 Influence of pH and preincubation on phosphomonoesterase activity of "crude" lingcod muscle extract. No preincubation of enzyme at pH indicated prior to addition of substrate - - -○-, enzyme preincubated 15 minutes at pH indicated prior to addition of substrate ●—●. Sodium succinate buffers used throughout, substrate as in Fig. 1.

The extreme lability of part of the activity of the "crude" extract at pH 5.0 or lower is emphasized. An examination of the course of the reaction at pH 4.9 without preincubation of enzyme before addition of substrate showed that the reaction was zero order for at least 30 minutes. Hence no inactivation of enzyme occurred in the presence of the substrate. The substrate thus protects the labile enzyme from inactivation at pH 4.9 and 25°C. The activity of the "crude" extract also decreases rather rapidly during the repeated freezing and thawing that occurs in the storing and use of such extracts (pH 6.5) from day to day.

Table I lists the influence of several substances on the activity of both the "crude" and "pH 4.5 purified" extracts. The most striking effect observed was the very high activation of the "pH 4.5 purified" preparation by Zn^{++} at pH 5.3, and the complete lack of effect of this ion at pH 4.08, suggesting the presence in this preparation of two phosphomonoesterases, one activated by Zn^{++} , the other not influenced by this ion. The difference in sensitivity of the "crude" and "pH 4.5 purified" extracts at pH 5.3 to tartrate, fluoride, formaldehyde, and 0.0002M copper sulfate, indicates the presence of two different enzymes in the "crude" extract, one of which is eliminated by the pH 4.5 treatment. Evidence will be presented below that this latter enzyme, too, is not identical with the Zn^{++} activated enzyme.

TABLE I - The influence of possible inhibitors or activators on the phosphomonoesterase activity of lingcod muscle extracts¹.

Addition	Concentration M	% activation (+) or inhibition (-)		
		"Crude" extract ²	"pH 4.5 purified" extract ³	
		(at pH 5.3) %	(at pH 5.3) %	(at pH 4.08) %
None	-	0	0	0
NaCl	0.01	+9	+9	-5
Natartrate	0.005	-26	-98	-100
NaF	0.0005	-23	-89	-100
Formaldehyde	0.17	-49	-18	0
CuSO ₄	0.0002	-71	-18	0
CuSO ₄	0.005	-93	-89	-61
ZnSO ₄	0.005	+194	+700	0
MgSO ₄	0.005	+13	+7	0
CaCl ₂	0.005	+3	+5	-8
Na ₂ SO ₄	0.005	0	+5	-3
EDTA	0.025	0	0	-4
(EDTA)	0.025)	+6	0	-8
(+CuSO ₄)	0.005)			

¹ With p-nitrophenyl phosphate as substrate.

² "Crude" extract 42 µg. protein N/0.33 ml. reaction mixture.

³ "pH 4.5 purified" extract 60 µg. protein N/0.33 ml. reaction mixture.

It was known as a result of the work done on the purification of ribonuclease described above, that heating a lingcod extract at pH 9.0 and 58°C. for 10 minutes ("pH 9.0 purified" extracts) would remove all evidence of phosphomonoesterase as measured in the absence of added "activators" and with either NPP or RNA as substrate. The effect of Zn^{++} on such a preparation was investigated. Table II shows that the addition of Zn^{++} does reveal the presence of phosphomonoesterase activity not otherwise detectable, and also shows that this activity is much more stable to heating at pH 7.0 than at pH 9.0. Heating "crude" extract at pH 7.0 (58°C., 10 minutes) was adopted as a step in the purification of this activity ("pH 7.0 purified" extract).

TABLE II - The influence of pH on the heat sensitivity of a Zn^{++} requiring phosphomonoesterase from lingcod muscle.

Treatment of "crude" extract	p-nitrophenol released ¹ ($\mu M \times 10^3$)	
	No Zn^{++} added	Zn^{++} added
None	188	540
Heated ² at pH 7.0	<1	138
" " " 8.0	<1	44
" " " 9.0	<1	18

¹ In 0.33 ml. of reaction mixture at pH 6.2/hour. Protein N in each 0.33 ml. reaction mixture: untreated extract, 112 μg . pH 7.0, pH 8.0 and pH 9.0, heated extracts, 58.5 μg ., 58.5 μg . and 61.5 μg ., respectively. Differences in protein N due to denaturation of protein during heating only.

² At 58°C. for 10 minutes in each case.

The use of heat at more acid pH's is not desirable, for traces of phosphomonoesterase activity not requiring Zn^{++} remains in such preparations. Table III records data indicating that 0.005M is about the optimum concentration of Zn^{++} for activation of phosphomonoesterase in the "pH 7.0 purified" extract. The following salts, each at a concentration of 0.005M, produced no activation of the "pH 7.0 purified" extract: $CaCl_2$, $CuSO_4$, $CdSO_4$, $NaCl$, Na_2SO_4 , KCl and $MgSO_4$.

TABLE III - Influence of $ZnSO_4$ concentration on phosphomonoesterase activity of "pH 7.0 purified" lingcod muscle extract.

$ZnSO_4$ M	p-nitrophenol released ¹ $\mu M \times 10^3$
0.05	20.5
0.025	57.5
0.01	148.5
0.0075	155.
0.005	158.
0.0035	154
0.002	148.5
0.001	122.
0.0005	90.
0.00025	57.5
0.	<1

¹ In 0.33 ml. reaction mixture/hour. 60 μg . protein N extract in each 0.33 ml. mixture. pH 6.2.

Figure 3 shows the influence of pH on the phosphomonoesterase activity of the three different preparations in the presence of Zn^{++} . An important feature of this figure is the fact that while the pH optimum of the "crude" and "pH 4.5 purified" preparations in the presence of Zn^{++} is near 6.2, the optimum for the "pH 7.0 purified" extract is near 5.5. While this difference might possibly be due to some alteration in the enzyme in the purification procedure, it is more likely that there are two Zn^{++} activated enzymes in the "crude" extract, one of which is more labile to heat at pH 7.0 than the other.

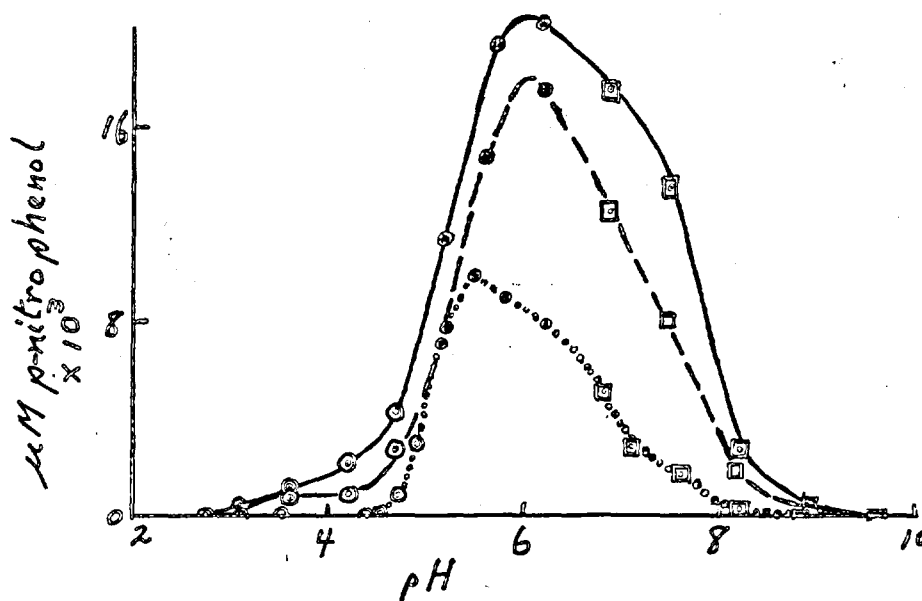


Fig. 3 Influence of pH on the phosphomonoesterase activity of variously treated extracts from lingcod muscle in the presence of Zn^{++} (0.005M). "Crude" extract ●—●, "pH 4.5 purified" extract — — —, "pH 7.0 purified" extract ○.....○. Buffers and substrate as indicated in Fig. 1.

Several lines of evidence indicate that the Zn^{++} activated phosphomonoesterase(s) is (are) not the same as the acid labile enzyme of the "crude" extract. (1) The pH optima for the activities are different. (2) Fifteen minutes' preincubation of the "crude", "pH 4.5 purified" and "pH 7.0 purified" extracts in the presence of Zn^{++} between pH 5.0 and pH 6.0 has little effect on the shape of the pH response curve of the phosphomonoesterase activity of these extracts, whereas this treatment has a very marked effect on the curves for the "crude" extract in the absence of Zn^{++} . (3) Zn^{++} does not activate any of the extracts at pH 4.4 or lower (at which pH the acid labile enzyme has high activity), whether these extracts are preincubated in the absence of substrate or not. In fact the activity of the crude extract (without preincubation) is actually inhibited at this pH by Zn^{++} (Table IV).

To sum up, it thus appears that the "crude" extract must contain at least four acid phosphomonoesterases, two of which are activated by Zn^{++} , and all of which, although having low pH optima, are active up to pH 7.5 or higher.

The course of the reaction with "pH 7.0 purified" extract has been examined at pH 4.9 and pH 6.2. The reaction is zero order for at least 30 minutes at either pH. Similarly, the reaction is zero order (at pH 6.2) with either 0.005 or 0.0005M $ZnSO_4$. The rate is thus proportional to Zn^{++} concentration over a limited range (see also Table III). It has also been found that preincubation in the presence of Zn^{++} without substrate added is not necessary for activation of the system, for when Zn^{++} is added to a substrate-enzyme mixture, maximum rate of reaction is reached almost instantly.

TABLE IV - Influence of Zn^{++} on the phosphomonoesterase activity of "crude", "pH 4.5 purified" and "pH 7.0 purified" extracts of lingcod muscle at pH 4.4.

Extract ¹	Zn^{++} ²	Preincubated ³	μM p-nitrophenol released ($\times 10^3$)/30 min.
"Crude"	-	No	51
"	+	No	39
"	-	Yes	28
"	+	Yes	27.5
pH 4.5 purified	-	No	19
"	+	No	18.5
"	-	Yes	18.7
"	+	Yes	18.5
pH 7.0 purified	-	No	<1
"	+	No	<1
"	-	Yes	<1
"	+	Yes	<1

¹ Protein N/0.33 ml. reaction mixture. "Crude" 37.5 $\mu g.$, "pH 4.5 purified" 20 $\mu g.$, "pH 7.0 purified" 60 $\mu g.$

² 0.005 M.

³ 15 minutes, 25°C., at pH 4.4 before substrate added.

(b) With AMP as substrate -

It was found that neither of the Zn^{++} activated phosphomonoesterases attacked AMP. Thus "pH 7.0 purified" extract released no Pi from AMP with or without Zn^{++} added, while both "crude" and "pH 4.5 purified" extracts released Pi, but Zn^{++} failed to increase the activity of these extracts (at pH 5.5).

The influence of pH on release of Pi from AMP was determined for both the "crude" and "pH 4.5 purified" extracts (Fig. 4), and was found to be essentially the same for both extracts (unlike the effect when NPP was substrate). The shape of these response curves was different from those of the same extracts with NPP as substrate. This would appear to indicate that the phosphomonoesterase attacking AMP is the same in both extracts, but that it is different from the two enzymes attacking NPP.

However, the data for inhibitors (Table V), while rather definitely ruling out the acid labile enzyme of the "crude" extract as one capable of attacking AMP, point to the acid stable enzyme as possibly the one in both preparations that attacks AMP, for the following reasons. First, while the effect of the inhibitors was practically identical with either preparation and AMP as substrate, their effects were very different with the two preparations when NPP was substrate. This is additional evidence (to that afforded by the influence of pH on the activity of the two preparations with AMP as substrate) that the same enzyme in both preparations attacks AMP, while, as indicated before, in the "crude" extract, two enzymes attack NPP. Second, in the preparation of the "pH 4.5 purified" extract there was a loss of 42.5% of the total activity vs. AMP, and this activity in both preparations was essentially completely inhibited by tartrate or fluoride, as was the activity of the "pH 4.5 purified" preparation toward NPP. If now the assumption be made that the activity of the "pH 4.5 purified" extract with AMP and NPP is due to a single enzyme, and that the second

enzyme attacking NPP in the "crude" extract is not inhibited to any marked extent by either tartrate or fluoride, then it should be possible to calculate (by applying the quantitative data for AMP activity in the two extracts to the NPP of the "pH 4.5 purified" extract), the expected inhibition of the "crude" extract vs. NPP by these two inhibitors. This was done, and the calculated values were 48% and 45% for tartrate and fluoride, respectively. These are in quite fair agreement with the experimental values of 46% and 36%, and thus the data are consistent with expectations if one relatively acid stable phosphomonoesterase attacks both AMP and NPP. There are, of course, objections to this. For example, the different shapes of the pH response curves suggest separate enzymes, but it is possible this effect is due to differences in the substrate rather than in the enzyme. Similarly, the extent of inhibition by formaldehyde is different with the two substrates, but this again may be related to substrate differences.

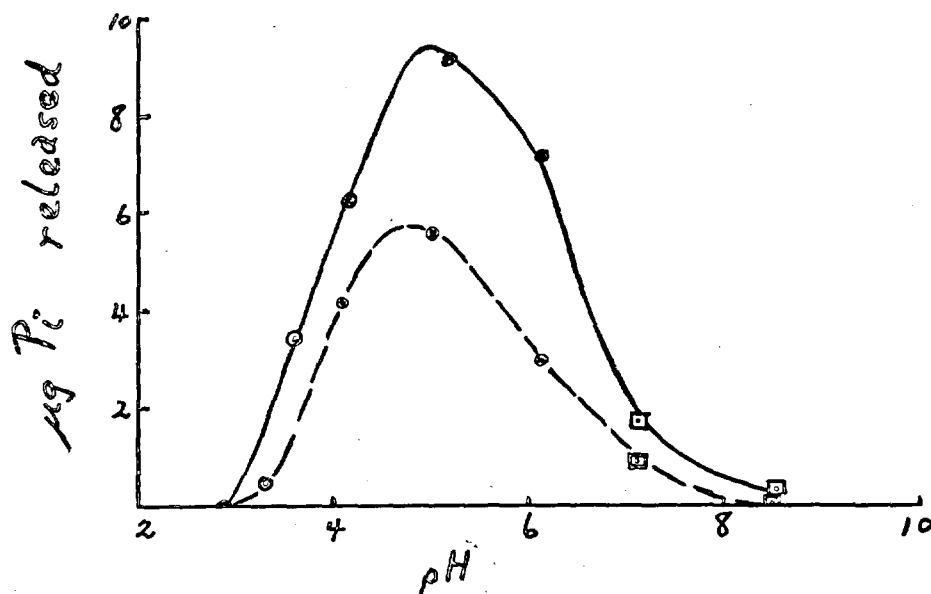


Fig. 4 Influence of pH on the phosphomonoesterase activity of "crude" —•— and "pH 4.5 purified" •- -•- extracts from lingcod muscle with adenosine -3'-phosphate as substrate. Buffers as indicated in Fig. 1.

Phosphomonoesterase activity in muscle extracts of marine fish

"Crude" extracts have been prepared from muscles of lemon sole, spring salmon, dog fish, sculpin (cabezon), herring, and Atlantic sword fish and have been examined for ability to degrade NPP at pH 5.5 and pH 8.5. The extracts from each of these fish possessed enzymic activity with NPP, all but one having much greater activity at the acid pH. The one exception was spring salmon, the extract from which had a little higher activity at the alkaline pH.

TABLE V - Influence of tartrate, fluoride and formaldehyde on phosphomonoesterase activity of "crude" and "pH 4.5 purified" lingcod muscle extract.

Substrate	Inhibitor ¹	% inhibition ²	
		"crude" ² extract	"pH 4.5 purified" ² extract
<u>AMP</u>	none ³	0	0
	Na tartrate	100	100
	NaF	96	98
	Formaldehyde	29	28.5
<u>NPP</u>	none ³	0	0
	Na tartrate	46	98
	NaF	36	93
	Formaldehyde	37.5	19

¹ Inhibitor concentrations: tartrate 0.005 M, NaF 0.0005M, formaldehyde 0.17M.

² Protein N in reaction mixture (0.33 ml): "crude" 375 ug., "pH 4.5 purified" 200 ug.

³ Pi released from AMP and μ M NP from NPP in absence of inhibitor: "crude" 6.2 μ g. and 0.48 μ M, "pH 4.5 purified" 3.6 μ g. and 0.133 μ M, respectively.

SUMMARY NO. 26

LINGCOD MUSCLE NUCLEOSIDE PHOSPHORYLASE

H.L.A. Tarr

Previous studies concerning this general problem were discussed in this Station's Annual Report for 1956-57, Summary No. 13, pp. 23-30. One short scientific paper has been published and another submitted for publication in which some of the information contained in last year's report and some of which is described in the present report has been included. The work on the enzyme has been completed and certain data concerning it which were not included in last year's report are given herewith.

Comparative activity of crude and purified preparations

It was found that liberation of orthophosphate from a ribose 1-phosphate in presence of hypoxanthine (10 μ M/ml. of each substrate) was linear when crude or purified enzyme was used in 0.05 to 0.75 mg. protein N/ml. concentration (37°C.; 0.05 M veronal buffer pH 7.4). Under these conditions the activity of the purified preparations was 32 μ M/orthophosphate/mg. protein N/hour, which compares favourably with several similar mammalian liver enzyme preparations. The XE 64 cation resin purification procedure removed about 95% of protein from crude preparations but occasioned a marked loss of the active enzyme component (about 75%). The purified preparations were only three times as active as the crude preparations, calculated on a protein N basis.

The pH-activity relationship of purified nucleoside phosphorylase was determined using succinate, veronal and "tris" buffers. The results showed that the buffers used had no noticeable depressing effect on the enzyme, which exhibited a very wide range of marked activity (pH 5.5-7.5). It was almost inactive at pH 5 and showed slow decrease in activity between pH 7.5 and 9.2.

Pentose phosphate specificity

The enzyme was fully active with α ribose 1-phosphate (both chemically synthesized and natural esters) and natural deoxyribose 1-phosphate, but showed no activity with chemically synthetic arabinose 1-phosphate (mixture of α and β isomers) or β ribose 1-phosphate.

Equilibrium

The equilibrium depended somewhat on the substrate concentration and, with the guanine-guanosine system, was undoubtedly greatly influenced by the great insolubility of guanine. With 10 μ M/ml. concentrations of reactants the equilibrium was 85% in favour of inosine synthesis in the hypoxanthine-ribose 1-phosphate system, and 75% in favour of guanosine synthesis in the guanine-ribose 1-phosphate system. With higher concentrations of reactants in the latter system (e.g. 50 μ M/ml.) the equilibrium was altered until it was greatly in favour of ribose 1-phosphate synthesis.

Proof of unity of the enzyme

Experiments were carried out in which, using the hypoxanthine-inosine system, it was shown that, when the enzyme was saturated with respect to one of its pentose-phosphate substrates (ribose 1-phosphate or deoxyribose 1-phosphate in 20 or 40 μ M/ml. concentration), the addition of an equivalent concentration of the other pentose phosphate did not accelerate the reaction. This can be regarded as strong evidence that these two pentose phosphates are activated by a single enzyme.

Heat lability

It was shown that purified nucleoside phosphorylase retained full activity for 3 hours at 50°C., but in 5 hours at this temperature this decreased to about 60%, and in 7 hours to about 20, of the initial activity.

Preparation of dicyclohexylammonium deoxyribose 1-phosphate

A further larger scale preparation of this very labile phosphate ester was prepared (as described in last year's report on page 24). In this experiment 2 mM of deoxyguanosine (536 mg.) yielded 440 mg. (1.07 mM) of pure dicyclohexylammonium deoxyribose 1-phosphate. C, 49.45; H, 9.20; calculated for $C_{17}H_{37}O_7N_2P$ (M.W. 412.46); C, 49.5; H, 9.04. The deoxyribose content (Dische method) was 103%, and the orthophosphate liberated in 100 minutes at 20°C. at pH 4.0, 98%, of the theoretical amounts.

The specific rotation of this compound $[\alpha]_D^{22.5} = +38.76^\circ$ (C, 2.57 in H_2O) was very similar to that which has been reported elsewhere for chemically synthesized dicyclohexylammonium α -D-ribose-1-phosphate $[\alpha]_D^{20} = +40.3^\circ$. This, in addition to its activity in this enzyme-system, suggests that natural deoxyribose 1-phosphate has an α configuration.

Dicyclohexylammonium ribose 1-phosphate

Some improvement in preparation of this compound has been effected by applying the procedure used in preparation of deoxyribose 1-phosphate, namely that in which the crude barium salt is changed into the cyclohexylammonium salt by passage through the cyclohexylammonium form of a cation exchange resin such as Dowex 50. As previously noted (p. 24-25 of the Annual Report for 1956-57) the yield on the basis of the guanosine used is poorer than with the deoxyribose compound, due to presence of a rather strong phosphoriboisomerase enzyme. In a typical procedure

2 mM of guanosine yielded 0.34 mM (145 mg.) of crystalline dicyclohexylammonium ribose 1-phosphate. C, 46.95; H, 8.64; P, 7.10. Calc. for $C_{17}H_{37}O_8N_2P$ (M.W. 428.46): C, 47.65; H, 8.71; P, 7.23. The ribose content was 98% of the theoretical and the preparation contained 0.7% of orthophosphate.

SUMMARY NO. 27

PHOSPHORIBOMUTASE AND PHOSPHOGLUCOMUTASE ACTIVITY OF LINGCOD MUSCLE ENZYME PREPARATIONS

H.L.A. Tarr

During work on lingcod muscle nucleoside phosphorylase it became evident that the crude enzyme preparation possessed other enzymic activities including those of phosphoribomutase and phosphoglucomutase systems. Initial studies have been made of these from the point of view of a general determination of the reactions involved, rather than from that of an immediate study of a purification of the enzymes, which it is hoped to carry out later.

The literature concerning similar mammalian and yeast enzymes is not extensive but contains useful information. The following reactions have been described and in general most investigators have found that the somewhat crude preparations used by them contained both phosphoglucomutase and phosphoribomutase activities, the former being about 100 times that of the latter as calculated on a protein nitrogen basis. In certain cases it has been shown that the phosphogluco- and phosphoribomutases are very probably different enzymes, but no clear-cut separation has been effected. The following reactions are known to occur:

- (1) $G\ 1-P + G\ 1,6 - P_2 \rightleftharpoons G\ 1,6 - P_2 + G\ 6 - P.$
- (2) $R\ 1-P + G\ 1,6 - P_2 \rightleftharpoons R\ 1,5 - P_2 + G\ 6 - P.$
- (3) $R\ 1-P + R\ 1,5 - P_2 \rightleftharpoons R\ 1,5 - P_2 + R\ 5 - P.$

(Where $G\ 1-P$ = glucose 1-phosphate; $R\ 1-P$ = ribose 1-phosphate; $G\ 1,6 - P_2$ = glucose 1,6-diphosphate, etc.).

Reaction (1) is catalyzed by phosphoglucomutase and (3) by phosphoribomutase. However, reactions such as (2) could presumably be catalyzed by an enzyme which could have both functions. All these "mutase" reactions are strongly stimulated by added "diphosphate" esters, and it seems that these are essential before the reactions can proceed actively. They have been considered as "co-enzymes" of these reactions, but are also able to enter into the reactions in concentrations of the same order as the monophosphate esters.

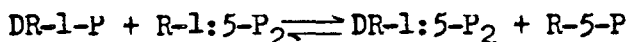
Isolation of deoxyribose 1,5-diphosphate

It was found that when deoxyribose 1-phosphate and ribose 1,5-diphosphate were incubated with crude lingcod muscle extract (the preparation used in the nucleoside phosphorylase studies), 6 different compounds could be identified in the mixture by paper chromatography. There were, in addition to the starting compounds, ribose 1-phosphate, ribose 5-phosphate, deoxyribose 5-phosphate and an apparently new compound. This compound was prepared in small amounts in a fairly high state of purity and was identified and described briefly for the first time. It is deoxyribose 1,5-diphosphate and the general method of preparation and analysis follows:

A number of preparations were made similarly and attempts were made to crystallize the compound. Thus the lyophilized dry powdered eluates from cyclohexylamine columns were taken up under as dry conditions as possible in absolute methanol, ethanol, n-propanol or n-butanol in different experiments. Cautious addition of anhydrous diethylether in the cold never caused crystallization, only an amorphous

mass precipitating. Needle crystals, similar to those obtained with the dicyclohexylammonium salts of ribose 1- and deoxyribose 1-phosphates have been seen in methanol solutions which have slowly dried over a desiccating agent, but attempts to obtain a pure crystalline compound have so far failed. Tetracyclohexylammonium deoxyribose 1,5-diphosphate is extremely hygroscopic and will undoubtedly be difficult to obtain in pure crystalline state.

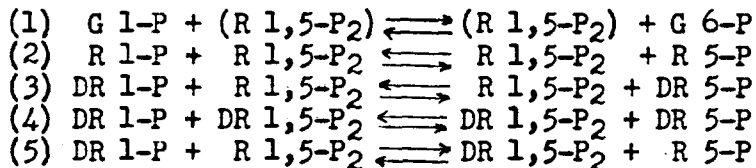
Incubation mixtures of equimolar quantities of dicyclohexylammonium DR-1-P and tetracyclohexylammonium R-1 : 5-P₂ with crude nucleoside phosphorylase yielded six compounds, as indicated by paper chromatography, and by colorimetric analyses of the eluates for deoxyribose, ribose and acid labile and comparatively acid stable phosphorus. Two of these compounds were the starting compounds, and others were ribose-1-phosphate, deoxyribose-5-phosphate and ribose-5-phosphate. In addition, an apparently new compound which moved slightly faster on paper chromatograms than did R-1 : 5-P₂ was detected. This compound gave a yellow zone with aniline hydrogen phthalate reagent, quite distinct from the red colour given by R-1 : 5-P₂, and this fluoresced strongly in ultraviolet light. Enzyme reaction mixtures containing 50-75 μ M of each of the starting compounds were incubated 3 hours at 30°C., and were then deproteinized with 50% propanol and subjected to preparative paper chromatography using Whatman 3 MM paper. The zones containing the new ester were eluted with ammonia water (pH 9.5-10), the solution concentrated to a small volume at low temperature and passed through a column containing a few ml. of Dowex 50,200-400 mesh, cation exchange resin in the cyclohexylamine form. The eluate and washings were evaporated to dryness in the cold and taken up in absolute methanol. On cautious addition of 3 volumes of diethyl ether a precipitate formed which, on drying for a few hours over phosphoric oxide in high vacuum, possessed properties which suggested that it was tetracyclohexylammonium deoxyribose-1:5-diphosphate (M.W., 690.76). Analyses of chromatogram eluates from several reaction mixtures indicated that about 30-35% of the R-1 : 5-P₂ used appeared as the corresponding deoxyribose derivative. In a typical experiment 50 μ M (35.3 mg.) of tetracyclohexylammonium R-1 : 5-P₂ yielded 12 mg. of tetracyclohexylammonium DR-1 : 5-P₂ which gave the following analyses (per cent. of theoretical) : deoxyribose, 100.5%; phosphorus labile in 100 minutes at pH 4.0 (measures DR-1-P phosphorus), 96.5%; and phosphorus labile in 60 minutes in 1N HCl at 100°C. (measures both DR-1-P and DR-5-P phosphorus), 98%. No orthophosphate was detected in this preparation. It is concluded that the following reaction occurs:



This work was made possible by donation of generous supplies of R-1 : 5-P₂ by Drs. Tener and Khorana.

Phosphoribomutase and phosphoglucomutase activity of lingcod muscle preparations

No purification of these enzyme activities has yet been attempted, all preliminary studies being conducted with lingcod muscle crude riboside phosphorylase which contains both enzymes. Initial studies have been entirely concerned with ascertaining which of the various possible "phosphoribomutase" activities are present in lingcod muscle and whether ribose 1,5- and/or deoxyribose 1,5-diphosphates act as "co-enzymes" in accelerating the reactions. The technique used involved measuring the changes in the amount of "labile" organic phosphorus by appropriate techniques as an indication of the changes in types of compounds initially present. These analyses were verified by qualitative demonstrations of the changes in the pentose or hexose phosphate esters. The results have shown that the following activities are present in lingcod muscles. In all experiments the monophosphate esters were used in 10 μ M/ml., and the diphosphate esters in 0.5 μ M/ml. concentrations.



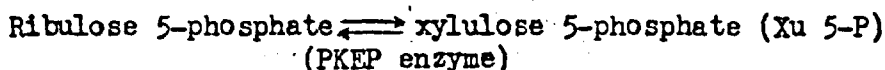
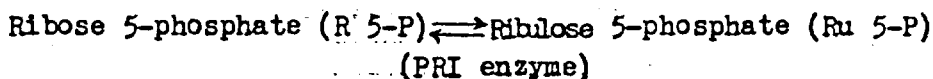
All four reactions proceeded in absence of added diphosphate ester, the final equilibrium, when it was attained, being in favour of formation of the more acid stable phosphate ester (G 6-P, R 5-P or DR 5-P). Since a crude enzyme preparation was used the results could mean that the reactions can proceed in complete absence of added diphosphate ester, or that they are present in very small amounts in crude enzyme preparations. Reaction (1) proceeded very much more rapidly than the other three reactions, but the comparative rates have not as yet been accurately measured. In experiments so far made reaction (1) has proceeded so rapidly that the possibility that R 1,5-P₂ acts as a co-enzyme in this system is still in doubt. Glucose 1,6-P₂, the ester which was first shown to be the natural co-enzyme of this particular system, has not yet been prepared in this laboratory and has therefore not been tested. While R 1,5-P₂ definitely accelerated reactions (2) and (3), DR 1,5-P₂ only accelerated the reaction between the deoxyribose phosphates (reaction 4). Attempts will be made to separate the various phosphoribomutase activities of lingcod muscle.

SUMMARY NO. 28

PHOSPHORIBOISOMERASE (PRI) AND PHOSPHOKETOPEPTIDOMERASE (PKEP) ACTIVITY OF LINGCOD MUSCLE

H.L.A. Tarr

Fairly recent work with certain mammalian and plant tissues have shown that both PRI and PKEP enzymes are present and are responsible for certain important enzymic steps in intermediary metabolism of the carbohydrates. The two activities concerned carry out the following general reactions:



During work on the phosphoriboisomerase activity of lingcod muscle extract, paper chromatographic procedures revealed the presence of certain apparently new sugar phosphate derivatives, which have subsequently been partially identified by paper and by ion exchange chromatography as almost certainly being Ru 5-P and Xu 5-P. So far work indicates that the main reaction products which result when R 5-P is incubated with crude lingcod muscle "nucleoside phosphorylase" is Xu 5-P and residual R 5-P, together with a little Ru 5-P. It would appear that the two enzymes mentioned above are present in lingcod muscle and crude Xu 5-P has been isolated as the barium salt. It is hoped that the enzymes responsible may be separated and their properties studied. So far, difficulties have been experienced in obtaining good yields of Xu 5-P and the preparations have not been very pure.

Preparation of D-ribulose and D-xylulose

In order to have a standard preparation of these rather unstable ketoses for reference purposes in colorimetric and spectrophotometric procedures use in identification of the products of the above reaction these two ketopentoses are being prepared.

Three g. of dry D-arabinose (Nutritional Biochem. Corp.) was refluxed with anhydrous pyridine according to the procedure of Glatthaar and Reichstein. Pyridine and arabinose were removed from the reaction mixture as described and the o-nitrophenylhydrazine was prepared both directly from the crude, syrupy ribulose, and also after first purifying the ribulose by quantitative paper chromatography, using 48-hour development in butanol/ethanol/H₂O : 10/1/2, and elution with 99% ethanol. A total of 300 mg. of D-ribulose o-nitrophenylhydrazine M.P. 165-166° and 170 mg. of a less pure product was obtained (M.P. 162°). This is being decomposed to obtain free ribulose as required and used directly in the cysteine-carbazole colorimetric procedure. The o-nitrophenylhydrazine used in the above reaction was prepared by the method of Bishler which involves preparation of o-nitroaniline hydrochloride, its diazotization with sodium nitrite and reduction with stannous chloride to yield the tin double salt of o-nitrophenylhydrazine hydrochloride. After removing the tin with H₂S the o-nitrophenylhydrazine hydrochloride is decomposed with sodium acetate and the red needle crystalline o-nitrophenylhydrazine recrystallized from benzene (M.P. 90°C.).

D-Xylulose has been prepared as the p-bromophenylhydrazone by the method of Schmidt and Treiber, which is in general closely similar to that used in preparation of D-ribulose o-nitrophenylhydrazine (M.P. 128°). It has been used successfully as a standard for Xylulose in the cysteine-carbazole reaction.

Preparation of 5-phosphoribonic acid

Since there was a slight indication that some 5-phosphoribonic acid might be formed from ribose 5-phosphate by lingcod muscle extract, a small amount of this material was prepared. It may be also useful as a specific inhibitor of phosphoriboisomerase.

The barium ions were removed from a watery solution of 250 mg. of commercial Ba R 5-P by a slight excess of the Dowex 50 H⁺ analytical grade cation exchange resin. The ester was oxidized by the alkaline hyperiodite method of Goebel. At the conclusion of the reaction Na⁺ ions were removed with Dowex 50 H⁺ (pH of solution 1.5) and the free iodine then removed by using a rotary evaporator at 37°C. The solution (5 ml.) was treated with powdered CaCO₃ to pH 5.0, cooled, filtered through a small sintered glass filter and the crude Ba phosphoribonic acid precipitated with 2 vols. of ethanol. The precipitate was washed with a little 65% ethanol, then absolute ethanol, and dried in vacuo over P₂O₅ (yield 135 mg.). This product was not completely soluble in water. The product was dissolved in 3 ml. of water and filtered to remove a gummy residue which proved to be almost completely insoluble in water. The clear filtrate, on treating with 2 vols. of ethanol, yielded 90 mg. of a dry powder which was apparently the neutral calcium salt of phosphoribonic acid (C₅H₈O₉P)₂ Ca₂ (M.W. 606.43). P, 10.23%; Found P, 9.72%. Paper chromatography, using acid and alkaline solvent systems which have been used for phosphate esters, gave 2 phosphorus zones (Hanes and Isherwood Spray) when the product applied to the paper was the neutral phosphoribonic acid from which Ca was removed by Dowex 50 H⁺ resin. When this solution was treated with 50% acetic acid before "spotting" for chromatography only a single zone appeared. This indicates that 5-phosphoribonic acid exhibits both free acid and lactone ring structures. Glucuronic and galacturonic acids exhibit similar lactone structures and paper chromatographic properties.

SUMMARY NO. 29

ANTIFUNGAL ANTIBIOTICS

B.A. Southcott
E.G. Baker

It has been established that sorbic acid is an effective agent in the control of mold growth on smoked fish (Summary No. 23 of this Station's Annual Report for 1954). Experiments have been carried out to assess the value of several antifungal antibiotics and to compare their activity with that of sorbic acid.

Antifungal antibiotics, in suitable solvents, were added to the 20% sodium chloride solutions in which the fish samples were to be soaked prior to smoking; chlortetracycline (CTC) was added to these solutions at a level of 10 p.p.m. to minimize bacterial growth on the samples during the storage period. Sorbic acid was used at a level of 1.0%, other test substances at .02%.

Pieces of fresh lingcod 2"x2"x1½", with skin on one side, were prepared; four pieces from different fish were soaked in each solution for one hour. The samples were subsequently drained 1½ hours. on wire racks, then smoked for 6 hours and cooled in a smoking tunnel. Each piece was placed in an individual plastic bag, heat sealed, and stored at 4°C. The samples were examined at intervals for evidence of mold growth.

Antifungal	Manufacturer	Number of samples showing mold growth after days					
		29	36	43	60	66	78
Control		4	4	4	4	4	4
CTC (control)		0	4	4	4	4	4
Acti-dione + CTC	Upjohn	0	4	4	4	4	4
Filipin + CTC	Upjohn	0	0	1	2	2	4
Mycostatin + CTC	Squibb	0	0	0	0	2	3
Ayfactin + CTC	Bristol	0	0	0	0	1	2
Amphotericin A+CTC	Squibb	0	0	1	1	1	1
Amphotericin B+CTC	Squibb	0	0	0	1	1	1
A 5283 + CTC	Lederle	0	0	0	1	1	1
Sorbic acid		0	0	0	0	0	0

The results show that, in the comparatively high concentration used, sorbic acid was more effective than the antifungal antibiotics. However, sorbic acid is permitted in 0.1% concentration in certain foods, and previous trials indicated that dipping fillets in a 1% solution is unlikely to cause the fish to contain more than 0.1% of sorbic acid.

SUMMARY NO. 30

FILLET PRESERVATION WITH TETRACYCLINE ANTIBIOTICS

B.A. Southcott
E.G. Baker

Fresh lingcod fillets of a uniform size were prepared and dipped for one minute in 5 p.p.m. solutions of chlortetracycline (CTC), oxytetracycline (OTC) or tetracycline (TC). The fillets were then drained for three minutes on wire racks, subsequently wrapped in individual plastic bags and stored at 4°C. At intervals, direct counts were made.

Kind of Dip:	Bacteria per gram of fish ($\times 10^6$)			
	CTC	OTC	TC	H ₂ O
Days stored				
7	fresh 4.8	fresh 77.4	fresh 66.3	sl. stale 133.6
8	fresh 31.3	sl. stale 160.6	fresh 94.0	sl. stale 332.8
10	fresh 64.7	stale 452.6	stale 381.6	extremely stale 482.3
13	sl. stale 228.9	putrid 373.1	putrid 428.2	extremely putrid 425.0

In another experiment, fillets were prepared from Red Spring salmon and were treated as above. Up to five days of storage there was no odour in any of the samples.

Kind of Dip:	Bacteria per gram of fish ($\times 10^6$)			
	CTC	OTC	TC	H ₂ O
Days stored				
5	very sl. stale 123.5	sour 297.3	putrid 301.0	putrid 312.2
6	very stale 148.4	putrid 328.6	putrid 332.8	ext. putrid 320.7
8	putrid 449.4	ext. putrid 484.4	ext. putrid 513.0	ext. putrid 502.4

It can be concluded that CTC is the best of the antibiotics tested for use in preserving the quality of lingcod fillets; its use on salmon fillets appears to be of limited value.

SUMMARY NO. 31

ANTIBIOTIC ICES

B.A. Southcott
E.G. Baker

In experiments extending the investigation of the use of antibiotic ices in preserving fresh fish (Summary No. 4 of this Station's Annual Report for 1956-57), crushed ices were used containing 5 p.p.m. CTC, OTC or TC and .01% CMC with .03% NaCl as dispersing agents.

Fresh eviscerated lingcod were stored in these ices in insulated boxes; at intervals fish were removed, skinless fillets prepared and incubated 18 hours at 10°C. before being counted by viable and total methods.

Days in ice	Type of ice	Odour after 18 hrs. at 10°C.	Bacteria per gram fish ($\times 10^6$)	
			Direct	Viable
14	Untreated	Stale	269	47
	CTC	Fresh	7	1
	OTC	Fresh	80	22
	TC	Fresh	90	72
17	Untreated	Stale	393	30
	CTC	Very sl. stale	30	4
	OTC	Sl. stale	235	17
	TC	Sl. stale	216	26
20	Untreated	Very putrid	422	46
	CTC	Sl. stale	86	10
	OTC	Sour	214	91
	TC	Putrid	255	115

At 17 days, samples were also taken which consisted of whole slices through the fish including skin and bone rather than the usual fillets; the slices were put through a grinder and incubated as above before being counted.

	Odour after 18 hrs. at 10°C.	Bacteria per gram ($\times 10^6$)	
		Direct	Viable
Untreated	Sour	1,116	312
CTC	Fresh	212	43

SUMMARY NO. 32

CHLORTETRACYCLINE RESIDUES IN HALIBUT FLESH AND SKIN

B.A. Southcott

In connection with this Station's engineering studies on the storage and transport of halibut in refrigerated sea water (Summary No. 39 of this Station's Annual Report for 1956-57), tests were made to determine the amounts of CTC which remained in the skin and flesh of these fish held under commercial conditions in sea water containing the antibiotic. Twenty samples were taken at random from fish which had been held 10 to 19 days in refrigerated sea water containing CTC. The sea water, after this 19-day storage period, contained 2.65 p.p.m. of the antibiotic when assayed by the usual method; extract of halibut muscle was used to prepare standard curves. The flesh and skin portions of each sample were assayed separately with the following results (p.p.m.):

Skin samples: <0.1, 0, 0, <0.1, 0, 0.33, 0.33, 0.12, 0.17, 0.22, 0, 0.35, 0.42, 0.13, 0.43, 0.7, 0.08, 0.52, 0.41, 0.74.

Flesh samples: 0.03, 0, 0, 0.03, 0.13, 0, 0.02, 0, 0, 0, 0.14, 0, 0, 0, 0, 0, 0, 0.06, 0.02.

Most of the skin samples had small amounts of CTC; 65% of the flesh samples contained no demonstrable antibiotic, and the amounts present in those flesh samples which did contain CTC would be destroyed by any normal fish-cooking procedure.

SUMMARY NO. 33

PRESERVATION OF BLACK COD WITH CHLORTETRACYCLINE

B.A. Southcott
R. Moyer

Tests carried out several years ago indicated that dipping whole, eviscerated salmon in chlortetracycline (CTC) solutions for one minute on board a trolling vessel prior to icing them with ordinary ice could cause a marked improvement in keeping quality (Summary No. 10 of this Station's Annual Report for 1953). For this purpose comparatively strong concentrations of CTC (e.g. 50 p.p.m.) were required.

Black cod (Sable fish) are frequently subject to considerable spoilage on fishing vessels, or before or during smoking. Arrangements were made whereby eviscerated black cod were immersed between 5 minutes and 1 hour in a sea water solution of 50 p.p.m. CTC (as "Acronize", American Cyanamid Co.) before they were iced with ordinary ice. For each 500 lb. lot of fish to be treated, 30 gallons of freshly-prepared CTC solution was used.

Samples were taken from the iced fish at the end of the 18-day trip; skinned fillets were prepared for 18-hour incubation at 10°C. and subsequent bacterial counts, while flesh and skin samples were taken separately for CTC assay. The assays were performed using black cod extract as a diluent in standard curves (Summary No. 31 of this Station's Annual Report for 1955-56).

Effect of transient immersion of black cod in a 50 p.p.m. solution of CTC followed by normal icing on their subsequent keeping quality and CTC content.

Post-mortem age when landed (days)	Odour after 18 hrs. at 10°C.	Bacteria per gram fish (x10 ⁶)	p.p.m. CTC in skin	p.p.m. CTC in flesh
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Controls

3	extremely stale	27	0	0
6	stale	27		
10	putrid	35	0	

Dipped Fish

12	fresh	6.9	0.22	0.09
17	slightly stale	4.2	0.15	0.15
17	slightly stale	5.8	0.18	0.14

These results show that the dipping procedure exerted a pronounced beneficial effect on the keeping quality of the fish after landing as shown in the accelerated spoilage tests.

The amounts of CTC present in the flesh and skin of the fish were small and would undoubtedly be undetectable after the fish had been smoked and cooked in the usual manner. It can be concluded that under the conditions of this experiment, very low concentrations of CTC are found in the skin and flesh of these fish.

SUMMARY NO. 34

BACTERIOLOGICAL STUDY OF FISH STORED IN
REFRIGERATED BRINES WITH AND WITHOUT CTC

B.A. Southcott
E.G. Baker

In a continuation of work described in Summary No. 4 of this Station's Annual Report for 1956-57, experiments were carried out on the bacteriological aspects of fish preservation in refrigerated brines.

An attempt was made to compare the spoilage patterns of aerobically and anaerobically stored fish. Fresh dressed lingcod were stored at 10°C. in four 128 oz. clear glass jars filled with 3% NaCl solution. Air was bubbled continuously through two jars (one with 5 p.p.m. CTC, one without); two identical jars were treated with N₂ under low pressure for 30 minutes then sealed off. Escape tubes for gases formed were led from the jars into H₂O. At intervals, individual fish were removed for sampling and the N₂ jars refilled. Fillets prepared from the fish were incubated overnight at 10°C. before total and viable counts were done; these counts gave irregular results. It was noted that the differences in viable counts of fillets from fish stored in jars with and without CTC was not as great as would have been expected from the marked variation in odour evident after the fillets had been stored overnight at 10°C.

Days in jar	Odour in jar when samples taken				Fillets after 18 hrs. at 10°C, Odour and bacteria per gm. (x10 ⁶)			
	Nitrogen		Air		Nitrogen		Air	
	No CTC	+CTC	No CTC	+CTC	No CTC	+CTC	No CTC	+CTC
15	putrid	fresh	putrid	fresh	stale	sl."off"	stale	fresh
					0.6	0.4	0.7	1.6
18	putrid	sl."off"	putrid	sl."off"	putrid	sl."off"	stale	sl."off"
					0.5	2.6	1.1	1.1
22	putrid	putrid	putrid	putrid	putrid	stale	putrid	stale
					1.0	1.5	0.7	1.4

Since difficulties were experienced in the above experiment with finding a suitable aeration technique, and since the size of the glass jars made impossible the use of a reasonably large number of fish for any one treatment, it was decided to use large plastic tanks containing 20 litres each of 3% NaCl. The tanks were either aerated or were kept as "still" tanks; each treatment was carried out with and without 1.5 p.p.m. CTC. At intervals fish were removed, filleted, and the samples incubated overnight at 10°C. before direct counts were made. (See Table I at end of Summary.)

Commercial samples of cod and salmon which had been stored on board a vessel in refrigerated sea water for 18 days were received at the Station; the complaint was that the fish had a "peculiar" odour. The colour and texture of these fish were excellent. Samples were incubated overnight at 10°C. and counts made. No unusual forms could be found on the total count slides; the counts were not considered to be high.

Bacteria per gm. (x10⁶)

	<u>Total</u>	<u>Viable</u>
Cod	168	0.2
Salmon	36	0.15

From the results of these experiments it is apparent that a distinctive type of odour occurs in fish which have spoiled in refrigerated brine or seawater. The presence of low concentrations of CTC in refrigerated brine does not usually effectively control bacterial growth as it does in ices and fillet dips, but it does reduce the odour produced.

Since the methods employed in these tests do not give conditions comparable to those occurring on vessels employing refrigerated seawater tanks, further experiments are to be carried out in small tanks constructed so that the refrigerated seawater will circulate in the manner of that in commercial installations. Anaerobic as well as aerobic methods are to be used for studying the bacteria involved in the spoilage processes and slightly higher CTC concentrations will be used.

TABLE I - Counts of fish stored in refrigerated brine.

Days	Brine *	Whole fish when sampled	Fillets after overnight at 10°C	Bacteria per gm. (x10 ⁶)
13	CTC air	fresh	fresh	3.1
	CTC still	fresh	fishy	4.5
	K air	strong "fishy" odour	putrid	153.7
	K still	putrid	ext. putrid	42.4
15	CTC air	fresh	sl. fishy	26.0
	CTC still	fresh	fishy	27.0
	K air	putrid	ext. putrid	46.1
	K still	ext. putrid	ext. putrid	29.2
16	CTC air	fresh	stale	15.4
	CTC still	fresh	very stale	21.2
	K air	putrid	ext. putrid	87.5
	K still	ext. putrid	ext. putrid	20.1
17	CTC air	fresh	stale	43.5
	CTC still	fresh	very stale	5.3
	K air	putrid	putrid	54.6
	K still	ext. putrid	ext. putrid	46.1
20	CTC air	fresh	fresh	129.9
	CTC still	fresh	stale	97.5
	K air	ext. putrid	ext. putrid	79.5
	K still	ext. putrid	ext. putrid	95.4

* K = Controls without CTC.

SUMMARY NO. 35

EFFECTIVENESS OF CTC TREATMENT IN WHALE TISSUES

R.H. Moyer

At the request of a local company, a conventional "t" test was applied to part of the data (bacterial counts) contained in Industrial Memorandum No. 20, 1957, of this Station. Tables I and II of this memorandum were compared, as were Tables VI and VII. The results are summarized in the following tables. It is readily apparent that the differences found in the 1956 trials are highly significant, while those in the 1957 trials are non-significant. Due to the sporadic nature of the data, the standard errors are very large, accounting for the fact that comparatively large differences between some of the means are not significant.

Comparison of Tables I and II

Part of whale	Difference between means (average)	Standard error of means	P.
A	152.12	51.01	$0.01 > P > 0.001$
B	154.00	38.03	$P < 0.001$
C	53.69	20.52	$P = 0.01$
D	39.20	19.45	$0.05 > P > 0.02$
E	304.12	96.35	$0.01 > P > 0.001$
F	197.06	82.40	$P = 0.02$
G	166.89	77.20	$0.05 > P > 0.02$

Comparison of Tables VI and VII

Part of whale	Difference between means (average)	Standard error of means	P.
A	69.20	55.62	$0.3 > P > 0.2$
B	5.51	46.16	$P > 0.9$
C	158.47	104.13	$0.2 > P > 0.1$
D	26.31	86.77	$0.8 > P > 0.7$
E *	153.09	66.36	$0.05 > P > 0.02$
F	-86.55	-	

* This is significant at the 5% level, but judging from the remainder of the data, this appears to be the one occasion in twenty that arises due to chance.

SUMMARY NO. 36

CONTROL OF POST-MORTEM BACTERIAL SPOILAGE OF WHALES WITH TETRACYCLINE ANTIBIOTICS

Jock Leung
D.W. Duncan
H.L.A. Tarr

The results of previous studies were reported in Summary No. 5 of this Station's Annual Report for 1956-57. During the past season further trials were made following a request by the whaling company concerned that the introduction of the antibiotic be made by incorporating it in the bomb head of the harpoon used to kill the whale. Station personnel, who had previous experience with preservation of whale carcasses, were very doubtful of the successful outcome of this method of introduction and their doubts were borne out by the results obtained. Two antibiotics were used, namely, chlortetracycline hydrochloride (CTC) and oxytetracycline hydrochloride (OTC); 50 g. contained in a polyethylene bag were placed in the bomb head of the harpoon beneath the charge of black powder. Direct bacterial counts of samples of muscle or liver tissue of untreated and treated animals were made as in previous trials. It is important to note that there was a 40% loss of antibiotic by this method due to the harpoons missing their targets. The results of direct bacterial counts (Table I), which have been analysed statistically in Summary No. 35 of this report, show that there was no significant difference between treated and untreated whales. On the other hand, significant improvement was found where CTC had been injected into the visceral cavities of whales in previous experiments (see Summary No. 35 of this report). The complete data of both 1956 and 1957 trials have been published in this Station's Industrial Memorandum No. 20 (see list of publications).

TABLE I - Millions of bacteria per gram of whale tissue.

Post-mortem age (hours)	No. of whales examined	T i s s u e*					
		A	B	C	D	E	F
From untreated carcasses							
< 20	18	0.24	0.31	9.2	12	12	92
21-25	10	38	31	73	130	120	190
26-30	12	18	7.4	150	110	190	140
31-35	3	360	5.5	620	370	30	700
36-40	3	40	19	390	360	150	570
41-50	5	260	250	150	800	710	-
< 51	2	1000	770	160	1100	1200	-
From carcasses treated with OTC introduced by the harpoon							
< 20	3	0.82	0	0.98	0	0.75	15
21-25	5	0.12	1.6	26	9.3	1.8	460
26-30	4	0.34	0.14	35	3.3	1.1	310
31-35	1	0.11	0	120	0.11	4.1	300
36-40	3	19	2.0	350	120	2.2	590
41-50	3	67	140	250	730	180	480
From carcasses treated with CTC introduced by the harpoon							
21-25	3	0	0	0.038	0.075	0.038	1.8
26-30	2	1.1	0.33	15	0.68	1.0	0.22
36-40	1	62	31	740	680	515	-
41-50	8	77	180	280	3200	78	600

* A, front surface muscle close to blubber; B, rear surface muscle close to blubber; C, front deep muscle near backbone; D, rear deep muscle near backbone; E, deep muscle from flank; F, liver tip.

SUMMARY NO. 37

DISTRIBUTION OF CTC IN ICE MADE FROM HARD WATER

R.H. Moyer
B.A. Southcott

There has been considerable work done concerning the suspension of germicidal substances, notably CTC, in block ices, and techniques for successful suspension have been worked out. (See Summary No. 29 for 1955-56 and Summary No. 6 for 1956-57 of this Station's Annual Reports) Although these methods provide good suspension in ices made from relatively soft waters, they are not applicable to some hard waters. Experiments are now in progress to determine the effectiveness of these methods as applied to a number of typical hard waters and to develop new methods or modify the existing ones where these prove ineffective. Artificial "hard" waters have been prepared and, after considerable technical difficulties, a method which it is hoped will prove reliable for determining CTC in melted samples of the experimental ices, has been worked out.

SUMMARY NO. 38

TASTE PANEL FOR THE EVALUATION OF QUALITY IN FROZEN FISH

R.H. Moyer

In connection with proposed tests on the suitability of a number of packaging materials for storage of frozen fillets (Summary No. 40 of this report), two 5-member taste panels were organized from among the Station staff. A ranking technique similar to that proposed by Reimer (Food Research, Vol. 2, No. 6, 1957) is used in assessing relative acceptability. This technique was adopted because it is particularly applicable in tests where the quality of all the samples decreases with time and where a relative, rather than an absolute, value is sought.

For any one tasting session, four frozen samples are partially thawed and five 15-gram aliquots taken from each. These are placed in individual petri dishes and permitted to stand until completely thawed. Four dishes, representing the four samples, are then presented to each judge for ranking of the raw samples. Following this, the dishes are partially filled with water and placed in a 350°F. oven for 15 minutes. The purpose of this method of cooking is to accentuate any "off" flavour that may be present. The raw samples are rated on appearance and odour alone, while taste and texture are considered in ranking the cooked samples. In order to help prevent taste "fatigue" and to aid in clearing the senses between samples, a beaker of apple juice is presented with the cooked samples.

SUMMARY NO. 39

ASCORBIC ACID CONTENT OF COMMERCIAL DIPPING SOLUTIONS

R.H. Moyer

It has been shown (Summary No. 15 of this Station's Annual Report for 1956-57) that the ascorbic acid concentration in a local fish plant's dipping solution decreased drastically in the course of seven hours of operation. It was assumed that this decrease was due to dilution by wet fillets. Subsequent observations, however, indicated that the fillets were not the only cause of depletion.

The dipping solution used at the cannery was made up to contain 0.3% ascorbic acid and 0.5% hydrocolloid (commercial carrageen, Kraystay "T"), i.e. 12.8 oz. ascorbic acid and 22.3 oz. Kraystay per 27 gallons water. A "blank run" was made using this solution in the cannery's dipping tank. The tank and accessories were kept in normal operation for four hours but only eight cod fillets were passed through the solution. The object of this "blank run" was to determine the extent of the ascorbic acid depletion which was due to mechanical dilution of the apparatus alone. The results are listed in Table I.

TABLE I - Concentration of ascorbic acid in a dipping solution and amount picked up on the fillets using Kraystay "T".

<u>Time</u>	<u>Dip (% ascorbic)</u>	<u>Uptake on fillets (% ascorbic)</u>
10:45	0.253	0.0172
11:45	0.226	-
12:45	0.163	0.0104
2:30	0.141	0.0078

The extremely low uptake of ascorbic acid on the fillets (from Table I) was probably caused by failure of the hydrocolloid to dissolve completely. Thus the initial level of hydrocolloid in the tank was rapidly depleted due to settling and the net effective level of ascorbic acid was not much more than would occur in aqueous solutions containing 0.25%. A later observation disclosed that the Kraystay was indeed settling and being carried off by the belt.

A similar "blank run" was made using ascorbic acid alone in a concentration of 1.5% (4-lb. ascorbic per 27 gallons water). The results are shown in Table II.

TABLE II - Concentration of ascorbic acid in a dipping solution
and amount picked up on the fillets without Kraystay "T".

<u>Time</u>	<u>Dip (% ascorbic)</u>	<u>Uptake on fillets (% ascorbic)</u>
9:45	1.45	0.147
10:45	1.26	0.115
1:15	1.23	-

Recommended amount of ascorbic in fillets for good protection is
0.04%-0.06%.

The ascorbic acid content of the fish was probably greater than is necessary for good protection. The immersion time in both trials was twenty seconds. Later trials in the laboratory showed that the uptake on fillets was almost as great after three seconds immersion as after ten. This indicates that the large dipping tanks now in use at this particular plant could be reduced in size without any great loss in efficiency.

At the request of the above company, a rather extensive experiment has been planned in order to assess the commercial value of isoascorbic acid in protecting frozen fillets. This experiment will involve taste panel tests and certain objective tests.

SUMMARY NO. 40

THE VACUUM AND CONTROLLED-ATMOSPHERE PACKAGING OF FRESH FISH

N.A.W. LePage

One way to insure a continued and expanding market for fresh fish is to make certain that oxidative rancidity is held to an absolute minimum until the fish reaches the consumer. Vacuum packaging in plastic pouches has been successful in controlling oxidative rancidity in other foods and there is no obvious reason why this technique should not give similar good results with fish.

This Station has recently acquired, on a one-year's lease, a "Flex-Vac" packaging machine, model 6-8-2R.G., from E.S. & A. Robinson (Canada) Limited of Toronto. This machine uses plastic pouches and is set to draw 28 in Hg vacuum (higher vacuums may be obtained). It is easily convertible to controlled-atmosphere packaging.

The investigation into the use of vacuum and controlled-atmosphere (nitrogen) packaging with fresh fish will begin as soon as an adequate supply of Spring salmon becomes available. Several different plastic laminations will be used in the pouches including mylar-polyethylene, cellophane-polyethylene and mylar-saran-polyethylene. Pouches made from a single film of Saran (as Cryovac) may be used but not with this

machine. After the fish has been packaged, it will be kept in refrigerated storage. Samples will be withdrawn at intervals to be tested for oxidative rancidity using both organoleptic and chemical tests.

Tentative arrangements have been made with one local company to carry out tests in which mutual observations will be made.

SUMMARY NO. 41

REFRIGERATED SEA-WATER VESSELS

J.S.M. Harrison

The work done in refrigerated sea water this year has been almost entirely in the field of consultation and observation of installations made or projected by industry and studies of materials and techniques which could be used to improve future systems. The following reports will deal with the knowledge gained or the experimental features of these many installations.

"SILVER VIKING"

The "Silver Viking" operated a full halibut and salmon season under practical fishing conditions. Two halibut trips were accompanied by an engineering staff member. These trips of three and four weeks' duration gave us a chance to assess the system under conditions existing in offshore fishing. The most notable point was that the equipment operated without breakdown beyond that which could be repaired with the meager materials and resources on board. This point is one that could only be proven in practical application and it must be appreciated that an extreme degree of reliability is required by refrigerated sea-water equipment as it is competing with ice, a non-mechanical system whose reliability is absolute. It is now proven as a result of these trials that the reliability of this equipment need no longer be a consideration in its use.

The performance of this system in extremely rough seas was demonstrated by these trips to the satisfaction of skeptics. Repeated violent gales had no effect on the fish in the tanks or the equipment.

Deficiencies in the system regarding sanitation were pointed out by one of these trials. It was shown that care must be exercised in the design of equipment to facilitate cleaning of the equipment and in the cleaning of equipment and the fish. No fish was lost however in any of these trips but better quality fish was shown to depend on sanitary methods of handling and especially on careful cleaning of tanks and heat exchange units.

The success of the "Silver Viking" experiment is shown in practical terms in that the vessel landed a quarter of a million pounds of halibut in three trips, a very high average, in some part due to the refrigerated sea-water equipment allowing longer trips. Also the owner, in building a second vessel, at his own expense entirely, shows his approval of the system. (See below.)

SUMMARY NO. 42

"PACIFIC OCEAN"

J.S.M. Harrison

This vessel is a 72-ft. steel-combination drum seiner, dragger, halibuter, built this year, employing a refrigerated sea-water fish-holding system. The refrigeration system was built to our recommendations in that it used two chiller units each with a separate compressor; the units being designed in accordance with that of the chiller system we developed experimentally and used on the "J.R.D." and

"Silver Viking". Hydraulic power is used here to drive compressors and circulating pumps and is a new feature in that to our knowledge it has not been used to operate compressors of this size. Hydraulic power is very desirable in many respects for this equipment as it is otherwise used on all large fishing vessels now for gear handling; hence, much of the cost is borne by that equipment. It has the positive and reliable features of mechanical drive but also permits constant speed operation which is desirable for compressors and necessary for pumps.

The builders of the vessel found what we believe and hope will be a good approach to the problem of carrying full herring loads in a vessel equipped with refrigerated sea water. Tanks are arranged on either side of a shaft alleyway as in the "Silver Viking". Sliding gates in each tank permit discharging of fish into the alleyway, which is of sufficient width to facilitate unloading from this one area and can also be used for additional herring storage.

SUMMARY NO. 43

NEW STEEL VESSELS

J.S.M. Harrison

A new vessel is now under construction for the owner of the "Silver Viking" - a 75-ft. steel-combination seiner-halibuter. Its tank and compressor arrangement are the same as the "Pacific Ocean". Because of the owner's satisfaction with the electrical system on the "Silver Viking", it is being used again on this vessel. This is a 120-208-volt, three-phase, alternating current system. It is of special interest to us in that we employed alternating current on the "Silver Viking", considering it the most practical method of driving the three compressors and seven pumps. After our success in developing chillers which reduced the required equipment to two compressors and two pumps, an alternating current system was no longer vital to the refrigeration system. However, it had been so adaptable to other equipment on the vessel that the owner and builders decided to expand its use to drive almost all auxiliary equipment. As the "Silver Viking" was the first fishing vessel in British Columbia to use an a.c. system, it may prove that our experiment may have contributed to a second major feature in the design of fishing vessels.

A new approach to the problem of insulating tanks has been developed by the builders in this vessel. The simplest approach, that of using a metal tank shell with insulation between it and the vessel's skin and bulkheads, presents certain problems in fabrication and heat transfer through structural members. To avoid this they are using an inner shell of Fiberglas reinforced plastic, which is applied to the insulation, which is adhered to the skin by adhesives and welded anchors. The insulation is styrofoam which is ideal in many respects. It has a very low conductivity; is highly moisture resistant; has good compressive strength and is easily cut and easy to work with. It does not lend itself to use with welded steel linings because of low melting point and inflammability. Its chief disadvantage for this use is its vulnerability to solvent attack and destructive solvents are used in the Fiberglas plastic application. The means of overcoming this problem is to coat the insulation with resorcinol resin prior to application of the Fiberglas plastic. The success of this method of building tanks will depend on the adequacy of this treatment and the water tightness and durability of the Fiberglas plastic. Should the method be effective, it will be a forward step in the as yet unsolved problem of insulating fishing vessels for refrigerated sea water or iced fish storage.

A new steel-combination troller-crab fishing vessel is under construction to be outfitted with refrigerated sea-water storage. We are collaborating with its owner and the Naval architects on design.

SUMMARY NO. 44

SHORE TANK INSTALLATIONS

J.S.M. Harrison

Two shore installations of refrigerated sea water were made this year, one for salmon and one for halibut. In this field refrigerated sea water offers great benefits at a low unit cost. Icing for short term holding is difficult, costly, and requires much plant area, making it prohibitive for salmon canning plants. Refrigerated sea-water tanks in providing a simple means of holding for short periods, could make great economies in smoothing the flow of fish to processing. The installations made this year have shown promise in that field.

Halibut - The tank installed at Prince Rupert for halibut has a capacity of 40,000 lbs. Refrigeration is supplied by the main ammonia system for the plant to iron pipe coils in the tank. Agitation of the brine is provided by a motor-driven propeller. Systems of this type provide storage at a low first cost where surplus refrigerating capacity is available in the plant. However, they do not take advantage of the high performance of refrigeration compression systems operating at high temperatures, since the plants other duties require low temperature refrigeration.

This tank has provided a useful service and the operators are considering further expansion in this field.

Salmon - A large installation for holding over 200,000 lbs. of salmon was made at a new cannery at Steveston, B.C. It has 10 steel tanks, each with a sliding discharge gate. The tanks were assembled as a unit and the outside walls were insulated with foamglass.

The refrigeration system comprised a 20 h.p. compressor, an air condenser and a double tube heat exchanger. A single 4-inch pump delivers brine to all tanks, which are suitably piped and valved to operate any or all tanks simultaneously.

The small salmon pack this year gave little opportunity to evaluate the use of this equipment for other than holding over weekends, which according to operators was quite satisfactory. However, the large salmon run anticipated this year should provide that opportunity.

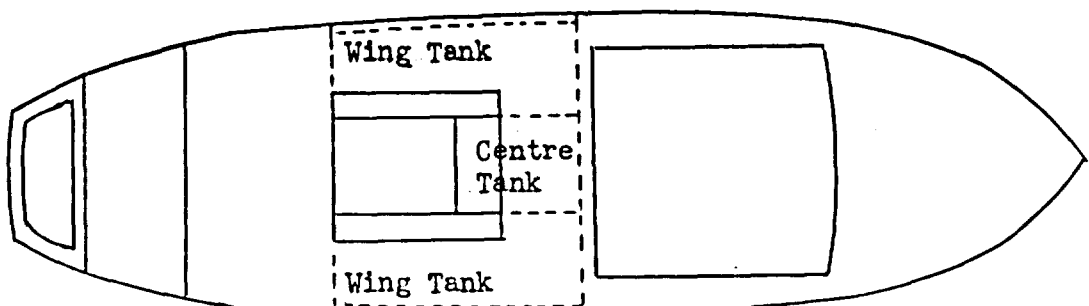
SUMMARY NO. 45

REFRIGERATED SEA WATER - "RUTH G"

S.W. Roach

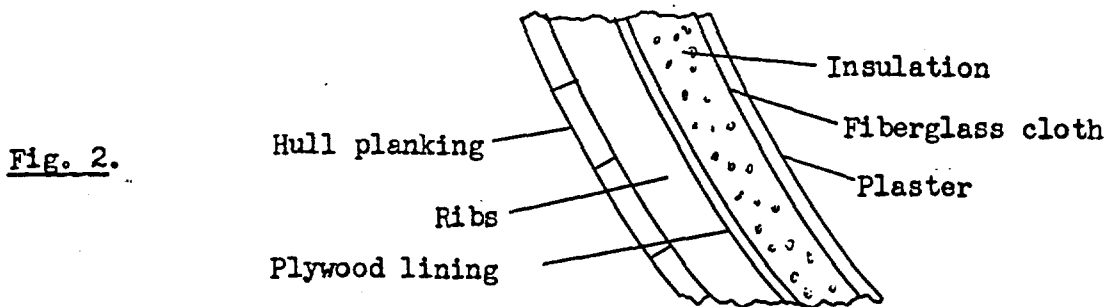
The main purpose of this project, for which funds were provided by the Industrial Development Vote, was to test a radically different method of installing refrigerated sea-water tanks in a fishing vessel. This 45-ft. wooden vessel was used as a salmon troller in the summer and for transporting live crabs in the winter. The "Ruth G" foundered in December of this year, fortunately with no loss of life, although the vessel itself was a total loss. However, enough was learned during several months of operation to enable us to assess the advantages and disadvantages of the tank installation.

Fig. 1.



The tanks were built in the hold as shown in Fig. 1 by installing a plywood floor, plywood bulkheads in the hatch opening and two steel bulkheads running for and aft to divide the hold into three compartments. The sides of the hold and under the deck were lined with plywood. This work was carried out in a shipyard at the vessel-owner's expense. No attempt was made to make these compartments water-tight up to this point, our plan being to install a waterproof insulated lining in the tanks. This lining was installed by a firm experienced in the application of insulation; however, they were not able to guarantee complete satisfaction as they had no previous experience in designing such a hydrostatically waterproof lining. The lining shown in X-section in Fig. 2, consisted of the following materials:

1. A plywood lining to give an initial smooth working surface.
2. A rubber-asphalt emulsion (Flintkote Type 32) used here as an adhesive for the insulation and as a sealer between the insulation blocks.
3. Three inches of waterproof insulation (Foamglas).
4. Fiberglas cloth glued to the insulation with an asphalt emulsion.
5. An asphalt-portland cement plaster trowelled onto the entire inside of the tanks. This plaster consisted of a mixture of sand, fibrated asphalt emulsion, Portland cement and some water.



When the tanks were put into service they were found to leak badly and considerable patching had to be done to make them reasonably water-tight. The initial failure was due to several causes, the most serious being:

1. The asphalt emulsion used as an adhesive and sealer for the insulation was unsatisfactory; it could not be dried sufficiently to become waterproof and in fact was actually washed away when exposed to water.
2. The asphalt-portland cement plaster should have been reinforced with metal lath since it tended to develop shrinkage cracks in the corners.
3. The design of the tank unloading doors and the door frames was faulty in that insufficient care was taken to prevent leaks in this region.

These faults were overcome by coating the inside of the tanks with hot asphalt. This proved to be an inexpensive and yet serviceable finish.

SUMMARY NO. 46

MATERIALS TESTING PROGRAM

P.J.G. Carrothers

Most of the fishing gear problems in the British Columbia fishing industry still involve the new materials continually being made available; whether or not they can be used, and if so, how they can be used to best advantage. Consequently, the Materials Testing Program previously described in Summary No. 41 of this Station's Annual Report for 1956-57 has been continued. The data presented in tabular

form with last year's summary have been expanded and were submitted, along with a discussion of what physical properties are significant to the design of commercial fishing gear, what procedures were used to measure these properties, and what mathematical relations were used to analyse the test data, to the Food and Agriculture Organization of the United Nations for the International Fishing Gear Congress held in Hamburg, Germany, in October, 1957. It is anticipated that this paper will be published in the FAO book on commercial fishing gear which is presently in preparation.

In addition to the above, the following materials received attention: "Nyak" seine web, "Marlon" seine and trap web, a new construction nylon twine marketed as size #28, monofilament nylon gill-net web, braided nylon twine for halibut long-line gangings, "Nylast" nylon dip agent, "Morrison" hollow plastic gill-net floats, and Japanese nylon gill-net web.

"Nyak" is manufactured in Britain and the United States by twisting spun acetate yarns with continuous filament nylon yarns to form the twine. The nylon gives strength and the spun acetate helps to hold the knots and reduce the sharpness of the twine. This material is designed and sold as a substitute for cotton seine and trap web. The properties of this material compared with those of cotton are as follows:

	<u>"Nyak"</u>	<u>Cotton</u>
Specific strength (k yd)		
Twine dry	40	13.1
wet	34	14.4
Single knot, dry	23	9.4
wet	20	12.6
S.K. mesh, dry	18.6	8.2
wet	16.7	9.6
Specific gravity	1.19	1.55

On an equal weight basis, "Nyak" has about 75% higher wet mesh strength than has cotton and, being synthetic, is not subject to bacterial or mildew damage and will probably retain more of its strength through use. However, it is relatively light in water (16-lb. per 100-lb. air dry as compared with 35-lb. for cotton) and may present handling problems on this account. A sample of "Nyak" treated with "Net-Set", a proprietary bituminous agent, weighed only 6½-lb. in water per 100-lb. air dry.

"Marlon" is manufactured in Japan by twisting vinylon yarns with continuous filament nylon yarns to form the twine. The nylon gives strength and the vinylon helps to hold the knots and reduce the sharpness of the twine. Unfortunately, the only sample available for test had been used for ten weeks in the salmon trap at Sooke. However, even after this exposure, its specific mesh strength was 12.9 k yd. dry and 11.9 k yd. wet. There was a tendency for the portions of the web which were continuously immersed to be stronger than those which were not continuously immersed, despite considerable fouling, the implication being that Marlon is more damaged by weather than by sea immersion. Like "Nyak", this material has relatively low density, weighing about 15 lb. in water per 100 lb. air dry, and hence may give handling problems in seines.

The #28 continuous filament nylon twines result from a "mislabelling" of some Japanese nets. These Japanese nets are, in fact, #33 construction, but are weaker than Canadian #33 nets. To meet competition from these #28 nets, certain British manufacturers introduced a 210d/4/2 nylon net, which was between their #23 and #33 nets in weight and strength, but the twine construction was a little too open. This year, for their #28 nets, they are trying a 210d/2/4 construction which

was the subject of present tests. Comparative physical properties of British and Japanese #28 nylon gill-net web are:

	<u>British</u>	<u>Japanese</u>
Twine construction	210/2/4	210/3/3
Yards per pound of twine	2450	2280
Twine diameter (mils)	18	22
Wet mesh strength (lb.)	22.3	20
Specific wet mesh strength (k yd)	27.3	23.1

The British #28 nets are thus finer, lighter, and stronger than the Japanese #28 nets, although their cost is higher.

Nylon monofilament is much less visible in water than is nylon multifilament, so there is continuing effort to manufacture nets from monofilament. However, monofilament nylon is quite springy and gives handling problems on the netting looms. Last year, manufacturing techniques had developed to the point where 0.35 mm. diameter nylon monofilament could be made into netting by machine. This material was found to have a mesh strength of about 12½ lb. dry and 10 lb. wet. This is still too weak for satisfactory service in our salmon gill-net fishery, but the corresponding specific mesh strength of 26.7 k yd. dry and 21.1 k yd. wet are comparable with those for type 6 nylon multifilament netting. That is, the strength per unit weight is reasonable so that the major problem is to produce heavier web.

Three sizes of braided nylon ganging twine were examined; one was without core, one had a core of 2-ply staple twine plus a continuous filament braided twine, and the other had a core of untwisted continuous multifilament yarn. In all cases the braid was quite hard and the twine quite stiff. This stiffness may be required for proper handling characteristics, but a softer braid would have superior wear resistance. The knot did not slip during the strength tests, but there was a tendency for the core to slip or to break within the braid before the twine as a whole ruptured. A better balance between the load-elongation characteristics of the braid and those of the core would be desirable.

Several proprietary agents are marketed to increase the life of nylon stockings, and one of these is apparently available in quantity at relatively low cost. On inquiry as to whether this agent would improve nylon gill nets, a few preliminary experiments were undertaken, but no significant advantage was observed. Apparently this agent applies a surface coating to the nylon filaments which functions in a manner similar to the "dip" agent presently applied at the factory to many nylon gill nets, further coating from this proprietary agent producing little change.

Tests were applied to the Morrison hollow plastic gill net floats and to Japanese nylon gill-net web to round out information for Industrial Memorandum 19 on "The Selection and Care of Nylon Gill Nets for Salmon". Tests had previously been applied to the more widely used cedar and sponge plastic floats. These hollow plastic floats seem to be adequately rugged, but, because of their relatively small size, they can support only about half as much weight as the other types of gill-net float. Whereas, relative to the sponge plastic floats, more hollow plastic floats are required per net, the total cost for hollow plastic floats is greater even though the unit cost is less.

Japanese nylon gill-net web had been tested previously, but data on the twine and on double-knot netting were not considered adequate for Industrial Memorandum No. 19, therefore, further tests were undertaken. Apart from data presented in the Memorandum, it is interesting that the Japanese nylon yarns measure about 203 denier compared with about 212 denier for Canadian and British nets, both nominal 210 denier. This probably indicates that the Japanese set their twist by a hot stretch treatment,

whereas Canadian and British manufacturers are more partial to a relaxed, moist heat treatment. The latter gives more stable mesh length.

SUMMARY NO. 47

TESTING AND INFORMATION SERVICE

P.J.G. Carrothers

The testing and information service reported in Summary No. 43 of this Station's Annual Report for 1956-57 has been continued as follows:

- (a) Used nylon netting returned with complaint, for Nelson Bros. Fisheries, Ltd. and C.P. Leckie, Ltd. Strengths were found a little low, but not seriously so considering nylon's characteristically large initial strength loss.
- (b) Japanese white and tarred cotton salmon and herring seine web for British Columbia Packers Ltd. and Western Leckie, Ltd. The quality was found generally to be quite good, even in those samples made of #10 yarn.
- (c) Identified a small sample of netting for C.P. Leckie, Ltd. It turned out to be nylon.
- (d) Examined a piece of tarred hemp crab warp which had been returned to British Columbia Packers Ltd. as defective. The damage was found to be purely physical - considerable abrasion but little if any chemical break-down.
- (e) Information to W.E. Drake re molded nylon stern bearings.
- (f) Information to Imperial Chemical Industries, Ltd., England, re synthetic fibres in seines and local trends in desired stretchability of gill nets. They are experimenting with Terylene in Norway and have volunteered to forward their results.
- (g) Information to Mr. Hazen Thompson, New Brunswick Fisherman, re nylon gill nets.
- (h) Information to the Royal Norwegian Consulate General, Montreal and Quint, Vancouver, re style of netting used in the British Columbia fishery.
- (i) Discussion with C.P. Leckie Ltd. re their new specifications for nylon gill-net web.
- (j) Information to Dr. A. v. Brandt, German Institute for Net and Material Research, re test procedures and local fishing-gear publications.
- (k) Information to the Board's Biological Station in Nanaimo to assist in their selection of gear for experimental herring fishing.

SUMMARY NO. 48

OXIDATIVE METABOLISM OF A MARINE BACTERIUM

I. STUDIES ON THE MECHANISM OF PROPIONATE OXIDATION

C.A. Claridge

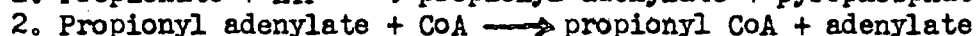
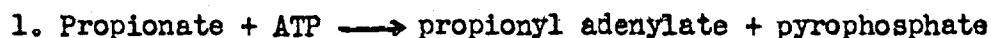
R.A. MacLeod

H. Hogenkamp

Studies reported last year on this topic (Summary No. 17 of this Station's Annual Report for 1956-57) have been continued and extended.

A. Propionate activation in a cell-free system

The activation of propionate (equations 1 and 2) has been demonstrated in an unidentified soil organism by Moyed and Lipmann (J. Bact. 73:117, 1957).



Attempts were made to determine if this system was present in cell-free extracts of marine organism B16. Pyrophosphate was precipitated from neutralized TCA extract of reaction mixtures as the manganese salt (J. Biol. Chem. 182:779, 1950) then determined colorimetrically (J. Biol. Chem. 211:791, 1954). Propionyl adenylate was estimated by a modification of the hydroxamic acid method of Lipmann and Tuttle (J. Biol. Chem. 159:21, 1945); the hydroxylamine concentration was doubled and incubation carried out for 10 minutes at room temperature.

The results of one of the best experiments can be seen in Table I.

TABLE I - Propionate activation by cell-free preparation of B16.

Buffer pH	Net μM formed per tube	
	Hydroxamate	Pyrophosphate
8.1 Tris	1.04	.156
7.0 Borate	.676	.624
6.0 Succinate	.390	.364
5.0 Acetate	.598	.520

Each tube contained: 0.5 ml. of enzyme preparation; 50 μM buffer; 10 μM ATP; 10 μM glutathione; 6 μM MgCl_2 ; 30 μM NaF ; 200 μM NH_2OH ; 40 μM K propionate; 10 units of CoA; total volume made to 1.3 ml. with water. Tubes were incubated at 37°C. for 90 minutes.

Sonic extracts prepared from B16 cells grown in nutrient broth, yeast extract medium gave the most satisfactory results.

Table I shows that with the exception of pH 8.1, the ratio of hydroxamate to pyrophosphate is 1:1. However, there did not seem to be any greater activation at the lower pH's where, with whole cells, propionate is oxidized to the greatest extent.

Attempts to increase the level of activation of propionate beyond that given in Table I were unsuccessful. The use of very young cells (4 hours) or the use of an increased hydroxylamine concentration had no effect.

B. Effect of fluoroacetate on propionate and succinate metabolism

It is known that marine bacterium B16 will oxidize succinate only partially under conditions that lead to complete oxidation of propionate. This fact led to the

conclusion that succinate is probably not an intermediate in propionate oxidation.

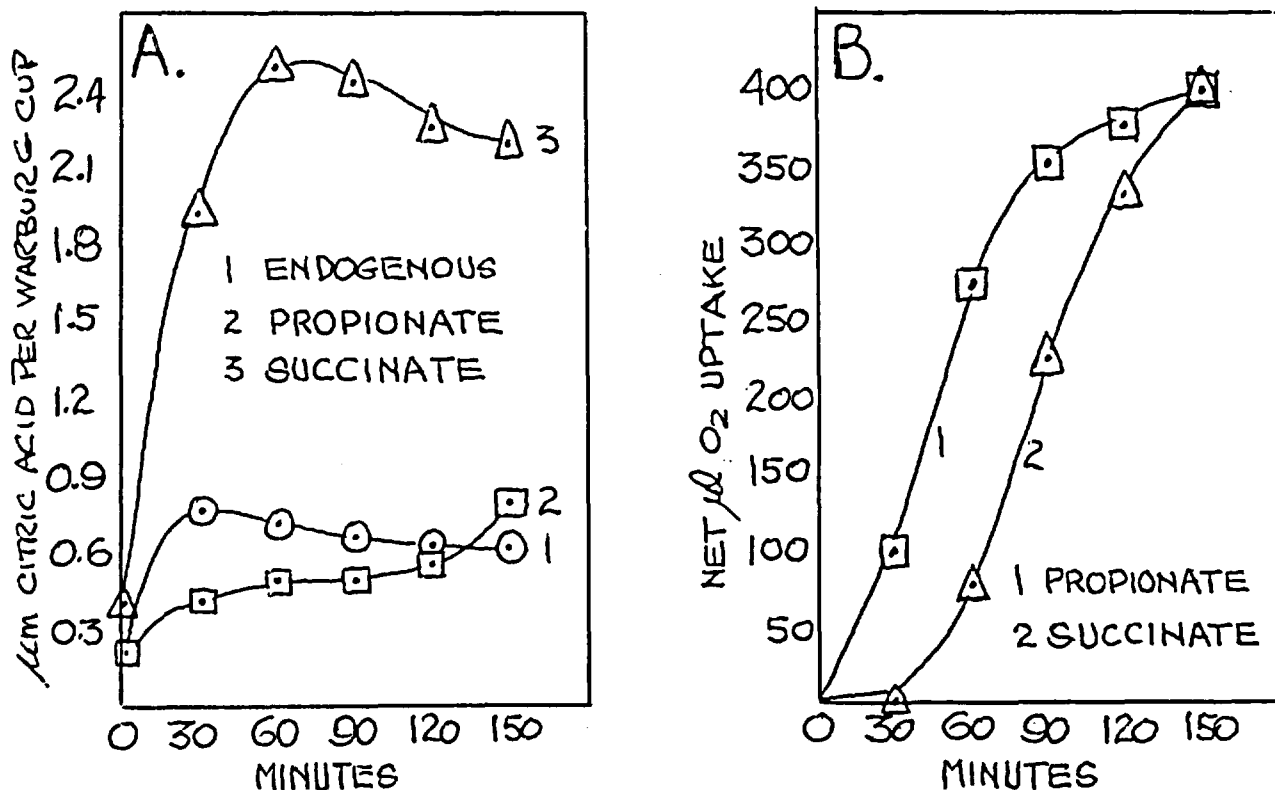
Sodium fluoroacetate has been found by other workers to exhibit the enzyme aconitase in many biological systems. Such inhibition would cause citric acid to accumulate when compounds giving rise to citric acid are oxidized. It was of interest to determine the effect of fluoroacetate on the extent of oxidation and on citrate formation when propionate and succinate were oxidized by whole cells of organism Bl6.

Cells were grown in nutrient broth, yeast extract for 18 hours at 25°C., centrifuged, washed thrice with a wash solution consisting of 2.34 gm. NaCl, 1.49 gm. KCl and 5.08 gm. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per 500 ml. of distilled water, and suspended in this wash solution at 13 mg. dry weight of cells per ml. for Warburg studies.

Sodium fluoroacetate was converted to the free acid and reprecipitated as the potassium salt. Citric acid was determined by the method of Natelson et al. (J. Biol. Chem. 175:745, 1948).

In the presence of fluoroacetate, substantial amounts of citrate accumulated with succinate as substrate, but essentially none with propionate as substrate. This would be expected if succinate is metabolized via a tricarboxylic acid cycle and propionate is not. Figure 1A demonstrates this and shows a peak of citrate accumulation at approximately 60 minutes, beyond which the level of citrate drops, probably through a slow metabolism.

Fig.1 Citrate accumulation in presence of fluoroacetate.



Each cup contained: 130 μM Tris phosphate buffer pH 7.0; 120 μM Na^+ with propionate; 520 μM Na^+ with succinate; 40 μM K^+ with propionate; 30 μM K^+ with succinate; 50 μM Mg^{++} ; 12 μM K fluoroacetate; 10 μM substrate; 0.2 ml 20% KOH; 14.6 mg. dry wt. of cells; total volume 3.0 ml; temp. 25°C; gas phase air.

Oxygen uptake data obtained in the same experiment (Fig. 1B) shows that succinate oxidation began immediately while propionate started only after a 30-minute lag period. After the lag, however, propionate oxidized as rapidly and extensively as succinate, yet during this time no citrate accumulated.

Various levels of fluoroacetate from 0 to 0.01M final concentration with both propionate and succinate as substrates showed that a level of 0.004 M was optimum for citrate accumulation. This was the level used in all experiments.

β -hydroxy propionate and acrylate have been shown by Giovanelli and Stumpf (J. Am. Chem. Soc. 79:2652, 1957) to be intermediates in propionate oxidation in peanut mitochondria. These two compounds were readily oxidized by Bl6 cells grown in nutrient broth, yeast extract medium, and these oxidations were markedly inhibited by fluoroacetate. However, no citrate accumulated (Table II). Accumulation of citrate would be expected if these compounds yield tricarboxylic acid cycle acids as postulated by these authors.

Thus, based on fluoroacetate inhibition studies, it is apparent that there is a difference in the metabolic pattern between succinate and propionate in Bl6.

TABLE II - Effect of fluoroacetate on suspected intermediates of propionate oxidation.

Substrate	Fluoroacetate	Percent theoretical O_2 uptake	Net citrate μM
β -hydroxy	+	11	-.02
Propionate	-	38	0
Acrylate	+	15	-.07
	-	54	0
Propionate	+	60	.08
	-	72	0
Acetate	+	59	.18
Pyruvate	+	16	.27

Each cup contained: 130 μM Tris phosphate buffer pH 7.0; 120 μM Na⁺; 50 μM K⁺; 50 μM Mg⁺⁺; 12 μM K fluoroacetate; 5 μM substrate; 0.2 ml. 20% KOH; 12.5 mg. dry wt. of cells; total volume 3.0 ml; temp. 25°C; gas phase air.

C. Fixation of $C^{14}O_2$ in the presence of propionate and succinate

The metabolism of propionate in animal systems is known to involve an initial carboxylation to yield succinate which is then metabolized by the conventional tricarboxylic acid cycle. An enzyme system has also been demonstrated in many bacteria which will catalyze the carboxylation of propionate.

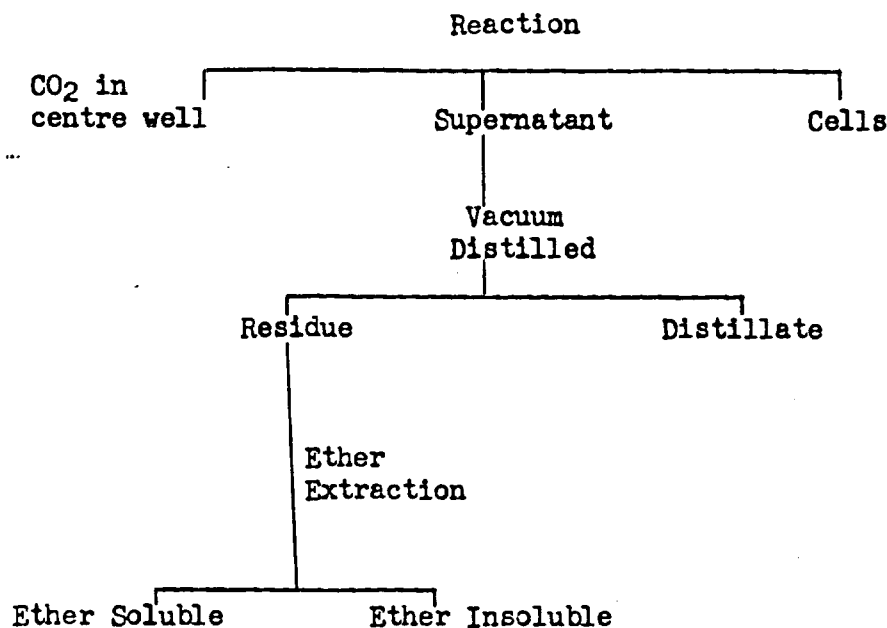
It became of interest to us to determine whether cells of Bl6 would fix a greater amount of $C^{14}O_2$ in the presence of propionate than in the presence of succinate. If they did, then it could be argued that perhaps this initial carboxylation may be an important mechanism for propionate oxidation by Bl6.

The method consisted of allowing Bl6 cells to metabolize the substrate in the presence of $NaHC^{14}O_3$ for a specified period, following which the cells were removed and the various fractions of the experiment analyzed for C^{14} content.

The cells were allowed to oxidize the substrate in 125 ml. erlenmeyer flasks which had been adapted with sidearms and a centre well as in a conventional Warburg flask. Rubber serum stoppers were used to cover the openings, thus permitting the injection of $\text{NaHC}^{14}\text{O}_2$ at the beginning of the experiment, HCl to stop the reaction and NaOH into the centre well to absorb residual CO_2 .

Each flask contained 0.5 ml. 0.52 M Tris phosphate buffer pH 7.0; 1.0 ml. Bl6 cells at 1 mg N per ml.; 320 μM Na^+ solution; 160 μM K^+ solution; 150 μM Mg^{++} solution; 1.5 to 2.0 ml. $\text{NaHC}^{14}\text{O}_3$ solution; 20 μM substrate in a total volume of 6.0 ml. The flasks were incubated at 25°C. on a rotary shaker for 50 minutes following which 1.0 ml. of 10% HCl was added to stop the reaction and 1.0 ml. 10% NaOH was added to the centre well to absorb residual CO_2 . Shaking was allowed to continue overnight to ensure complete CO_2 absorption.

Each cup was then fractionated as follows:



The results of the experiments are given in Tables III and IV.

TABLE III - $\text{NaHC}^{14}\text{O}_3$ in presence of propionate.

Fraction	Exp. 1	Exp. 3	Exp. 4	Exp. 5
Initial	1,614,000	916,640	2,940,950	3,855,000
Centre well	1,733,000	1,000,600	2,710,300	2,987,300
Supernatant	34,868	33,276	56,648	110,800
Cells	10,960	5,600	27,920	13,900
Residue	18,500	26,320	43,000	108,300
Distillate	1,340	2,390	2,400	1,300
Ether Soluble	1,368	704	800	2,830
Ether Insoluble	14,220	15,190	38,240	47,680

Values expressed in counts per minute.

TABLE IV - $\text{NaHC}^{14}\text{O}_3$ in presence of succinate.

Fraction	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Initial	1,547,000	916,640	2,940,950	3,855,000
Centre well	1,674,000	918,600	3,086,600	2,781,500
Supernatant	28,670	13,924	60,552	37,580
Cells	6,500	2,100	10,180	6,000
Residue	27,440	13,200	52,280	31,280
Distillate	0	350	230	240
Ether Soluble	1,120	224	400	390
Ether Insoluble	19,940	10,770	51,140	29,980
Valves expressed in counts per minute.				

If the carboxylation of propionate is an obligatory step in its metabolism, then more C^{14}O_2 should be fixed with propionate as substrate than with succinate as substrate. Thus the supernatant fraction should be more radioactive in the presence of propionate than in the presence of succinate. This is borne out very nicely in Experiments 3 and 5. However, when Experiments 1 and 2 are compared and Experiment 4, it can be seen that the supernatants here contain about equal activities. Thus there does not seem to be any definite pattern of CO_2 fixation.

The ether soluble fractions, which would contain the tricarboxylic acids, had very little activity. The ether insoluble fractions, which would contain amino acids and sugars, had considerable activity. These fractions have yet to be examined in detail.

Thus, as would be expected, there is a significant fixation of C^{14}O_2 by B16 cells when the substrates propionate or succinate are oxidized. However, there does not appear to be a greater amount fixed consistently with propionate as substrate as compared to succinate as substrate. Therefore, we are unable to determine by this method whether the carboxylation of propionate is a necessary step in its metabolism.

D. Separation of intermediates of the tricarboxylic acid cycle on celite chromatographic columns

Because of the remarkable success of other workers in separating members of the tricarboxylic acid cycle on chromatographic columns packed with celite (Swim and Utter Methods in Enzymology IV, p. 584, Academic Press, New York, 1957), it was decided to investigate this method in studying the metabolism of propionate- 1-C^{14} by organism B16. In Summary No. 17 of this Station's Annual Report for 1956-57, we reported on a separation procedure which involved ethanol and ether extractions followed by paper chromatography. Certain difficulties were encountered which lead to the search for a more desirable separation procedure.

Johns-Manville Celite Analytical Filter-Aid, washed with ether and dried, was used to pack the columns. Elution was carried out by chloroform-n-butanol mixtures equilibrated with 0.2 N H_2SO_4 . Several gradient elution techniques were tested but none proved as satisfactory as a step-wise elution starting with chloroform and proceeding to a final solution of chloroform containing 60% n-butanol.

The following known acids were passed through the columns singly and in combination so that peaks of known acids could be identified from future experiments. 50 μM sodium propionate; 50 μM sodium pyruvate; 20 μM fumaric acid; 10 μM succinic acid; 10 μM α -ketoglutaric acid; 20 μM malic acid; 10 μM citric acid.

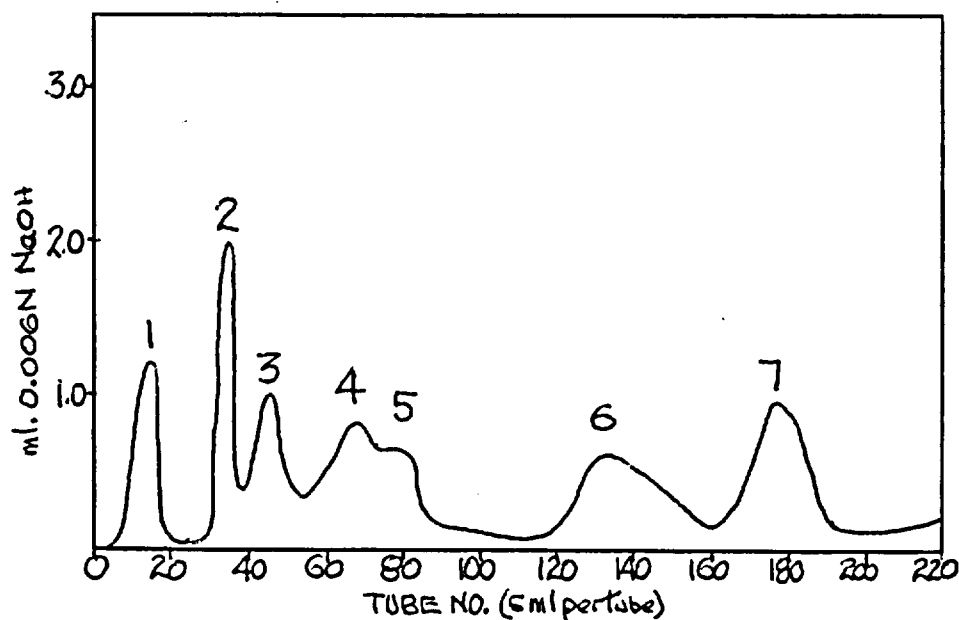
Samples of 5 ml. were collected in an automatic fraction collector and titrated with 0.006 N NaOH using phenol red as an indicator. Fig. 1 represents a typical chromatograph of a mixture of known acids.

The experiment, using propionate- l - C^{14} , was carried out in a 2.8-litre Fernbach flask fitted with a 3-hole rubber stopper, containing a separatory funnel and an inlet and outlet tube. The flask was continually flushed with CO_2 -free air by drawing the air through two bead towers containing 3 N NaOH, a tower of water, then through the reaction flask, followed by two small bead towers containing 3 N NaOH to remove any $C^{14}O_2$ produced.

The flask contents were as follows: 22 ml. 1 M phosphate buffer pH 6.4; 4000 μ M Na propionate (187 μ M Na propionate- l - C^{14} + 3813 μ M cold Na propionate); 77 grams Bl6 (grown in Sw-1 medium) suspended in artificial sea water to 200-ml. volume; water to a total volume of 500 ml.

The cells were collected by centrifugation and washed with 250 ml. distilled water.

Fig. 2 Typical chromatograph of a mixture of known acids.



- | | |
|--------------|---------------------------|
| 1. Propionic | 5. α -ketoglutaric |
| 2. Pyruvic | 6. Malic |
| 3. Fumaric | 7. Citric |
| 4. Succinic | |

After washing, the cell-paste was acidified to pH 2 with 2 N HCl and lyophilized.

The supernatant, after removal of the cells, was reduced to approximately 80 ml. by direct distillation then acidified to congo red with H_2SO_4 . Fifteen volumes were steam distilled.

The distillates were neutralized and taken to dryness on a flash evaporator. The residue was further reduced by direct distillation to 10 ml., mixed with 50 grams celite, packed in a column and extracted with 1500 ml. ether.

This ether extract was taken to dryness and chromatographed on a 10-gram celite column. Tentatively the peaks for pyruvic, fumaric, succinic or lactic,

α -ketoglutaric and malic acid were found. However, when the radioactivity of these fractions was determined, the peak of C^{14} content did not coincide with any of the major peaks as determined titrimetrically. These unknown radioactive compounds have yet to be determined.

The cells, following lyophilization, were mixed with 40 gm. celite, adjusted to pH less than 2 with H_2SO_4 , and packed in a column. The organic acids are eluted with 3 l. of ether followed by evaporation of the ether. The cell-celite mixture (after removal of the ether) was thoroughly extracted with water yielding a water soluble fraction. The residue remaining after extraction with water was suspended in 200 ml. 10% HCl and autoclaved at 15-lbs. pressure for 12 hours. The undissolved material was removed by centrifugation and the residue washed several times with water. The supernatants were combined and evaporated to dryness and this is referred to as the water insoluble fraction.

It is extremely interesting to note that the peaks of titratable acidity and radioactivity do not coincide in the fractions that have thus far been analyzed. It thus appears that at least C-1 of propionate-1- C^{14} does not become part of the tricarboxylic acid cycle acids. What will happen when this experiment is repeated using propionate-2- C^{14} or succinate-1- C^{14} should produce interesting data and will help to elucidate some of the pathways of intermediary metabolism of B16.

SUMMARY NO. 49

OXIDATIVE METABOLISM OF A MARINE BACTERIUM II. STUDIES ON TRICARBOXYLIC ACID CYCLE ENZYMES

R.A. MacLeod
C.A. Claridge
A. Hori

As part of the program to elucidate the mechanism of terminal respiration in one of our marine bacteria, evidence for the existence of the enzymes of the conventional tricarboxylic acid cycle was sought. Succinic dehydrogenase - cell-free extracts were prepared by sonic disruption of cell suspensions washed free of Na^+ and K^+ by a solution containing Mg^{++} ions. Succinic dehydrogenase activity was measured manometrically using a Warburg respirometer by measuring the rate of uptake of O_2 on the addition of succinic acid as substrate. KCN was added to inhibit cytochrome oxidase and methylene blue was included as an autoxidizable hydrogen acceptor. It was found that oxygen was taken up in the presence of KCN and in the absence of M.B., indicating either that the succinic dehydrogenase of these cells could couple directly with O_2 or a natural KCN insensitive autoxidizable hydrogen acceptor was present in the cells. The formation of fumaric acid in reaction systems containing added succinic acid was demonstrated by paper chromatography.

Fumarase - An active fumarase was demonstrated in cell-free extracts of the organism. The enzyme reaction was carried out in a spectrophotometer cuvette and the rate of formation or disappearance of fumaric acid was followed by observing the change in absorption in a spectrophotometer at a wavelength of 240 m μ . The formation of malic acid from fumaric and the reverse was demonstrated by paper chromatography.

Malic dehydrogenase - This enzyme was shown to be present in cell-free extracts of the organism. The reaction was followed by observing the rate of reduction of triphosphopyridine nucleotide in the presence of malic acid. Increase in absorption by the reaction mixture due to the reduction was followed at 340 m μ in a Beckman spectrophotometer. The system was found to require TPN rather than DPN and to require a 167 mM concentration of K^+ for maximum activity. Reversal of the reaction was achieved with oxalacetate rather than with pyruvate and CO_2 , indicating that a malic dehydrogenase rather than a malic enzyme was present.

We were very interested in the effect of Na^+ and of K^+ on the activity of these enzymes since we had shown previously that there was a requirement for Na^+ and for K^+ for oxidation of substrates by whole cell suspensions of the organism. It had furthermore been shown that the di- and tri-basic acids required three or four times as much Na^+ for maximum rate of oxidation as the monobasic acids.

The effect of Na^+ and K^+ in various combinations and concentrations on the activity of enzymes in a cell-free extract is shown in Table I.

TABLE I - Activity of succinic dehydrogenase, fumarase and malic dehydrogenase in cell-free extracts of a marine bacterium at various concentrations of Na^+ and K^+ .

Ions added		Succinic dehydrogenase	Fumarase	Malic dehydrogenase
Na^+	K^+	Relative activity (%)*		
mM				
0	0	100	100	8
200	33	65	25	37
200	-	55	36	5
33	-	88	97	8
-	33	70	77	82
-	200**	68	38	100
33	200**	58	30	100

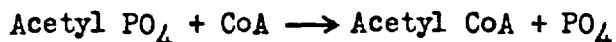
* For each enzyme the maximum activity attained was assigned the value of 100. The activity under other conditions is expressed as percent of the maximum activity.

** For malic dehydrogenase the level of K^+ for optimum rate of oxidation (167 mM) was used.

The lack of a requirement for Na^+ for activation of the three enzymes tested in the cells, coupled with evidence that the substrates involved required more Na^+ for maximum rate of oxidation by whole cells than did monobasic acids, led to the conclusion that Na^+ is involved in transport of the di- and tri-basic acids across the cell membrane. Since di- and tri-basic acids can form chelates and since molecular spin resonance techniques have shown that complexes between Na^+ and common metabolites will form, it is concluded that di- and tri-basic acids must form a complex with Na^+ before crossing the cell membrane.

Condensing enzyme - This enzyme is a most important one where the tricarboxylic acid cycle is the terminal respiratory pathway. This enzyme catalyzes the condensation of acetyl coenzyme A and oxalacetate to form citrate.

Acetyl CoA + oxalacetate $\rightarrow$ citrate. Coenzyme A is generated through the action of transacetylase which catalyzes:



A reaction system consisting of acetyl PO_4 , CoA, transacetylase (commercial preparation), oxalacetate and cell-free extract of the organism was prepared and allowed to incubate for 60 minutes at 25°C . At the end of this time a quantitative analysis for citric acid was performed. The results and the effect of deleting various components from the complete system are shown in Table II. Removal of any one of the components caused a marked reduction in citrate formation. ATP and acetate could not replace acetyl PO_4 . The absence of oxalacetate completely prevented citrate formation.

TABLE II - Effect of various components of the reaction system on the formation of citrate by condensing enzyme from marine bacterium B-16.

Component deleted*	Citrate formed μ per ml.
None	365
Cysteine	170
Transacetylase	112
Acetyl PO ₄	70
Acetyl PO ₄ (acetate + ATP)	23
CoA	70
Oxalacetate	0

* Complete system contained cysteine, transacetylase, acetyl PO₄, CoA, oxalacetate and cell-free extract of B-16.

Isocitritase - This enzyme has recently been found to be present in a number of bacteria and has been ascribed an important role as a source of glyoxylate for the formation of glycine in cells. The enzyme catalyzes the conversion of isocitrate to succinate and glyoxylate. Attempts to demonstrate the formation of glyoxylate from isocitrate by cell-free extracts of marine bacterium B-16 were unsuccessful. Since a sensitive test for the determination of glyoxylate was used, and since a variety of test conditions was employed, it seems reasonable to conclude that this enzyme is not present in our organism at least under the conditions under which it was grown in these experiments.

Cytochrome oxidase - Of interest in connection with the function of Na⁺ and K⁺ in metabolism is the observation of Baxter and Gibbons that the cytochrome oxidase of a halophilic bacterium was found to be activated by relatively high concentrations of Na⁺ and K⁺ ions. Since the cytochrome system has been postulated to play a role in ion transfer in many organisms, they concluded that their observation of the salt requirement of cytochrome oxidase supported this conclusion. To see if cytochrome oxidase in our marine bacterium was similarly dependent on ions for maximum activity, a test system for this enzyme was investigated. Reduced cytochrome C was used as the substrate for the reaction and the rate of oxidation was followed by determining the rate of change in absorption at 550 mμ in the Beckman spectrophotometer. The effect of various concentrations of Na⁺ and K⁺ on the reaction are shown in Table III.

TABLE III - Cytochrome oxidase activity of a marine bacterium at various concentrations of Na⁺ and K⁺.

Ions added		Activity O.D. change/2 min.*
Na ⁺ mM	K ⁺	
0	0	.094
200	33	.090
200	-	.087
33	-	.077
-	33	.066
-	200	.069
33	200	.072

* At 550 mμ.

As in the case of succinic dehydrogenase and fumarase, the activity of cytochrome oxidase was greater in the absence of added Na⁺ and K⁺ than in the presence of any one or combination of the two ions tested.

SUMMARY NO. 50

THE SODIUM AND POTASSIUM CONTENT OF
FISH FROM THE BRITISH COLUMBIA COAST

R. Jonas
R.A. MacLeod

This is a continuation of work reported in Summary No. 23 of this Station's Annual Report for 1956-57. It concerns a further investigation of the variations in the Na^+ and K^+ content of the flesh of herring and lemon sole. Results on salmon, since they are a part of a study of biochemical changes during salmon migration, are reported separately (Summary No. 8 of this report).

Most fish were sampled according to the modified A.O.A.C. method, although variations from this routine were made from time to time and will be noted. Five-gram samples were solubilized by nitric acid as previously described. A Beckman DU spectrophotometer with a flame photometer attachment and photomultiplier was used in all analyses. Spectral lines 589 mμ for sodium and 768 mμ for potassium were employed. Moisture and nitrogen were determined by the same methods that have been previously described. Fat was determined by the Damberg method as it was found to be rapid and accurate and the results compared very favourably with those of other methods.

Herring (*Clupea pallasii*)

Because of the size of the fish, herring were occasionally sampled by removing fillets from the sides rather than by pooling flesh obtained from slices cut according to the A.O.A.C. method. When the same batch of herring was sampled by the two procedures, variations in the Na^+ and K^+ content of the flesh were noted. This led to the suspicion that different levels of sodium and potassium may exist in different parts of the body of the fish. To check this possibility further a group of herring caught in February (1957) was sampled for analysis. Five fish of the same approximate size were chosen from the group. The long muscles on either side of the dorsal fin of each fish were removed and pooled. Another pool of muscles from the left and right ventral sides of the same fish were prepared. A third pool was made of milt and a fourth of roe. Each was homogenized separately in a Serval Omnimix homogenizer. Aureomycin at a level of 1 part per million was incorporated into each of the samples as a preservative. This procedure was repeated on a group of herring obtained in March of the same year. The results of the Na^+ and K^+ analyses, together with information on the fat and protein content of the muscle tissues are recorded in Table I.

TABLE I - Na^+ and K^+ content of different parts of the herring.

Flesh	Date	% H_2O	Fat	Protein	mg./100 g. flesh	
					Na^+	K^+
Dorsal) muscle)	February 1957	75.83	6.50	17.55	43.3	529
Ventral) muscle)		72.10	7.90	17.32	103.3	445
Dorsal) muscle)	March 1957	78.65	4.10	15.43	92.0	459
Ventral) muscle)		73.90	4.50	15.38	92.8	404
Milt)	February	-	-	-	125.7	320
Roe)	1957	-	-	-	112.6	287

In the February sample Na^+ in the dorsal muscle tissue was less than half the level in the ventral muscle while K^+ was correspondingly high. This situation did not apply in the March sample where values for Na^+ in both muscles are very nearly the same. There is at present no explanation for the marked difference in Na^+ and K^+ values in dorsal muscle tissue in February and March. March is the month these herring spawned and results obtained with salmon showed marked increases in the Na^+ content of the muscle tissue just prior to spawning (Summary No. 8 of this report). It is not unlikely that a similar phenomenon occurs in herring but it is not clear why only the dorsal muscle should be involved. There is no obvious correlation between the Na^+ and K^+ values obtained and the H_2O , fat and protein content of the flesh. It is of interest that a group of investigators in the U.S. have reported variations in the Na^+ content of flesh taken from different sections of the body of pink salmon.

Lemon sole (Parophrys vetulus)

Studies reported last year (Summary No. 23 of this Station's Annual Report for 1956-57) had shown that Na^+ levels in the flesh of lemon sole caught in one fishing area were markedly different from those from fish caught in another area. It was decided that a further observation should be made on the same species of fish caught at a different time of the year. It was not possible to obtain fish from Area 21 again but fish from Areas 17 and 18, which are not too far distant from Area 21, were sampled. Fish were obtained from Area 12 as before. The results, Table II, show that in March there was very little difference between the flesh levels of Na^+ of fish caught in the two fishing areas. Furthermore, if the results obtained for fish caught in Area 12 in March are compared with those for fish caught in the same area in July of the previous year, it is apparent that there is a considerable difference in values for levels of Na^+ in the flesh. What appeared previously to be a variation in fish from one area to another would now seem to be the result of a seasonal variation within an area. It is becoming clear from these studies that Na^+ and K^+ levels in the flesh of any given species of fish are by no means constant quantities and the levels are subject to wide variations due to factors about which we have little knowledge.

TABLE II - The Na^+ and K^+ content of the flesh of lemon sole caught in two different fishing areas in March.

Area where caught	Month caught		Na^+	K^+	Fat	H_2O
			mg./100 gm. flesh*			
No. 12	March	Average	83.2	394	2.02	79.1
		Range	71.7-98.0	362-410		
Nos. 17 and 18	March	Average	93.7	345	1.20	81.2
		Range	81.9-107.2	330-370		

* 6 fish from each area were sampled.

MISCELLANEOUS

ANALYSES

A.P. Ronald

Iron-pickling effluent - Analyses were requested by Mr. M.P. Houghton, Fisheries Inspector, on samples from an iron-pickling process at Burrard Dry Dock Co., North Vancouver. The following results were obtained:

<u>Sample</u>	<u>Iron (Fe+++)</u>	<u>HCl</u>
#1 ex tank	1.00%	0.84%
#2 ex ejector hose end	0.59%	0.52%
#3 ex sump at tide water	0.15%	0.48%

Iron in sodium chloride - Samples of salt suspected of containing iron were received from the Queen Charlotte Cannery Ltd., accompanied by a request for analysis. Two samples were sent, one from a new bag, the other from the drier. The sample from the drier looked dirty and gave a cloudy solution. Examination showed that the impurity was organic. Both samples had a low iron content. Salt from the new bag had 0.00005% iron as against 0.0004% for the salt from the drier.

Black spots on inner surfaces of cans containing crab meat - Queen Charlotte Cannery Ltd. requested that we identify black spots which had appeared on tins of a special pack of crab meat. The black spots were shown to be iron sulphide and it was also shown that much of the zinc in the enamel had been converted to zinc sulphide.

Iron in boat's water tanks - The Department of Fisheries requested analyses on samples of water taken from tanks on a boat. The water was found to contain a rather high level of iron. Miss B.A. Southcott identified an iron utilizing bacterium in the water.

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14. Dogfish. (For Pacific Prog. Rept., No. 110)
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STAFF OF THE VANCOUVER TECHNOLOGICAL STATION

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Executive Assistant (Admin. Officer, Grade 2)	J.W. Kilpatrick, <u>M.C.</u>
Secretary (Stenographer, Grade 3)	Nellie E. McBride
Stenographer and Clerk (Stenographer, Grade 3)	Cecilia L. Robertson

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Junior Scientist	Eva Onofrey, B.Sc.(Manitoba)
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Associate Scientist	P.J. Schmidt, B.E.(Sask.)
Assistant Scientist	U.H.M. Fagerlund, M.Sc.(Abo, Finland)
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Associate Scientist	J.S.M. Harrison, B.A.Sc.(Brit.Col.)
Associate Scientist	S.W. Roach, B.A.Sc.(Brit. Col.)
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Assistant Scientist	Burnett A. Southcott, B.S.A.(Brit.Col.)
Assistant Scientist (from May 6/57)	Rudolph H. Moyer, (Brit. Col.)
Junior Scientist (from June 28/57)	Vera M. Creelman, B.A.(Brit. Col.)
Junior Scientist (resigned June 29/57)	Helen M. Bluhm, B.S.A.(Brit. Col.)

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Librarian (Asst. Technician, Grade 3) part time	Alix G.A. Richards, B.A.(McGill)
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Caretaker (Grade 2)	P.E. Enright
Cleaner & Helper (part time)	Rita Reichert
Cleaner & Helper (part time)	Agnes M. Hunter

TEMPORARY STAFF

S.O. Akintobi, B.Sc. (Tennessee), Student Assistant. Term May 13 to July 15. (Chemistry).

I.I. Bitners, Student Assistant. Part time Jan. 1 to March 29/57. Term May 1 to Sept. 30. Continuing part time. (Chemistry)

R.J. Bose, M.S.A. (Brit. Col.). Student Assistant. Term May 6 to Sept. 3. (Biochemistry).

D.B. Clement, B.Sc. (Oregon). Student Assistant. Term May 13 to Sept. 3. Part time Oct. 1 to Oct. 12. (Chemistry).

R.B. Colman, Student Assistant. Term, July 18 to Sept. 18. (Chemistry).

D.W. Duncan, B.S.A. (Brit. Col.). Student Assistant. Term April 29 to Sept. 16. (Microbiology).

H. Hogenkamp, B.S.A. (Brit. Col.). Student Assistant. Part time Jan. 1 to March 15/57. Term May 1 to Aug. 23. (Biochemistry).

E.J. Hrushowy, B.A. (Brit. Col.). Technician, Grade 1, part time Jan. 2 to March 30/57; also part time from November, continuing. (Chemistry).

Clare Hucal, B.A. (Manitoba). Asst. Technician, Grade 3, part time Librarian from Oct. 28/57 to Jan. 31/58.

R.E.E. Jonas, B.A. (Madras). Asst. Scientist. From November 16, 1956, continuing. (Biochemistry).

J. Leung, Student Assistant. Term May 21 to Sept. 23. (Microbiology).

L.J. Lukey, Asst. Technician, Grade 3. Part time from Nov. 1, continuing. (Biochemistry).

A.M. MacQuillan, B.S.A. (Brit. Col.). Technician, Grade 1. Part time from Oct. 16 to November 1. (Biochemistry).

E.L. Maddex, B.S.A. (Brit. Col.). Asst. Technician, Grade 3. Part time from Dec. 2 to Dec. 27. (Biochemistry).

C.R. Muirhead, B.A. (Brit. Col.). Student Assistant. Part time from Jan. 1 to March 29/57. Term May 6 to July 15. (Bacteriology and microbiology).

C.J. Tai, B.Sc. (Alberta). Jr. Scientist from Dec. 1/56 to April 30/57. Student Assistant, term May 1 to Oct. 11. (Biochemistry).