CONFIDENTIAL

FISHERIES RESEARCH BOARD OF CANADA

ANNUAL REPORT

for

1958 - 59

of the

TECHNOLOGICAL STATION
Vancouver, B. C.

H. L. A. TARR, Director

SH
223
A119
1958/59

FISHERIES RESEARCH BOARD OF CANADA

TECHNOLOGICAL STATION
VANCOUVER, B.C.

INTRODUCTION

At the time of the preparation of last year's report a new building to replace greatly inadequate quarters was being erected on the University of British Columbia campus. At the time of completion of the present report the transfer to the new Station is almost finalized.

Since its small beginning in 1926, the Station has grown slowly but strongly, and the theoretical and practical contributions to fishery knowledge made by the staff have found wide appreciation and application, both nationally and internationally. The more modern building and closer association with academic research should provide an even better environment for original contributions to branches of the fisheries where chemical, microbiological and engineering possibilities have been inadequately explored.

H. L. A. Tarr,
Director.

GENERAL SUMMARY OF INVESTIGATIONS

TABLE OF CONTENTS

Biochemistry of Migrating and Maturing Salmon

Studies continued on the Stuart Lake sockeye captured in 1957. Cholesterol, fat, protein and water were determined in the body of the standard fish. The changing composition of the body exclusive of flesh was compared and contrasted with the changes occurring in the flesh. It was shown that the flesh contributed little toward maintaining the body weight during the river migration, whereas the remainder of the body took on considerable water. The cholesterol levels in the remainder of the body of the female decrease only slightly while those of the male increase. The flesh was the major source of fat for both sexes during the early phase of the river migration but the remainder of the body was the major source of fat for the greater part of the journey. The flesh was the major source of protein for both sexes and the remainder of the body actually had more protein at the end than at the beginning of the migration. The energy consumed from the body of the standard female over the entire migration was 1.1 cal/kg/km as compared to 0.84 cal/kg/km for the male.

The androgen content of the male testes was 41 µg/kg at Lummi Island, 106 µg/kg at Lillooet, and 133 µg/kg at Forfar Creek as determined by bioassay.

The distribution of phosphorus compounds, creatine, inositol and fat in all major organs and tissues of the Stuart Lake samples was studied. One of the more interesting observations was the marked decrease in the sugar phosphate content in the flesh of both sexes at Lillooet. The decrease in total phosphate of the female from Lummi Island to Forfar Creek more than accounts for the gain in total phosphate in the roe and remainder of the body. However, the gain in phospholipid phosphate in the roe and remainder of the body is not accompanied by a compensatory loss of this constituent in other tissues. On a unit weight basis roe and liver are the tissues most rich in phospholipid, while flesh is most abundant in inorganic and nucleotide phosphorus. The head, skin, bones and tail are devoid of nucleotide phosphate and are a poor source of sugar phosphates. However, these tissues are second only to flesh as a source of creatine. Acid-soluble inositol is found in high concentration in the

kidney and also in the alimentary tract during the early phase of the river migration. The head, skin, bones and tail contain the largest amount of acid-soluble inositol based on the standard fish. The phospholipid content of the alimentary tract, particularly of the female, increases during the river migration, but due to the extensive wasting of these tissues, all components of the standard fish decrease in absolute amount. The sugar phosphate concentration in the kidney of the female increases appreciably during the migration. The nucleotide concentration in the kidney of both sexes reached a maximum at Lillooet. The kidney of the female is rather unique in that the fat concentration increases in the later phase of the migration.

Cortisone, which is apparently not present in detectable concentration in the blood of mammals, was isolated from sockeye salmon plasma. Cortisol, the principal adrenal cortical hormone in the peripheral plasma of the normal human, was also isolated from sockeye plasma. Another steroid, 17α-hydroxyprogesterone, previously undetected in blood, was isolated from plasma taken from pre-spawning sockeye salmon. Plasma from pre-spawning female salmon contained double the concentration of cortisone and cortisol of the male plasma, 41 μg and 26 μg/100 ml respectively as compared to 22 μg and 11 μg/100 ml. The same plasma from the female contained 19 μg/100 ml of 17α-hydroxyprogesterone. Corticosterone, 11-desoxycorticosterone and aldosterone were not present in identifiable amounts in the plasma from more than 100 fish. Cortisone, cortisol and total 17-hydroxysteroids were quantitatively determined in plasma samples obtained from two pure races of sockeye captured at various stages of sexual maturity. The combined concentration of cortisone and cortisol which, at the mouth of the river was three times that of the normal human, increased to as high as seventeen times this level following spawning. Cortisol is the major steroid of both sexes following spawning but cortisone predominates during the later phase of the river migration. It was also shown that the major increase in plasma steroids almost certainly occurs post-spawning, and the results are discussed in relation to the degeneration and death of the fish after spawning. The probable physiological

significance of the results is discussed.

Capilano river coho salmon were analyzed at the request of the Department of Fisheries.

It was found last year that free histidine in the muscles of migrating sockeye salmon decreased to one-fifth the initial level during the early stages of migration. This year it has been found that this is also true with the alimentary tract of these fish and to some extent with liver and gonads. A detailed investigation has been made of the amount of deoxyribonucleic acid and ribonucleic acid in all tissues of migrating sockeye salmon, and on the nucleotide composition of the RNA. The DNA content of the male gonads increased by about 60% and the RNA content of the roe nearly doubled during migration. There was a net loss in RNA and a net gain in DNA in the whole fish during migration. The ratio of the four nucleotides which result from alkaline hydrolysis of the RNA was with few exceptions quite similar in the different tissues and again, except in one instance, did not change to any important extent during migration.

To determine whether the collagen changes observed in herring during maturation were unique for this species, analyses were made of collagen levels in tissues of migrating sockeye salmon. Collagen increases in the salmon were noted in the flesh and in a group of tissues which consisted of the head, skin, bones and tail but these increases were restricted to males.

Analyses of the tissues of a group of coho salmon which had been held to ripen in tanks at the Vancouver Aquarium last year were completed. Although the small number of fish which could be handled in the tanks available made it difficult to assess some of the results obtained, a few general trends were evident. Analyses of blood for non-protein nitrogen, amino acid nitrogen, total protein, glucose, sodium and potassium, revealed that, except for the amino acids, the blood components were maintained at remarkably well-elevated levels over the whole three-month period the fish were held without food. In the case of the amino acids a drop in blood level to half the previously prevailing value occurred at the end of the second month. Analyses of skeletal muscle, lateral line muscle, liver, kidney and heart for fat, protein and

water showed that the fat levels of the tissues remained remarkably constant over the whole period the fish were without food. There was also considerable nitrogen loss from the liver, indicating that this organ may well be an important depot for nitrogen compounds for the milt and roe.

A study of these components was also made in sockeye salmon blood at various points on the path of spawning migration to determine if parallel changes occurred in the two species of fish. Only in the case of the amino acid levels were the changes occurring obviously different from those found in ripening coho.

Steelhead trout (Salmo gairdnerii gairdnerii) were used as a readily available source of tissue for a study of the oxidative and glycolytic pathways in fish tissues. The general oxidative and glycolytic patterns in steelhead were shown to be the same as had been previously observed in coho. The inability of homogenates of tissues, other than heart muscle, to convert glucose to lactic acid was found to be due to a lack of hexokinase in the homogenates. For skeletal muscle, an active apyrase also interfered with primary glucose phosphorylation. Homogenates retained glycolytic activity if kept frozen in the presence of ethylenediaminetetraacetic acid. An active phosphoglucomutase was demonstrated.

Fresh Fish Preservation

Studies on preservation of fresh fish have continued. Experiments with eviscerated dogfish which were held under ideal conditions in refrigerated-sea-water and in ice with and without added chlortetracycline antibiotic have not borne out findings of previous investigators with this type of fish. Thus the flesh did not become appreciably alkaline nor did the total volatile base content rise very significantly until the fish had been held for at least 18 days. Bacterial counts tended to rise sharply after about 18 to 20 days. The results indicated that bacterial counts increased more steadily during spoilage than did either trimethylamine or total volatile base content of the muscle of these fish. Further studies with dogfish are in progress and it appears that these fish spoiled no more rapidly than do most food fish

previously studied at this Station. So far in these experiments with dogfish, CTC antibiotic treatment has not given the spectacular improvement which has usually been evidenced with salmon, lingcod and other food fish.

Further detailed investigations with lingcod stored in refrigerated-sea-water with and without added CTC antibiotic have again indicated a very marked improvement in quality of the antibiotic-treated fish. The results have shown that the total bacterial numbers in the flesh of fish held in refrigerated-sea-water is very much greater than that of the living or viable bacteria. There was no indication of development of strictly anaerobic bacteria in the sea-water. Undesirable coliform bacteria, which are usually indicative of sewage or faecal pollution, were not found in CTC-treated harbour sea-water but were found and definitely persisted in untreated sea-water. Reports from certain localities have indicated that there is considerable loss of bacteriostatic activity in CTC-antibiotic ice which has been prepared from hard rather than from soft waters. This has been shown to be due to a complex which is formed between calcium or magnesium salts, the colloid which is used for distributing the antibiotic, and the antibiotic itself. The curd-like precipitate which forms as such ice melts is relatively insoluble and, although it contains active antibiotic, this is not readily available for desired preservative purposes. The obvious, and apparently only practical solution, is demineralization of the water by accepted, simple, practical techniques.

Fish Solubles and Liquefied Whole Fish

A consideration of the factor responsible for high viscosity and gelling of late season herring solubles led to determinations of the gelatin content of solubles prepared from fish caught at approximately monthly intervals during the year. The close parallel observed between changes in physical properties and gelatin content of these solubles strongly suggested that gelatin was responsible for the seasonal variations in viscosity.

Since collagens yield gelatins on heating, it was of interest to know if observed changes in the gelatin content of solubles were due to changes in the collagen concentrations in the fish, or to changes in the solubility of these collagens with season. Fish caught at times when the gelatin in the solubles was at its highest and lowest concentrations were analyzed for total collagen, and for collagen extractable with salt solutions and acid buffer. February fish, which produced solubles with a high gelatin content, contained about twice as much total collagen as fish caught in June when the gelatin level in the solubles was low. The solubility characteristics of the collagens were not appreciably different in the two groups of fish. Examination of individual tissues of February and June fish showed that the increased collagen in February fish was due principally to increases occurring in the skin and scales, the head and the flesh. With skin and scales an increase in the amount of the tissue present in the fish contributed to the increase in collagen. The same phenomenon was noted to a lesser extent in the case of the head.

The preparation of liquefied whole fish is being studied. Liquefaction following adjustment of a whole herring homogenate to acid pH values was shown to be due to natural enzymes present in both the flesh and the viscera of the fish (see also Summary No. 2). Digestion was more rapid at pH 2.0 than at 4.5 and at 37°C than at 25°. At 37°C and pH 2.0 digestion was essentially complete in 24 hours. Much more rapid digestion occurred if homogenates were supplemented with commercially available proteolytic enzymes. Enzymes with a low pH optimum were more effective than those with a high one. Pepsin was the most active for with this enzyme digestion of a preheated homogenate was essentially complete in 90 minutes.

Packaging and Antioxidants

Studies initiated last year concerning application of various packaging materials, vacuum packaging and antioxidant treatment with isoascorbic acid are continuing. It is becoming increasingly evident that the vacuum packaging technique normally used does not remove all the oxygen from the fish but that the technique is

desirable for it produces a close cling of the material to the surface-treated samples. The results have already shown the superiority of certain of the more oxygen impermeable films used and that extrusions are preferable in general to laminates for low-temperature storage. The tests are continuing.

Experiments on nutritive value of antioxidant-treated herring meals have continued. The antioxidants "Santoquin" and butylated hydroxytoluene, both of which have been studied extensively on the east coast of the United States, and one of which is used commercially for fish meal there, have continued. It has been found that, as judged by appearance and by the objective thiobarbituric acid test, treated meals show considerable improvement over the untreated meals. However, chick nutrition tests have not presently indicated that the antioxidants protect the protein nutritive value of the herring meals. In view of these results the possible value of the antioxidant treatment in protecting the nutritional value of the usually considerable amount of oil present in herring meals is being investigated. Difficulty is being experienced in evolving suitable nutritional tests for the total lipids extracted at various storage intervals.

Muscle Enzymes and Deterioration

It is now well established through work carried out at this Station or elsewhere that various tissue enzymes are often involved in occasioning or accelerating certain of the undesirable processing changes such as browning and oxidative rancidity in fish processed by different methods. In view of this a considerable amount of basic work is being carried out in order to ascertain distribution of some of the enzymes which may be involved in catalyzing these changes in fish muscles. It has been known for some years now that the five carbon atom sugar ribose is one of the important agents which contributes to browning of dried and heated fish muscles, and it has been postulated that this arises either from fish muscle ribonucleic acid and/or adenosine triphosphate. Enzyme preparations have now been obtained which will together carry out complete degradation of ribonucleic acid to yield the free sugar

ribose and purine or pyrimidine bases. These enzymes occur in the muscles of at least six different species of Pacific coast fish. Thus a highly active ribonuclease capable of splitting native ribonucleic acid has been isolated. This enzyme will degrade the nucleic acid as far as the mononucleotide stage. Fish muscles also contain a number of phosphomonoesterase enzymes which are capable of splitting phosphorus from these nucleotides to yield nucleosides which are then split by previously described nucleoside hydrolase enzymes to yield the free ribose responsible for browning. It was previously shown that, though ribose is active as such, through complicated enzyme changes which probably occur post mortem in fish muscles it can be changed to a simple ribose 1-phosphate ester and thence to ribose 5-phosphate. Work conducted during the present year has shown that the picture is even more complicated than was first assumed since enzymes have been isolated and described which will transform the rather stable ribose 5-phosphate ester into keto pentulose sugar phosphates, namely ribulose 5-phosphate and xylulose 5-phosphate. The present and previous studies have shown that certain fish muscles form an excellent source of enzymes which may be used for preparation of certain of these rare and interesting biochemical pentose phosphate esters.

Work carried out elsewhere has indicated that the presence of free fatty acids arising from hydrolyzed triglyceride fats may hasten development of oxidative rancidity either directly or through rendering added or natural antioxidants less effective. Work carried out at the Halifax Station has shown that free fatty acids do arise in muscles of frozen fish during storage. At this Station a lipase enzyme capable of hydrolyzing a number of fairly readily solubilized triglyceride esters have been prepared from lingcod muscle. A special method of preparation had to be evolved since the enzyme is rather unstable at 0°C though may be kept in the frozen state for some weeks without loss of activity.

It was demonstrated several years ago that considerable amounts of the six carbon atom sugar glucose were present in fish muscles post mortem, and that these

could contribute to undesirable browning changes in processed muscles. It has now been found that enzymes capable of carrying out glycolytic transformations of certain simple hexose or glucose phosphate esters such as glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate are present in fish muscles. Certain of these enzymes have been concentrated and studied in some detail.

Refrigerated-sea-water Experiments

When sockeye salmon, halibut, herring, lemon sole and brill were stored in refrigerated-sea-water for a week, analyses of the flesh revealed that the rise in Na^+ and fall in K^+ and weight increases previously recorded for other species had occurred in these fish as well. Solutions containing Na^+ and K^+ at concentrations approximating those calculated for the total tissue water of the fish prevented the ion changes, while the addition of polyoxyethylene sorbitan macromolecules to the immersing solution prevented the weight increases.

Sterilizing Brine by Ultraviolet Radiations

Investigations were made this year on the use of ultraviolet radiation for sterilizing brines, having in mind its application to the treatment of refrigerated-sea-water in holding tanks, and the treatment of wash water on brine dips in processing plants. Some work was done on this problem by this station 20 years ago but the development of small, efficient, commercial sterilamps and the availability to us of apparatus for determining the transmission of ultraviolet radiation in brines have made application and design of equipment feasible.

Our apparatus consists of a 15-watt fluorescent-type sterilamp enclosed in a quartz glass tube, further enclosed in a jacket through which the brine passes at rates of the order of 5 US gpm. Although it has been found that the transmission of ultraviolet radiation is extremely low (5% in 3 mm) in some brines, significant kills of bacteria have been obtained with this apparatus. One test gave a kill of 87% at a flow rate of 5 US gpm. This intensity (about 1200 μwatts seconds per sq. cm.) is much lower than the lethal rates reported for most bacteria, but these tests indicate

that volumes of brine of commercial significance could be treated with small, low-cost apparatus.

Refrigerated-sea-water

Vessels - Consultation with builders of new vessels installing refrigerated-sea-water equipment has continued this year. The steel troller "Ruth G II", completed this year, shows promise of proving a satisfactory technique in building tanks into steel vessels. In this vessel, rigid, foamed glass insulation was applied directly to the vessel's plates over which a steel lining was applied, depending entirely on the insulation for support. The welds were tested by vacuum. Testing has been the chief difficulty in this type of construction. This vessel seems so far to have proven the adequacy of this technique.

The use of fibre glass plastic tank lining as tried in the vessel "Silver Viking II", on the initiative of the ship-builders, has not been satisfactory. The problems which were anticipated, of the attack of the plastic solvent on the polystyrene insulation, and the water tightness of the fibre glass plastic, proved too much for this application. These tanks have been dismantled and reinstalled by the builders using a new plastic and technique which it is hoped will be effective.

Shore tanks - Surplus refrigerated-sea-water equipment removed from the "A.P. Knight" has been installed on shore to study the problem of cannery salmon holding, which is chiefly handling of the fish rather than preservation because of the short term required. Results so far have indicated economies can be effected in labour and ice costs.

Gear

With the Board's decision to reorganize fishing gear research on a broader basis, the gear research unit at this Station was transferred to the Nanaimo Station in November, 1958. A thorough study of nylon monofilament material used to make gill nets was carried out and useful data was obtained on wet mesh strength and it was found that knots in the web had been set in order to prevent slipping. A study was

made of the possible use of a Type 719 nylon in replacing the 300 Type nylon now used for Canadian salmon gill nets. Though the type 719 is definitely a stronger material, it has other undesirable properties which indicate that it may not have an overall advantage for fishing gear. Studies have been carried out on British, Japanese and Canadian nylon twines. It appears that Japanese nylon material has improved and is now similar to the British and Canadian materials. It was also established that currently produced British and Canadian nylon twines are at least equivalent in their properties to materials compared in Industrial Memorandum No. 19 previously published from this Station. Much of the gear research effort was expended on providing information to fishermen, fishing companies, government specifications, boards and industrial manufacturers of fishing gear.

Biochemistry of Invertebrates and Fish

A hitherto undescribed sterol, 7,24(28)-ergostadien-3β-ol, has been isolated from a starfish. It is suggested that the sterol is an intermediate between triterpenes and 24-methylenecholesterol which has been previously identified as a major sterol of several molluscs. A number of marine invertebrates were collected and the fat, phospholipid, non-saponifiable and sterol content determined. Data were obtained on the sterols of some of the species. Experiments were conducted which establish the ability of starfish to metabolize cholesterol *in vivo*. Conversion of cholesterol to 24-methylenecholesterol is probable but not proven. The synthesis of sterols from acetate by molluscs was demonstrated. The rate of synthesis appeared to be much slower than that found for warm-blooded animals, whereas the incorporation of acetate into other non-saponifiable materials was much more rapid. Squalene was converted to sterol by a clam *in vivo*.

The *in vivo* metabolism of 2-C^{14}-myo-inositol in coho salmon was investigated and the distribution of C^{14} determined in all major tissues. The liver was the richest source of C^{14} nucleotides and it was investigated in detail. Four nucleotide fractions contained radioactivity. The radioactivity in one fraction was all located in the

glucuronic acid. A highly unstable inositol compound(s) was present in one fraction. Succinoadenine and AMP-2'3' were identified as components of salmon liver during the course of this study.

Oxidative Metabolism of a Marine Bacterium

Studies on tricarboxylic acid cycle enzymes showed that all seven enzymes of the cycle were present in cell-free extracts of the marine bacterium under investigation. Because sodium and potassium ions are required for oxidation of various tricarboxylic acid cycle intermediates by whole cells of the organism, the effect of these enzymes on the individual enzymes in the extract was demonstrated. Aconitase was found to be almost inactive in media of low ionic strength. Isocitric dehydrogenase required not only a medium of appropriate ionic strength for activity but also a wash solution for the preparation of cell suspensions prior to sonic treatment which did not contain a halide as the only anion present. α-Ketoglutaric dehydrogenase was more active in the absence than in the presence of added sodium and potassium ions and was found to require DPN and Coenzyme A for activity. Isocitritase was demonstrated in cells grown on acetate as the sole source of carbon. All of the enzymes were present in a soluble form in the extract and were inactivated by dialysis.

Studies on the mechanism of propionate oxidation by the organism have involved allowing the cells to attack C^{14}-labelled succinic and propionic acids and isolating from the cells the intermediates formed during oxidation. When propionate 1 and 2 C^{14} were oxidized and the extracted acids separated on celite columns, it was found that both propionate 1 and 2 C^{14} formed one volatile and three non-volatile compounds which were radioactive and which did not coincide with the positions of known acids in the tricarboxylic acid cycle on chromatograms. Succinate and malate were found not to be radioactive in these cells. When succinate C^{14} was oxidized, the malate isolated was radioactive and the unknown compounds resulting when propionate was oxidized did not appear.

INDEX

INVESTIGATORS' SUMMARIES

INDEX

INVESTIGATORS' SUMMARIES (Cont'd.)

INDEX

INVESTIGATORS' SUMMARIES (Cont'd.)

SUMMARY NO. 1

PROBLEMS IN HERRING REDUCTION
I. GELATIN AS THE AGENT RESPONSIBLE FOR SEASONAL VARIATIONS IN THE VISCOSITY OF SOLUBLES

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

As previously reported in this Station's Annual Reports for 1955-56-57-58, gelling in herring solubles prepared via the acidulation process is first noted in solubles prepared from herring caught during the late fall. This gelling effect in solubles becomes more pronounced when winter herring are used and reaches a maximum level during the early spring. Immediately following the spawning period in late March all traces of the gel effect in the herring solubles disappear until the following fall.

Since proteolytic enzymes were effective in breaking the gel (Summary No. 22 of this Station's Annual Report for 1957-58), it seemed reasonable to conclude that the gelling agent was proteinaceous in nature. Also, due to the process followed in solubles production, the protein would have to be one which was soluble in water and dilute salt solutions, and was not coagulated by heat or acid. Gelatin, a denatured protein derived from connective tissues, fitted all these qualifications. Furthermore, the gelling capacity of gelatin is well known and, indeed, this protein, because of its gelling capacity, is of considerable commercial value.

In order to check the possibility that the gelling effect was due to the presence of gelatin in the solubles, an investigation was carried out in which herring solubles were prepared from fresh herring via the acidulation process. The viscosity levels of these solubles were then compared to the gelatin levels of the corresponding solubles.

The initial work concerning the methods of solubles preparation via the acidulation process and some of the chemical analysis used in this study have been reported previously in Summary No. 27 of this Station's Annual Report for 1956-57. The extraction and determination of the gelatin in solubles was carried out as follows: an accurately weighed 2-gm sample of solubles was placed in a 50-ml steel centrifuge tube along with 20 ml distilled water. The steel cylinder plus contents were then autoclaved for 3 hours at 15-lb pressure. The extraction suspension was then centrifuged and the supernatant decanted into a labelled beaker. The extraction procedure was repeated a second time, the second extraction supernatant being pooled with the first. Following the addition of trichloracetic acid to a level of 5% (w/v) to the extract, the extract was filtered and the precipitate of non-gelatinous protein discarded. The supernatant was next evaporated to dryness by directing a stream of air into the beaker placed in a boiling water bath. The dried solids were redissolved in distilled water and diluted to a known volume. Hydrolysis was accomplished by placing a 2-ml aliquot of the sample in a combustion tube along with 2 ml of 12 N HCl. The tube was sealed and heated at 110°C for 12 hours. The hydrolysate was filtered, dried as previously outlined, redissolved in distilled water and diluted to a known volume.

Gelatin determinations were based on determining the level of hydroxyproline present in each hydrolyzed sample. These hydroxyproline values were than converted to gelatin on the basis of the percent hydroxyproline in a sample of pure gelatin from the original raw material. Since no values on the hydroxyproline level in pure gelatin from herring were available, a sample of pure dry, ash-free gelatin was prepared, using herring scales as the raw material. The value of 8.55% for hydroxyproline obtained in this sample was in good agreement with other values reported in the literature for fish gelatins.

In Table I the chemical analysis and the viscosity values of solubles prepared from herring caught at monthly intervals via the acidulation process are shown. The values given are based on a solubles total solids level of 50%.

TABLE I - Gelatin content and viscosity of herring solubles prepared from herring caught at monthly intervals.

Month herring caught	Gelatin content %	Stormer Values* (25°C)	
		2 hr	24 hr
August	1.06	20.8	21.8
September	1.50	28.0	42.1
November	2.03	63.0	94.0
January	2.41	274.0	406.0
March	2.18	strong gel	strong gel
April	0.89	23.0	23.6
May	0.89	20.3	20.7
June	0.82	23.3	24.2

*Sec/100 revs/200 gm load

The data in Table I show that very nearly the maximum viscosity level of 274 sec and 406 sec at 2 hours and 24 hours, respectively, and the maximum gelatin level of 2.41 gm percent both occur during the month of February. Similarly, both the minimum viscosity values and gelatin levels occur during the months of April, May and June. Furthermore, as the viscosity levels increased, the gelatin levels displayed a corresponding increase. The results obtained would strongly suggest that the gelling in herring solubles is due to the presence of increased amounts of gelatin in herring caught during late fall, winter and early spring.

Once it had been established by investigation that significant changes occur in the gelatin levels of solubles prepared from herring caught at different seasons of the year, it was of interest to know whether this variation could be attributed to a seasonal change in the total gelatin present in herring, or whether the differences existed in the degree of solubility of the gelatin, or both. In order to test these possibilities, herring caught during February and June were obtained fresh and analyzed for their total gelatin and for their collagen fractions.

Collagen, the natural precursor of gelatin, exists as three different fractions, each extractable under a different set of conditions. The salt soluble collagen fraction was obtained by extracting a weighed sample of whole herring homogenate suspended in five times its weight of 0.2M NaCl. The extraction was carried out at 4°C, the herring suspension being under continual agitation during extraction. At the end of each 24-hour period the herring suspension was centrifuged, the supernatant being retained at 0°C and the tissue residue being re-extracted with fresh 0.2M NaCl as before. At the end of the fifth extraction the pooled salt extract was dialyzed for 24 hours against running cold tap water. The extract was then autoclaved for one hour at 15 lb pressure to convert the collagen to gelatin. In order to remove non-gelatinous protein, trichloroacetic acid was added to a level of 5% (w/v). The precipitate formed was removed by filtration and discarded.

The tissue residue remaining after completion of the salt extraction was then extracted with citrate buffer pH 3.5 to obtain the acid soluble collagen fraction. The procedure followed for acid extraction was exactly as outlined for the salt extraction. Again, all five acid extracts were pooled and the total

extract prepared for analysis as outlined for the salt extract except that the acid extract was not dialyzed.

The third fraction, the insoluble collagen, was obtained by autoclaving the tissue residue left after completion of the acid extraction with 20 ml of distilled water for three hours at 15-lb pressure. This extract was prepared for analysis as outlined for the acid extract.

All three extracts following trichloracetic acid precipitation and filtration were dried and redissolved in distilled water to a known volume. A suitable aliquot was taken from each and hydrolyzed for gelatin analysis as described previously.

In Table II the gelatin levels of the various collagen fractions are given in conjunction with separate total gelatin values determined on the same groups of fish used for the collagen fractionations.

TABLE II - Levels of salt, acid and insoluble collagen in February and June whole herring.

Month herring caught	Total collagen and collagen fractions	Gelatin mg%	% of total extracted gelatin
February	Independent total	927.0	
	Salt soluble collagen	210.0	23.9
	Acid " "	274.0	31.3
	Insoluble "	394.0	44.9
	Total:	878.0	
June	Independent total	495.0	
	Salt soluble collagen	151.5	31.5
	Acid " "	151.0	31.4
	Insoluble "	199.0	41.3
	Total:	501.5	

The results obtained show that the total gelatin content of February herring is approximately double the total gelatin level of June herring. Although significant increases in the gelatin levels of the three collagen fractions of February herring over the corresponding June levels are evident, the percent composition of the total gelatin of these three fractions is approximately the same for both months.

As significant differences in the total gelatin content of herring were noted between the months of February and June, four fresh whole herring caught during each of these two months were selected in an attempt to determine in which tissue or tissues of the herring these changes were taking place.

Following the weighing of each whole fish the major tissues were removed with due care by dissection and weighed separately. The weight of each tissue was then expressed as the percent of the total body weight. Following this, each tissue was analyzed separately for gelatin.

The results of this study given as the average values are listed in Table III.

TABLE III - Comparison of February and June herring with regard to gelatin content and amount of various tissues present.

Tissue	February herring*		June herring*	
	Gelatin content gm %	% of body weight	Gelatin content gm %	% of body weight
Head	1.50 ± 0.17	18.8 ± 2.1	1.10 ± 0.17	15.4 ± 1.2
Tail	3.67 ± 0.27	1.3 ± 0.2	2.18 ± 0.25	1.3 ± 0.4
Skin & bones	3.37 ± 0.29	9.9 ± 0.9	1.92 ± 0.13	5.9 ± 0.8
Bone	1.66 ± 0.28	4.2 ± 0.4	1.85 ± 0.13	3.7 ± 0.3
Flesh	1.49 ± 0.16	48.9 ± 3.5	0.75 ± 0.12	63.3 ± 1.6
Viscera	.36 ± 0.07	2.5 ± 0.6	0.19 ± 0.02	10.1 ± 1.1
Milt or roe	.06 ± 0.006	14.5 ± 4.9	-	-

* Fish caught in February and June of 1958. Results based on 4 fish in each group. Deviations recorded are average deviations of the mean.

Examination of the results given in Table III indicate that significant differences exist in the percent body weight and gelatin content of skin, flesh and viscera between the months of June and February. While the skin contributes an average of 5.90% of the total body weight during June, this same tissue makes up an average of 9.90% of the total body weight during February. Also, the gelatin level of the skin during February is approximately 1½ times that of the June skin gelatin level. On the other hand, while both the flesh and viscera make up a greater average percentage of the total weight of the June herring, i.e., 63.32% and 10.11%, respectively, than they do in the February herring, i.e., 48.9% and 2.5%, respectively, the gelatin levels of both these tissues during February are about double the values found during June for the corresponding tissues. The drop in the percent of the total body weight of viscera and flesh noted in the February herring is probably due to the fact that rapid gonad maturation is occurring at a time when the fish has stopped feeding. Although the reasons for the significant increase in the percent body weight of skin found in the February herring are unknown, it is of interest to note that similar findings have been found in migrating male sockeye salmon (see Summary No. 3 of this Annual Report).

In order to establish a quantitative picture of the amount of gelatin each tissue would contribute to the total fish for each of the two months, the tissue gelatin contents were calculated on the basis that each fish weighed exactly 100 gm. The findings of this study are given in Table IV.

The data recorded in Table IV show that considerable differences exist in the amount of gelatin contributed by certain corresponding tissues in June and February whole herring. The head and skin of the February herring contained, on the average, over 1.5 times as much gelatin as the corresponding tissues in the June herring. Furthermore, although the difference was not so marked as in the case of the head and skin, the flesh of February herring contained a significantly higher level of gelatin than did the flesh of the June herring. The gelatin levels of the bone and tail, however, did not differ significantly between June and February. On the other hand, the amount of gelatin contributed by the viscera in June was about double that amount present in the same organ in February herring.

TABLE IV - Comparison of the contribution made by various tissues to the total gelatin content of herring caught in February and June. Calculations in each case based on a 100-gm fish.

Tissue	Gelatin content (mg) February	June
Head	278 ± 23	163 ± 15
Tail	46.9 ± 2.7	31.1 ± 6.8
Skin and scales	332 ± 27.5	110 ± 14.3
Bone	65.5 ± 2.5	68 ± 9.3
Viscera	35.9 ± 6.5	78 ± 13
Flesh	176 ± 16.3	120 ± 12.8
Milt or roe	9.3 ± 0.4	-
Total	943.6	523.4

The results also show that although the head and skin together comprise less than 30% of the total body weight of either June or February herring, these two tissues together contribute well in excess of 50% of the total gelatin present in the fish in either of these two months. Furthermore, although the flesh makes up approximately 50% or more of the total fish weight during June or February, this tissue contributes less than 20% of the total gelatin in either sample. Of added interest is the very low gelatin levels noted in the milt and roe of the February samples. This last result would explain the previous finding that little, if any, difference in the viscosity of solubles is found when, in one case, the milt and roe are removed from the herring, and in the second case, the whole fish taken from the same batch as in the first case is used to produce solubles under identical conditions (see Summary No. 15 of this Station's Annual Report for 1955-56).

Since the presence of increased amounts of gelatin in the solubles from late season herring appears to be responsible for the high viscosity of solubles prepared by acid treatment, methods other than treatment of the stickwater with proteolytic enzymes might conceivably be effective in reducing the viscosity. It has been demonstrated by other investigators that the capacity of pure gelatin to produce a stable gel can be reduced by prior heat treatment of the gelatin solution. Consequently, the application of heat to stickwater before the preparation of solubles was investigated as a possible means of reducing the viscosity of the latter. Primary late-season stickwater, pH 5.0, was heated to 100°C for periods of less than 1 minute, 30, 60 and 120 minutes. The heated stickwater was cooled and then centrifuged at 12,000 rpm in a Servall SS-1 centrifuge for 20 minutes. To avoid using different amounts of heat during concentration of the heat-treated samples of stickwater, the application of heat at this step was avoided by concentrating the samples at room temperature. This was accomplished by the use of a rotary evaporator with the receiver for the distillate chilled in an acetone dry ice mixture. The results of this investigation are given in Table V.

It is evident from a comparison of the viscosities of Sample No. 1, where no heat was applied, with Sample No. 2, which had been heated for less than 1 minute at 100°C that the most pronounced reduction in viscosity occurs during the initial few seconds of heating. Further heating at the same temperature had little additional effect during the following 60 minutes. A further decrease in viscosity was noted after applying heat for another 60 minutes (Sample No. 5). Even heating at 100°C for 2 hours, however, failed to bring the viscosity of the solubles to the acceptable level of 30 seconds by the Stormer viscometer test.

TABLE V - Effect of heating primary stickwater for varying periods of time on the viscosity of the corresponding solubles.

Sample No.	Total solids (gm %)	Treatment	Stormer values 25°C
1	47.2	Control - no heat applied	62.1
2	46.5	Heated to 100°C for less than 1 min	43.3
3	47.6	" " " " " " 30 "	41.8
4	46.8	" " " " " " 60 "	41.3
5	46.7	" " " " " " 120 "	33.2
6	47.1	" " " " " " 240 "	30.8

The specific problem under investigation in this report was the nature and source of the substance remaining in stickwater after acid treatment which caused gel formation in the solubles prepared from it, particularly at certain seasons of the year. The results presented here leave little doubt that this substance is gelatin.

Means of overcoming the effect of the gelatin, although given some consideration in this report, were dealt with in greater detail in a previous report (Summary No. 22 of this Station's Annual Report for 1957-58). As previously reported, various commercially available proteolytic enzymes, especially Rhozyme B-6 and Bromelin, were shown to be able under controlled conditions to split effectively the gelatin and to break the gel in herring solubles.

SUMMARY NO. 2

PROBLEMS IN HERRING REDUCTION
II STUDIES ON THE PREPARATION OF LIQUID FISH

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

Approximately 200,000 tons of herring were landed during the past calendar year and almost all of the catch was converted to animal feed. The wet reduction process is the one currently used in British Columbia to bring about this conversion. In this process the herring is cooked and then pressed to remove most of the oil and much of the moisture. The press juice after separation of the oil is either reincorporated into the press cake in whole or in part, treated and concentrated to a product called solubles or is discarded. The press cake, with or without the addition of the press juice or stickwater, is finally dried to the product called fish meal. If much stickwater or solubles is added back to the press cake, the resulting product is often referred to as "whole" meal.

Herring meal is of high nutritive value and is widely used as a protein and growth factor source in animal feeds. There are certain advantages, however, to having a supplement to animal rations in a liquid rather than in a solid form. In a liquid form it can be more readily transported in bulk in tank cars than is the case with the dry product. Secondly, there are certain advantages to having such a substance as a binder in otherwise dry rations. Thirdly, it should be possible, theoretically, to prepare a liquid product more cheaply than a dry one since less steam would be required for its preparation.

Three methods have been proposed in the past to liquefy fish. In the ensilage process, first introduced in Finland in 1920, minced fish is acidified to pH 2.5 to 4.0 and allowed to stand for a period of approximately two weeks during which time

liquefaction occurs. This process, although referred to loosely as ensilage, is actually, as will be shown in this report, a liquefaction of fish resulting from natural enzymes present in the fish. True ensilage differs from this in that the breakdown is accomplished by the action of lactic acid bacteria in a process strictly comparable to the fermentation which takes place in a silo. The true ensilage procedure as applied to fish has been studied to a limited extent in France.

In another process patented in 1952, fish and fish waste is liquefied by the application of steam under high pressure. The use of proteolytic enzymes to digest the fish has also been suggested but no report of a thorough investigation using these enzymes has been made.

Since most of the liquefaction procedures have been applied to relatively non-fatty fish, and since no systematic study has yet been made of their application to British Columbia herring, our previous studies of problems associated with herring reduction have been extended to include an investigation of the preparation of liquid fish products from B.C. herring. This again is a joint project involving personnel of the biochemistry, chemistry and chemical engineering sections of the Station.

To approach the problem systematically it was decided to first prepare products from B.C. herring by the established procedures, to evaluate these products and then to determine if necessary how such procedures could be modified or new procedures devised which would give the best liquid fish product from B.C. herring from the standpoint of cost of production, nutritive value and physical properties.

Because a small autoclave permitting the application of high pressure steam on a laboratory scale was not immediately available, studies were begun of the "ensilage" and commercial proteolytic enzyme digestion procedures.

"Ensilage" herring

This has been a popular product in Europe, especially the Scandinavian countries, for many years. Published reports indicate that the process is cheap and the product is of good quality.

In order to ensure uniformity for all studies, a large sample of fresh, whole November herring was kindly supplied and stored at -20°C by British Columbia Packers Ltd. Table I lists the procedures followed and the rate of solids breakdown as measured by Stormer viscosity tests on the products during ensilage. In each study the herring, whether whole or eviscerated, were ground in a meat grinder to a crude pulp. In order to facilitate pH adjustment 1/5 volume of distilled water was added and thoroughly mixed into the homogenate. The pH in each instance was adjusted at 24-hour intervals to the zero time level until the termination of the experiment. Two procedures have been recommended for the liquefaction of herring by this procedure. In one, the pH is adjusted to 2.0 with a strong acid such as HCl or H_2SO_4. In the other, the pH is adjusted to and maintained at 4.5 with formic acid. Both procedures were investigated here.

At the completion of each experiment the product was filtered through two layers of cheese-cloth and the solids removed by filtration were then dried and weighed. After the filtrate had settled overnight in a separatory funnel, the top layer of oil was removed and discarded. The sample was then concentrated in a vacuum flash evaporator, the distillate being trapped in a flask placed in a dry-ice acetone bath. In order to minimize the effect of heat on the final product the temperature of the water bath was kept within the range of 25°-30°C.

In the first experiment, two products, Samples 1A and 1B, were prepared by the established procedures, that is, whole ground herring were held at 25°C at

pH 2.0 and pH 4.5. It is evident from an examination of the rate of digestion of these two samples listed in Table I that the solids breakdown initially, at least, is greater when the sample is held at pH 2.0 than at pH 4.5. At the end of the 20th day, however, the viscosity levels of the two samples are about equal. In order to test the effect of a higher temperature on the rate of solids breakdown, a second experiment was carried out using whole fish at the same pH levels used in the first experiment at a temperature of 37°C. In this study, Sample 2B displayed a greater initial rate of protein breakdown at pH 2.0 than did sample 2A at pH 4.5. Both samples, however, showed considerable solids destruction at the end of the first 24 hours of incubation. Again, by the 20th day of digestion, little if any differences existed in the viscosity of the two samples.

TABLE I - Effect of temperature and pH on the rate of solids breakdown in the production of whole and eviscerated herring ensilage.

Sample No.	Ensilage Treatment	Stormer values during ensilage[1] Time (days)								
		0	1	2	3	4	5	10	15	20
1A	Whole herring, pH 4.5, 25°C	*	*	*	29.6	17.8	17.1	15.5	14.8	14.1
1B	Whole herring, pH 2.0, 25°C	*	32.8	16.5	15.5	15.4	14.2	14.0	13.1	13.1
2A	Whole herring, pH 4.5, 37°C	*	21.2	16.8	15.1	14.8	14.2	13.4	13.3	13.3
2B	Whole herring, pH 2.0, 37°C	*	15.1	13.1	13.1	13.0	13.1	13.1	13.1	13.1
3A	Whole herring, pH 4.5, room temperature (21°C)	*	99.0	28.6	24.5	21.1	20.0	17.0	16.5	15.1
3B	Whole herring heated to 95°C for 15 min before incubation conditions same as 3A	*	*	*	*	*	*	*	*	*
4A	Whole herring, pH 4.5, 25°C	*	*	*	76.1	32.1	21.1	15.5	15.0	14.0
4B	Eviscerated herring, pH 4.5, 25°C	*	*	*	*	46.4	27.7	17.2	16.1	15.5

[1] Sec/100 revolutions at 25°C.
* In excess of 150 seconds.

It is evident from an examination of the results of Sample 3A and 3B listed in Table I that the ensilage of herring is due to the natural enzymes of the herring. When as in Sample 3B the herring homogenate is first heated to 95°C for 15 minutes to destroy any naturally occurring enzymes, there is little or no change in the viscosity of the resulting product on incubation. On the other hand in Sample 3A, where no heat was applied, there is a continual breakdown of the protein during the course of the incubation.

In order to ascertain whether the protein digestion was due solely to visceral enzymes or whether flesh enzymes also took part in the solids breakdown, an experiment was conducted in which the rate of breakdown of whole herring was compared with that of herring of the same batch from which the viscera had been removed. The results, experiment 4, Table I, show that although the whole herring liquefied faster than the eviscerated ones, there was surprisingly little difference between the two samples. Much proteolytic enzyme activity must thus reside in the flesh. Although the liquefaction of fish was proceeding at such a low pH that it was unlikely that bacterial action could have contributed significantly to the process involved, in view of the name attached to the process and of the possibility of confusing the digestion with a true ensilage fermentation, bacterial counts were made on the digestion mixture at the beginning of a digestion at pH 2, during the course of the digestion and at the end. The system was discovered to be essentially free of bacteria

on each test. It has thus been established that under the conditions used bacteria play no part in the liquefaction process.

The products obtained following digestion contain undigested solids and have a relatively high water content (70-80%). Both are undesirable from a commercial standpoint. In an attempt to overcome these disadvantages the products following complete digestion were filtered and then concentrated to a level of 45-50% total solids.

The proximate analysis and viscosity values of the concentrates along with the dry weight of the solids removed by filtration are shown in Table II. The Stormer determinations were carried out following a 30-minute standing period at 25°C, while the Pour Residue Test, expressed as % retained (Summary No. 14 of this Station's Annual Report for 1955-56), was determined after a setting-up period of 24 hours at 10°C.

TABLE II - Proximate analysis and viscosities of herring ensilage concentrates.

Sample No.	Filtered solids gm*	Analysis of concentrate				
		Total solids	(1) Stormer	% Retained	% Protein	% Oil
1A	19.82	45.1	16.5	100.00	27.5	10.20
1B	15.31	45.2	16.5	2.8	29.4	4.74
2A	25.61	48.0	17.7	5.3	33.4	3.68
2B	20.88	50.6	19.2	11.5	31.0	5.84
3A	21.16	44.4	17.3	100.00	26.2	9.05
3B	**	**	**	**	**	**
4A	20.05	44.6	48.8	98.3	30.0	6.55
4B	54.14	43.9	35.1	91.7	25.0	15.80

(1) Sec/100 revolutions at 25°C.
* Dry wt.
** Sample not concentrated.

The amount of solids removed by filtration of the ensilage digest, except for Sample 4B, were all within the range of 15-25 gm. In the case of Sample 4B, where eviscerated herring was used, considerably more undigested solids were removed by filtering.

Although all the concentrates listed in Table II except samples 4A and 4B indicated satisfactory Stormer values of under 30 seconds, only samples 1B, 2A and 2B gave good pour residue tests. The high % retentions found in the samples 1A, 3A, 4A and 4B can be explained in part by the high levels of oil in these samples. Herring oil, present as a protein-oil emulsion in these samples, solidifies at 10°C, the temperature used for pour residue determinations.

Although the initial results obtained to date would suggest that this procedure has many possible merits it has, however, one obvious demerit from a commercial viewpoint. The length of time required to obtain sufficient digestion would make it necessary to have a considerable number of large capacity digestion tanks for an operation of any considerable size.

Liquefaction of Fish by Commercial Proteolytic Enzymes

The use of the naturally occurring proteolytic enzymes of the herring to bring about its liquefaction has been shown to give rise to a product which has some very desirable characteristics. Unfortunately, however, the process is slow. The most obvious way of increasing the rate of digestion is to add more enzymes. This has been considered and tested in the past by others but no report of any detailed study has been made.

The initial phases of this study were focussed on determining the capacity of several commercially available proteolytic enzymes to reduce whole herring with maximum solids breakdown to a suitable liquid product.

In an attempt to maintain constant investigation conditions throughout all the different enzyme studies, the following factors were kept constant: 500 gm of a whole herring homogenate were used as the substrate in each case. To this was added 225 ml of distilled water to facilitate pH adjustment. The enzymic level selected was 0.5% of the wet weight of the undiluted herring homogenate, and the digestion time was held to 180 minutes. In all instances the substrate was agitated continuously by stirring and the pH maintained at the desired optimum level during digestion. The pH level and temperature of digestion selected for each enzyme were the optimum levels indicated by the manufacturer of the enzyme under investigation.

Since it is generally accepted that proteolytic enzymes other than collagenases do not attack collagen but do attack gelatin, and since collagen is converted to gelatin by heat, it was of interest to determine the relative effect of each enzyme on heated and unheated herring. Thus in one case the substrate was preheated to 95°C in a boiling water bath for 10 minutes while in the other the substrate was not heated prior to enzyme digestion. The course of enzyme digestion was followed by noting the amount of acid or alkali required to maintain the pH constant. Following the completion of enzyme digestion each substrate was heated in a boiling water bath for 15 minutes to insure enzyme deactivation. Upon cooling the substrate was filtered through two layers of cheese-cloth. The solids removed by filtration were dried and weighed. The filtrate was then allowed to settle overnight and the top oil layer removed. Again, in order to minimize the effect of heat on the final product, the filtrate was concentrated in a flash evaporator under vacuum. The distillate was collected in a flask placed in an acetone dry-ice bath while the temperature of the water bath was kept within the range of 25°-30°C.

The course of digestion as indicated by the amount of acid required to maintain the pH constant during digestion of both heated and unheated herring is shown in Fig 1, using pepsin as the test enzyme. It is evident that for this enzyme proteolysis was both more rapid and more complete when pre-heated herring was used as the substrate. With the pre-cooked substrate, digestion was essentially complete in 90 minutes, while with the unheated fish, levelling off of digestion occurred only after 170 to 180 minutes of incubation. The other enzymes tested, Rhozyme B-6 and protease, gave similar pH titration curves and digestion appeared to be complete in similar lengths of time. When the products from the different enzymes were examined further and concentrated, however, differences in the effects of the enzymes were apparent. Before concentrating, each of the digests was filtered through cheese-cloth to remove undigested material. This was dried and weighed. The amounts of material recovered from each digest are recorded in column 4 of Table III. When the digests from the unheated fish were filtered the Rhozyme B-6 digest showed the greatest, and pepsin the least, amount of undigested material. Pre-cooking increased the amount of digestion in all cases except protease.

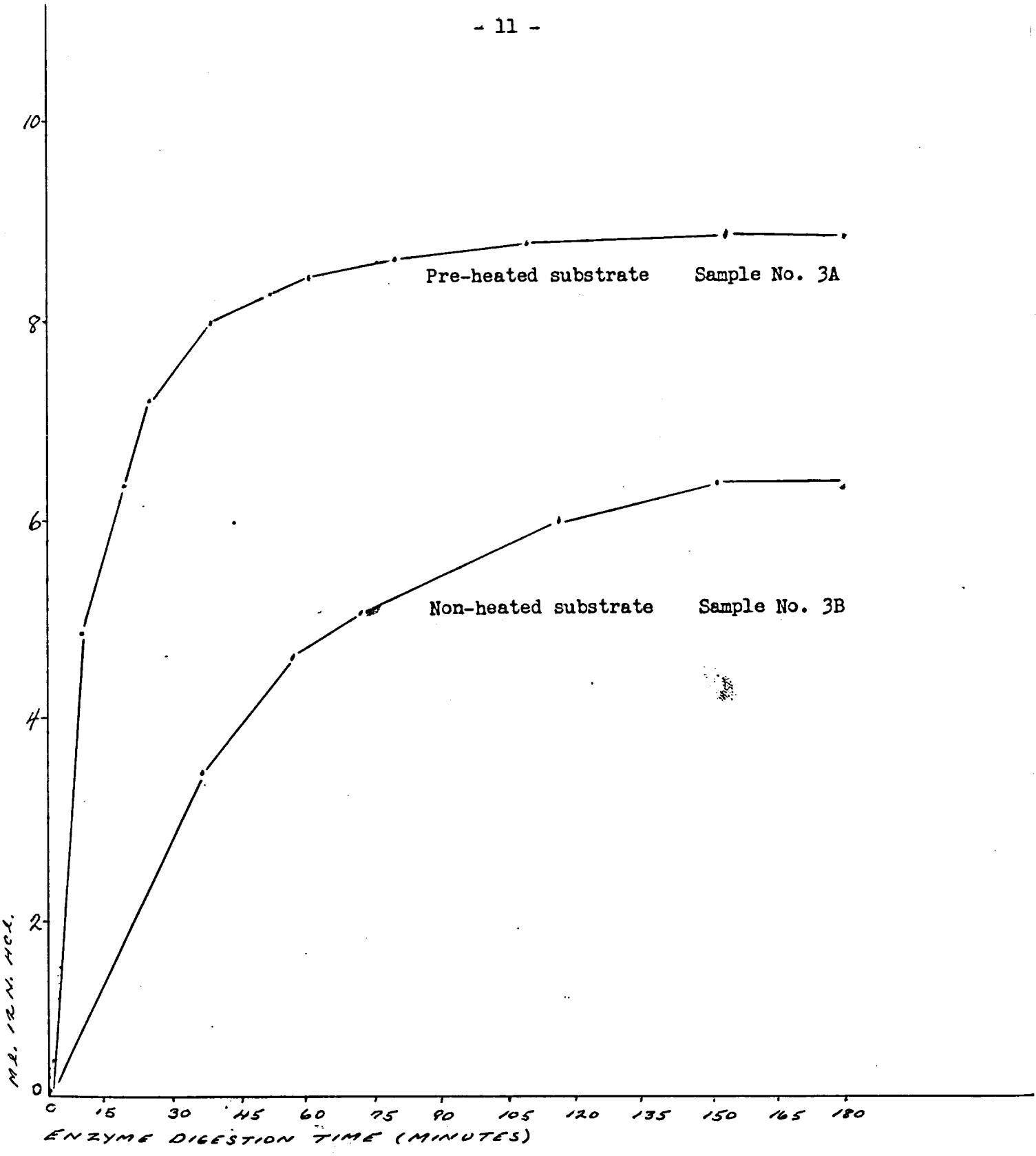


Fig. 1 Rate of pepsin proteolysis in pre-cooked and non-cooked herring homogenate.

TABLE III - The effect of several commercial proteolytic enzymes in the reduction of whole herring to liquid fish.

Sample No	Enzyme(1)	Substrate treatment	Filtered solids (gm)†	Analysis of concentrate (% wet wt) Total solids	Stormer *	% Retained	% Protein	% Oil
1A	Protease	Pre heated	16.70	31.2	21.7	22.1	15.10	11.6
1B	"	Not "	14.94	30.6	**	100.0	14.9	11.5
2A	Rhozyme B-6	Pre heated	77.88	29.6	16.9	1.49	13.9	10.1
2B	"	Not "	49.94	30.8	17.7	13.2	17.8	9.0
3A	Pepsin	Pre heated	10.12	33.2	15.3	2.87	14.6	12.3
3B	"	Not "	20.91	33.4	13.7	2.19	14.1	13.6

(1) Enzyme digestion at the following pH and temperature: Protease pH 8.0, 50°C; Rhozyme B-6 pH 6.0, 60°C; and Pepsin pH 2.0, 37°C.

* Sec/100 revolutions.

** In excess of 150 sec.

† Dry wt.

Although the ultimate total solids level of the final product from a commercial standpoint should be at least 50%, the products in this study were concentrated only to 30-33% to make direct comparisons between concentrates possible since some of the less well-digested products could not be concentrated beyond this solids level. As the results in Table III show, the only product to possess a poor viscosity from the standpoint of the Stormer test in this investigation was the one using the enzyme protease on an unheated sample of herring. Not only was the Stormer value of this product, Sample 1B, in excess of 150 seconds, but also it gave a 100% retention in the pour residue test at 10°C. Viscosities of the remaining products measured by the Stormer viscometer test were all below the maximum commercially acceptable viscosity levels for solubles. The pour residue test paralleled the Stormer results except in the case of Rhozyme B-6 acting on unheated herring. The retention in this case reveals a greater difference in the physical characteristics of the product from heated and unheated herring than was evident from the Stormer results.

Although the data obtained to date is only of an exploratory nature and incomplete in that other enzymes remain to be tested under identical conditions, the results do indicate some general trends. First, complete oil separation is not being achieved with the procedures followed. All the concentrates have high oil levels. While the oil levels at these ranges of 30-33% solids of concentration do not appear to affect the viscosities adversely, it is known from the ensilage studies that at higher total solids levels of 45-50% these levels of oil would tend to solidify the product at low temperatures. Also, the desirability of having products with such high oil levels from a nutritional standpoint is questionable.

Of the three enzymes tested, pepsin, when used in a preheated substrate, appears to give the best performance so far as completeness of solids breakdown and viscosity of the concentrated product is concerned. The advantage to be gained from precooking the substrate would appear to depend on the enzyme used. In general, however, a precooked substrate was digested faster and more completely than one that was not preheated.

This program will be continued with special emphasis being placed on devising ways of reducing the oil content of the product. Products obtained will also be compared with those produced with the aid of steam under pressure. All products will finally be evaluated as supplements in animal feeds.

SUMMARY NO. 3

BIOCHEMICAL STUDIES OF SOCKEYE SALMON DURING SPAWNING MIGRATION

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

The Quantitative Distribution of Gelatin in Several Major Tissues of the Standard Fish

Collagen, the native precursor of gelatin in living matter, is usually considered to be the main protein component in connective or supporting tissue such as skin, tendon, bone and scales. Our interest in collagen changes in sockeye during spawning migration arose from the observation that in herring a considerable deposit of collagen occurred during the final stages of maturation before spawning (Summary No. 1 of this Annual Report). It was of interest to know whether a similar phenomenon could be observed in salmon.

Although collagen itself is relatively insoluble it is readily extractable from tissues if converted to gelatin. This is accomplished by heating. The chemical composition of collagen and of the gelatin derived from it are identical, only the physical properties differ.

As gelatin concentrations are calculated from the amount of hydroxyproline present in each tissue extract, it was first necessary to establish the hydroxyproline level of a pure sample of dried ash-free gelatin from sockeye. Such a sample was prepared from sockeye scales and it was found to contain hydroxyproline at a level of 8.66 percent. This value is in good agreement with those for hydroxyproline found in gelatins from other sources. The gelatin content of each sample was calculated using the experimentally determined value for the hydroxyproline content of sockeye gelatin.

The tissue samples used for these analyses were the same as those collected in connection with other studies on biochemical changes in migrating salmon. Details of the collection and sampling procedures used have all been described (Summary No. 1 of this Station's Annual Report for 1957-58).

The gelatin levels found in the various tissues of the sockeye salmon analyzed are recorded in Table I. These results give no indication of the overall changes in gelatin levels in the fish during migration, unless changes in the weights of the tissues of the fish are also considered. This consideration gives rise to the data in Table II. From the changes in weight of the tissues previously recorded (Summary No. 1 of this Station's Annual Report for 1957-58), and the data in Table I, the contribution which each tissue or group of tissues made to the total gelatin content of the fish at each point in the migration, has been calculated. Since these tissues together do not make up the total fish, it was not possible to determine what overall changes occurred in the gelatin content of the salmon at the various points in the migration. Certain of the changes in the tissues, however, can be compared with changes in the corresponding tissues of herring (Summary No. 1 of this Annual Report). In the case of flesh, gelatin increases occurred only in male sockeye salmon. In herring gelatin increases in flesh were recorded but no attempt had been made to differentiate males from females. Head, skin, bones and tail were grouped in the salmon analyses and considered separately in the case of herring. The sum of the values in herring showed that this group of tissues not only contributed the most gelatin to the fish but also showed the greatest increase on maturation. For the sockeye, increases were again recorded only in the male. Since several organs had been removed from the viscera prior to analysis in the case of salmon, the values recorded for the alimentary tract are not comparable to those obtained on herring viscera.

TABLE I - Gelatin levels in various tissues of sockeye salmon at points on their spawning migration path up the Fraser River.

Location	Sex	Flesh	Alimentary tract	Head, skin etc.
			gm/100 gm tissue	
Lummi Is	Male	.320 ± .023	1.200 ± .028	2.980 ± .157
	Female	.358 ± .001	1.161 ± .029	2.990 ± .062
Lillooet	Male	.364 ± .017	2.179 ± .088	2.989 ± .176
	Female	.375 ± .008	2.182 ± .072	2.920 ± .048
Forfar Cr	Male	.404 ± .012	2.639 ± .088	2.980 ± .138
	Female	.424 ± .012	2.661 ± .101	2.841 ± .055

TABLE II - Changes in the total gelatin contributed by each tissue to the standard fish at various points on the spawning migration path.

Location	Sex	Gelatin (gm) Flesh	Alimentary tract	Head, skin, bones, tail
Lummi Is	Male	4.679	1.085	26.220
	Female	4.788	0.909	23.420
Lillooet	Male	4.747	0.654	26.098
	Female	4.261	0.572	22.290
Forfar Cr	Male	5.261	0.432	31.448
	Female	3.945	0.342	23.672

Until data has been obtained on the extent of the collagen changes in herring with sex, it will not be possible to determine how closely the species compare as regards collegen changes with maturation.

SUMMARY NO. 4

BIOCHEMICAL CHANGES IN RIPENING SALMON
I BLOOD AND TISSUE CHANGES

R.E.E. Jonas
E. Onofrey
R.A. MacLeod

During the summer and fall of 1957 a group of adult coho salmon which had been captured at a point in fresh water on the route of their migration to the spawning grounds were held in tanks at the Vancouver Aquarium. It was the purpose of the study to remove fish at intervals to determine by analytical and enzymatic methods the extent of the changes taking place during maturation.

Since fresh tissues were required for enzymatic studies, the enzyme studies were conducted at the time of sacrificing the fish and were reported last year (Summary No. 9 of this Station's Annual Report for 1957-58). Due to limitations in numbers of personnel, samples of tissues were frozen for chemical analysis when time permitted. These

analyses were completed during the past year, and are reported at this time.

Blood Analyses

From the coho salmon which were sacrificed for enzyme studies, blood was collected and a portion used for the preparation of heparinized plasma and another portion for serum. The plasma and serum were frozen immediately after preparation and analyzed subsequently. Plasma was analyzed for glucose, non-protein nitrogen, amino acids and total protein. The serum was analyzed for Na^+ and K^+.

Glucose was measured by a highly specific method employing the enzyme glucose oxidase. Total protein and non-protein nitrogen were determined by standard methods. Amino acids were estimated as a group by measuring the colour produced between amino acids and β naphthoquinone-4-sulfonic acid in alkaline solution. Na^+ and K^+ was determined on aliquots of serum by methods described previously (Summary No. 24 of this Station's Annual Report for 1956-57).

Since the fish were not feeding but drawing upon their body storage products and tissues for energy and gonad development, it was felt that changes in the tissue components being utilized at different stages during maturation might be reflected in the levels of various components in the blood. Also, as only a limited number of fish could be held in the tanks available, it was necessary to determine whether the changes in levels of blood components taking place would be sufficiently greater than the natural variation between samples to permit any indication of progressive changes in the levels to be detected.

The results of the blood analyses are presented in Table I. The N P N values except for four unusually high ones (Fish #1, 2, 13 and 14) remained well within the range observed in the other species recorded and there appeared to be no tendency for the values to change with maturation. No explanation can be offered for the four unusually high values recorded since none of the other components of these particular blood samples appeared to be abnormal in any other way.

The amino acid results show that blood levels of this group of compounds remained within the range recorded for fish blood of other species for the first 60 days. After this the level dropped to approximately half of the average of the preceding values.

Although blood glucose concentrations were extremely variable from fish to fish, it is evident that fish at the end of the experiment had levels as high as the average of those at the beginning.

Total protein remained almost constant except for the last fish in which blood protein dropped to about one-quarter of the value in the other fish on experiment. This happened in the case of the single fish in the experiment which appeared to be completely matured and which would likely have spawned within hours of the time of sacrifice had it been kept alive to do so.

Serum Na^+ remained very constant throughout the experiment while K^+ was so variable that no conclusions regarding the possibility of progressive changes in its concentration with maturation could be drawn.

It can be concluded from this study that except for the amino acids, the blood components investigated were maintained at remarkably well-elevated levels even in the final stages of maturation. It is also evident that again excepting the amino acid results, no inferences can be drawn from the data recorded regarding the nature of the tissue component or components being utilized for energy and gonad development during maturation of the fish.

TABLE I - Levels of various components of coho salmon plasma or serum at different times during maturation of the fish in an aquarium tank.

Fish No	Date killed 1957	Days on experiment	Sex	Wt in gm	Gonads %	Plasma N P N mg %	Plasma Amino Acid N mg %	Plasma Glucose mg %	Plasma Total protein %	Serum Na^+ mg/100 ml	Serum K^+ mg/100 ml
1	July 25	0	F	2370	5.1	70.5*	17.1	208.9	5.43	313	3.98
2	" 30	5	F	3324	3.1	-	-	-	-	-	-
3	Aug 2	8	M	3570	3.3	97.5*	22.6	96	5.47	354	4.92
4	" 6	12	F	2599	4.8	39.6	18.4	193	5.10	352	5.10
5	" 8	14	M	2654	3.8	36.1	19.9	103	5.36	363	5.93
6	" 13	19	F	1681	6.3	41.2	19.6	22	6.00	368	7.76
7	" 15	21	M	1679	5.4	40.0	17.4	82	4.86	366	8.78
8	" 20	26	M	3943	2.6	34.2	16.1	167	5.30	373	2.97
9	" 22	28	M	1295	7.8	39.8	16.2	73	6.00	377	16.10
10	" 27	33	M	1195	6.0	39.0	18.5	53	4.20	360	7.77
11	" 29	35	M	1945	6.2	44.9	18.8	90	6.06	319	35.00
12	Sep 4	40	M	4000	5.3	37.5	15.8	76	5.58	387	5.39
13	" 6	42	F	2055	8.9	62.2*	22.2	75	6.44	352	9.50
14	" 10	46	M	2588	3.5	91.8*	24.7	100	5.05	163*	10.42
15	" 17	53	M	712	8.1	-	-	-	-	-	-
16	" 19	55	F	2795	17.7	34.2	16.4	123	5.51	337	4.36
17	" 24	60	F	2843	16.6	35.3	17.7	91	4.55	370	15.10
18	Oct 2	68	M	2145	3.8	35.4	8.1	140	4.50	359	10.30
19	" 4	70	M	580	6.9	-	8.1	128	-	-	-
20	" 8	74	F	1505	11.6	38.4	9.1	105	5.05	351	18.30
21	" 25	91	F	2480	22.1	24.2	8.1	99	1.16	367	6.16

* Expressed as percent of body wt.

	Plasma N P N mg %	Plasma Amino Acid N mg %	Plasma Glucose mg %	Plasma Total Protein %	Serum Na^+ mg/100 ml	Serum K^+ mg/100 ml
In normal human blood:	25-35	5-8	70-100	5-8	300-330	16-22
rat blood:	34.5-40.2	12.6 -16.2	-	-	-	-
carp blood:	26.5-36.6	15.2 -20.61	57.3-230	3.25-4.75	292.316	17.5-26.9
trout blood:	25.4-35.4	17.95-20.95	51.4-111	2.94-4.12	348-373	16.9-25.8

Tissue Analyses

Since the fish were without food during the maturation period it was of interest to know which of several tissues were drawn upon to the greatest extent to supply the requirements for energy and gonad development. Since similar studies were being conducted on sockeye salmon migrating up the Fraser, it would also be possible to compare the results obtained on fish maturing in a relatively sedentary state with those of fish maturing while migrating.

The results of analyses of kidney, liver, skeletal and lateral line muscle, milt and roe of each fish on experiment are presented in Table II.

TABLE II - Proximate analysis of various tissues from coho salmon maturing in an aquarium tank.

Fish No.	Days on expt.	Sex	Kidney			Liver			Skeletal Muscle					Lateral Line Muscle			Milt or Roe		
			Fat	Protein	H_2O	Fat	Protein	H_2O	Fat	Protein	H_2O	Na^+	K^+	Fat	Protein	H_2O	Fat	Nitrogen	H_2O
			Percent			Percent			Percent			meq/kg*		Percent			Percent		
1	0	F	2.15	14.0	79.8	3.68	17.0	74.5	2.03	20.8	77.2	9.35	121	25.2	16.7	58.2	13.0	4.23	57.3
2	5	F	2.77	14.0	79.5	4.00	17.3	73.7	3.01	21.7	74.7	7.22	123	13.7	18.2	65.3	14.3	4.14	56.3
3	8	M	2.32	14.2	79.4	3.22	15.7	75.3	1.51	22.6	73.9	9.61	119	11.5	15.5	68.4	1.65	3.21	78.5
4	12	F	3.05	14.4	79.5	2.84	17.2	74.5	2.31	22.3	73.3	9.95	129	17.3	16.7	64.1	14.0	4.33	59.4
5	14	M	2.73	15.7	78.3	3.00	16.2	75.7	1.69	22.4	74.0	10.0	127	15.9	16.8	64.4	1.71	3.59	75.2
6	19	F	2.54	14.9	78.1	4.70	17.7	76.2	1.35	23.6	73.3	9.56	130	24.4	15.3	57.6	13.6	4.34	55.9
7	21	M	3.27	14.7	78.7	3.64	16.9	74.7	2.42	22.6	72.8	9.95	129	20.9	15.4	60.7	.99	3.96	74.6
8	26	M	3.37	15.4	77.1	3.61	16.5	75.2	2.30	23.7	71.2	10.9	126	30.0	13.2	52.2	1.87	2.76	80.2
9	28	M	2.26	14.3	78.9	2.91	15.7	76.3	3.11	21.1	73.3	29.8	111	-	-	-	1.73	3.50	76.8
10	33	M	2.51	13.7	79.9	2.49	15.5	76.2	3.14	21.6	73.3	10.2	123	14.8	12.8	66.5	2.29	4.14	73.9
11	35	M	2.34	16.2	77.4	3.03	17.5	71.5	2.79	22.3	71.4	9.78	126	24.5	14.3	58.0	2.35	4.61	72.1
12	40	M	2.08	16.8	78.6	2.70	17.5	73.6	1.94	23.3	72.4	10.4	121	16.3	15.0	65.0	1.99	3.62	75.8
13	42	F	2.98	16.5	77.3	3.07	17.7	73.9	2.24	22.2	73.2	9.83	132	16.2	15.5	64.7	9.29	4.62	55.0
14	46	M	2.66	14.2	78.4	2.96	16.9	71.7	2.09	21.8	74.1	9.92	126	17.4	15.6	64.4	2.24	5.48	67.7
15	53	M	-	-	-	-	15.4	71.2	2.76	22.1	73.5	14.1	130	27.3	14.0	56.6	2.17	3.65	75.2
16	55	F	3.22	12.4	80.1	5.55	18.5	74.2	2.12	19.5	75.0	10.8	122	10.7	15.1	69.4	13.0	4.54	55.3
17	60	F	3.20	15.2	78.0	3.93	16.4	76.1	2.55	19.5	76.0	11.3	129	19.1	13.7	62.0	11.6	4.74	55.3
18	68	M	2.00	15.2	77.7	4.56	16.1	74.0	1.92	20.2	75.7	11.9	126	11.5	16.6	67.8	2.83	5.50	67.9
20	74	F	1.59	15.0	77.5	3.66	17.4	76.4	1.89	20.8	75.7	11.0	134	18.6	15.7	61.8	12.14	4.70	54.3
21	91	F	2.41	12.4	81.4	4.67	14.9	77.6	1.42	15.9	80.8	14.6	123	4.4	14.2	77.4	9.97	4.16	59.6
22	95	M	2.26	14.3	78.3	3.93	13.7	74.4	1.92	20.0	76.4	11.3	122	13.4	16.0	67.1	2.93	5.49	68.1
23	111	F	2.25	12.9	80.2	3.04	13.7	80.9	3.17	16.6	78.5	8.3	112	15.4	13.3	67.8	11.4	4.65	55.7

* Milliequivalents/kg.

In the kidney of both male and female fish, fat, protein and water remained almost constant over the 111-day period. In the liver the fat level did not change in either male or female fish but the total protein showed a drop toward the end of the experiment. Skeletal muscle showed no consistent drop in fat content but definite evidence of protein depletion with maturation in the female. For the male, neither fat not protein showed what could be construed to be a significant change over the period.

Na^+ and K^+ analyses were also carried out on skeletal muscle tissue since extensive studies of the changes taking place in these components in migrating sockeye and spring salmon have been conducted at this Station (Summary No. 8 of this Station's Annual Report for 1957-58 and Summary No. 24 for 1956-57). The Na^+ levels are lower than any that have been recorded for mature spring or sockeye salmon in fresh water and are almost identical to the levels found in juvenile coho migrating to sea. Similarly, K^+ values are lower than were found for spring and sockeye and are in the same range as previously found for juveniles of the species. No evidence of a sharp rise in Na^+ and fall in K^+ was observed toward the end of the maturation period, as had been found to take place in migrating sockeye and spring salmon.

The lateral line muscle can be seen to be very rich in fat. The range in values of fat concentration found was so great throughout the experiment, however, that no conclusions regarding fat in this tissue as an energy source could be drawn. The trend of protein concentration in this tissue was downward with maturation in the female but almost constant in the male. An inverse relationship between the concentration of fat and water was observed.

The fat level in the roe showed some signs of decreasing slightly toward the end of the experiment while that of the milt appeared to increase. The nitrogen level in the roe changed little, if any, during the period, while in the milt its concentration increased. No effort was made to express the nitrogen as protein in these tissues since much of it would occur as purines and pyrimidines.

The results of the tissue analyses bring out several interesting points. In the first place, the fat levels of the tissues remain remarkably constant over the whole period the fish are without food. This of course does not mean that fish do not use fat as a source of energy since there are fat depots in these fish which probably together contain considerably more fat than the tissues analyzed and this could be drawn upon first. It is evident, however, that considerable energy reserves were still available to the fish after nearly 4 months without food. Also of interest is the extent of the nitrogen loss from the liver, an indication that this organ may well be an important depot for nitrogen compounds for the milt and roe. As the liver represents only 1% of the weight of the fish while the flesh represents 50-60%, quantitatively the amount of nitrogen supplied by the liver is small compared with that from the skeletal muscle tissue. Since the largest losses in nitrogen in these two tissues coincides with the large increases in the total amount of the roe and with the increase in the amount of nitrogen contained in the milt, it seems likely that both liver and skeletal muscle are sources of nitrogen for the compounds required by the developing milt and roe.

Since analyses of the corresponding tissues in migrating sockeye, except for skeletal muscle, have not been completed, it is not possible to compare the extent of the changes taking place in the two groups of maturing fish. In skeletal muscle of migrating sockeye, considerably more fat was lost than was noted in samples from the coho held in the Aquarium. The fat content of the muscle tissue of the sockeye at the mouth of the river, however, was higher than that of the coho (Summary No. 5 of this Station's Annual Report for 1957-58).

SUMMARY NO. 5

CHANGES IN VARIOUS COMPONENTS OF THE BLOOD OF SOCKEYE SALMON DURING MIGRATION

R.E.E. Jonas
R.A. MacLeod

The levels of some constituents of the blood of sockeye salmon during migration have been reported (Summary No. 1 of this Station's Annual Report for 1957-58). The components examined in this study were followed since it was felt that their levels might well give some indication of the type of metabolism taking place in the tissues of the fish at various stages during the migration. The same components were tested for in the blood of coho salmon maturing at the Aquarium (Summary No. 4 of this Annual Report), to see if any changes which might occur during maturation paralleled each other in the two groups of fish.

The results obtained on the plasma of sockeye salmon of the Stuart Lake run (1957) are recorded in the following Table. The blood collected from approximately 8 fish of the same sex at each check point was pooled to give one sample. Four samples of each sex were obtained at each check point.

Levels of various constituents in the plasma of sockeye salmon during migration. (Stuart Lake Run 1957)

Area	Date 1957	Sex	No. of fish pooled	N P N mgN/100 ml plasma	Amino acid N mg/100 ml plasma	Glucose mg/100 ml plasma	Total protein in plasma %
Lummi Is	July 3	M	23	36.7 ± 3.4†	14.72 ± .43	206.5 ± 15.5	6.21 ± .38
"	"	F	35	35.0 ± 2.9	14.9 ± 3.8	209.3 ± 19.8	7.24 ± .18
Lillooet	July 13	M	40	29.2 ± 0.4	14.1 ± 0.4	181.3 ± 11.8	5.25 ± .22
"	"	F	40	29.7 ± .52	14.5 ± .77	186.3 ± 6.9	6.48 ± .20
Forfar Mouth	July 31	M	25	39.1 ± 2.22	15.2 ± 1.05	170.5 ± 6.0	4.67 ± .35
"	"	F	55	38.9 ± 1.68	15.2 ± .45	186.5 ± 5.0	5.45 ± 0.22

† The deviation recorded is the average deviation of the mean.

The methods used to analyze for each constituent were the same as those employed in the analysis of blood from the Aquarium coho (Summary No. 4 of this Annual Report).

Because of the large number of fish obtained for analysis, the uniformity of their size and the purity of their race, variations between samples at any one collection point were very small. The N P N values (see table) were essentially the same in both sexes and showed a drop at Lillooet. Amino acid levels remained remarkably constant throughout, showing no tendency to drop in the final stages of maturation, as had occurred in the coho salmon in the Aquarium.

Glucose showed a downward trend in the male fish during the whole migration while in female fish a drop was observed in samples collected at Lillooet and the level reached at that point was still the same as Forfar Creek.

Total protein in the plasma showed a small but significant downward trend during migration in both male and female fish. At each point female plasma contained more total protein than the male.

SUMMARY NO. 6

BIOCHEMICAL CHANGES IN RIPENING SALMON
II ENZYME STUDIES

E. Onofrey
R.E.E. Jonas
R.A. MacLeod

When the rate of oxygen uptake by slices of the liver, kidney and brain of coho salmon held in the Aquarium were determined, it was found that the Q_{O_2} values varied depending on the amount of tissue present in the Warburg flask (Summary No. 9 of this Station's Annual Report for 1957-58). As the weight of tissue used was increased, the Q_{O_2} value increased. Since this variation was not evident in the first one or two fish examined and appeared to increase the longer the fish were on experiment, the results were tentatively interpreted as indicating the possibility that a co-factor required for tissue respiration was becoming depleted over the lengthy period the fish were without food.

Since only one or two fish at the beginning of the experiment did not show the Q_{O_2} variation with amount of tissue, it was considered desirable to get more data on Q_{O_2} changes with tissue level in normal well-fed fish. No feeding coho salmon were available, but a supply of steelhead trout (Salmo gairdnerii gairdnerii) kindly made available to us by the Fish and Game Department were obtained. These two-year old growing fish were maintained on a pellet diet used at the Vancouver Aquarium. Q_{O_2} values were determined on liver slices at different tissue levels. The results obtained on four different fish are recorded in Table I. Only one of the fish, Fish #1, showed a relatively constant Q_{O_2} over the range of weights of tissue tested. The other three fish exhibited a progressive increase in Q_{O_2} as the weight of tissue used increased in a manner analogous to the changes previously observed in the coho salmon. It would thus appear that the phenomenon observed is more the rule than the exception in fish tissue, and makes it unlikely that the effect was due in coho to a depletion of a co-factor required for respiration. No mention has been made of such a phenomenon in tissue slice studies with animal tissues. Since the conditions used were those which had been found to be optimum for studies with animal tissues, it is not unlikely that the results with fish reflect the fact that the conditions used (such as co-factor concentrations, etc.) were not really optimum for fish tissues. It was thus evident that a great deal more basic information was required about the metabolism of fish tissues and optimum conditions for measuring it. For this reason a detailed investigation was begun of glycolytic and oxidative pathways in tissues of fish. Although we are ultimately concerned with the application of this information to an understanding of biochemical processes in migrating salmon, the latter are not available for study on a year-round basis. It was decided to work out the problem on more readily available experimental material and then by the judicious selection of experiments to relate the findings to the species of fish in which we were most interested. As steelhead trout were readily available and easily maintained and had some of the behaviour characteristics of the Oncorhynchus, these fish were selected for study. That coho and steelhead tissues behaved similarly as regards oxygen uptake was evident from Table I. A comparison of glycolytic activity of homogenates of various tissues of coho and steelhead is shown in Table II. The results are typical of those obtained in a number of different fish of both species. It is evident from the results that only heart muscle homogenates in either species showed any appreciable response to added glucose. All of the other tissues, however, did contain lactic acid in excess of that present in zero time controls. Results obtained last year with coho (Summary No. 9 of this Station's Annual Report for 1957-58) indicated that this lactic acid arose from the hexose diphosphate included in the incubation medium.

The inability of homogenates other than heart muscle to form lactic acid from glucose was investigated first. Two components of the incubation mixture, KCl and DPN, have been reported to have the most effect on glycolysis. Accordingly, both

were increased appreciably in the medium and the effect of the new concentrations compared with the old. These changes did not affect glycolysis.

TABLE I - Variation in the Q_{O_2} value of liver slices from steelhead trout (Salmo gairdnerii gairdnerii) with weight of tissue used.

Fish (1)		Fish (2)		Fish (3)		Fish (4)	
Wt of tissue*	Q_{O_2}	Wt of tissue	Q_{O_2}	Wt of tissue	Q_{O_2}	Wt of tissue	Q_{O_2}
mg		mg		mg		mg	
8.7	5.55	9.7	3.69	8.8	2.22	8.3	4.68
8.9	4.28	11.2	5.07	14.4	2.29	13.0	6.40
15.1	4.52	17.3	5.11	15.7	3.82	16.5	7.15
22.7	4.17	22.3	6.10	25.8	4.85	25.7	6.42
30.3	5.18	27.2	6.36	33.1	5.74	34.5	7.45
46.7	5.44	41.6	7.82	52.5	5.37	46.0	7.83
55.6	5.74	62.0	8.20	63.7	7.22	62.8	7.91
						92.1	8.37

* Dry weight of tissue. Tissue introduced as slices.

TABLE II - Glycolytic activity of the homogenates of various tissues of steelhead trout and coho salmon.

Tissue	Steelhead		Coho	
	Q lactic acid*			
	No glucose	+ glucose	No glucose	+ glucose
Skeletal muscle	.469	.515	.736	.786
Liver	.155	.216	.185	.198
Kidney	.527	.837	.219	.179
Heart	.850	2.84	.875	2.53

* Micromoles lactic acid produced per mg dry weight of tissue per hour.

To localize the deficiency in the glycolytic system, HDP was removed from the incubation medium and the effect of various compounds on lactic acid production compared. The results, Table III, show that glucose-6-PO_4 but not glucose can give rise to lactic acid and that it actually formed more lactic acid than an equimolar concentration of HDP.

Since glucose-6-PO_4 but not glucose formed lactic acid, the primary lesion preventing glycolysis with glucose appeared to be the hexokinase system. To confirm this, the effect of supplementing the system with a source of hexokinase was studied. Purified yeast hexokinase at a level of 50-100 micrograms per 3 ml flask permitted glycolysis with glucose by homogenates of all tissues. The results with skeletal muscle are shown in Table IV. The lactic acid production from added glucose was in this case about 70% of theoretical. The homogenizing medium used was isotonic KCl. Previously, when isotonic sucrose was the homogenizing medium, it had been observed that more than the theoretical amount of lactic acid formed from the added glucose. This discrepancy was traced to the presence of sucrose introduced with the homogenate into the incubation medium. It therefore became necessary

to know whether the fish tissue homogenate had the capacity to metabolize sucrose or whether the yeast hexokinase preparation was responsible for the sucrose breakdown. Careful studies indicated that neither the tissue homogenate nor the yeast hexokinase had either invertase or sucrose phosphorylase activity measurable by the methods used. It was found, however, that at a fixed level of homogenate the amount of lactic acid formed from sucrose increased as the hexokinase level increased and that more hexokinase was required for maximum activity with sucrose than with an equimolar concentration of glucose (Table V). This suggested that in the presence of sucrose, more than hexokinase activity was being supplied by the hexokinase preparation and that it was the yeast preparation rather than the homogenate that was making it possible for the tissue homogenate to form lactic acid from sucrose. Thus it was unlikely that fish tissues had a capacity to metabolize sucrose.

TABLE III - Comparison of the capacity of glucose and various glycolytic intermediates to cause lactic acid formation in homogenates.

Substrate	Skeletal muscle	Liver
	Lactic formed (μmoles/3ml)*	
0	2.83	1.82
Glucose (30 μmoles)	2.47	2.33
Glucose-6-PO_4 (12 μmoles)	10.30	8.78
Fructose-6-PO_4 (12 μmoles)	6.88	5.40
Hexose di phosphate (12 μmoles)	6.25	4.25

* Incubation time = 100 min.

TABLE IV - Effect of added hexokinase on lactic acid production by skeletal muscle homogenate of steelhead trout.

Hexokinase μg/flask	Glucose added 0	20 μmoles
	Lactic formed (μmoles)*	
0	7.01	6.98
5	7.00	9.19
10	6.77	12.17
25	6.78	25.43
50	7.03	35.65
100	7.01	34.09

* Incubation time = 90 min

It was also observed that with skeletal muscle homogenates the glucose had to be together with the other flask components for lactic acid to form. If glucose was introduced from the side-arm after equilibration of the flask in the Warburg bath, no lactic acid formed. Further tests revealed that the formation of lactic acid from glucose depended upon the time between the addition of the homogenate to the other components in the flask andthe addition of glucose. If five minutes elapsed between the two additions, only 10% of the glucose was utilized, while if 10 or more minutes passed, no glucose was broken down. When both ATP and glucose were introduced into the reaction mixture after the latter had been incubated for a

length of time (15 min), which would have ensured no glucose utilization if glucose had been added alone, nearly full utilization of glucose occurred. This strongly indicates that an ATP-ase in the muscle homogenate was destroying ATP in the original reaction mixture so rapidly that not sufficient was left after equilibration in the Warburg bath to initiate the phosphorylation of glucose.

TABLE V - Comparison of requirement for hexokinase for maximum lactic acid production from glucose and sucrose.

Hexokinase (µgm/3ml)	0	Glucose (20 µmoles)	Sucrose (20 µmoles)
		Lactic formed (umoles)*	
0	7.62	6.93	6.00
50	7.28	33.20	8.77
100	7.02	31.10	19.90
300	6.90	21.10	36.50
500	7.48	17.00	39.10

* KCl homogenate. Incubation time = 90 min.

Storage Studies

It was of considerable interest to know how long a homogenate could be stored and still retain glycolytic activity since, if a fresh homogenate was required for each experiment, a very large number of fish would have to be sacrificed. Accordingly, homogenates were prepared and stored for 2 weeks at 16°F. Since ethylene diamine tetraacetic acid has been found to be an active enzyme stabilizer for animal tissues, a parallel experiment was run including this compound in part of the homogenate. The results are recorded in Table VI. Whereas the activity in the presence of EDTA did not change appreciably in the first week, it dropped to 59% of the initial value over the same period in its absence. Even after 2 weeks, 72% of the activity still remained when EDTA was present.

TABLE VI - Glycolytic activity of a skeletal muscle homogenate after storage at 16°F in the presence and absence of EDTA.

Storage time (weeks)	No EDTA		+ EDTA	
	No glucose	+ glucose	No glucose	+ glucose
	Lactic formed*			
0	279 ± 14	1669 ± 77	196 ± 18	1606 ± 96
1	146 ± 10	976 ± 4	282 ± 2	1557 ± 77
2	236 ± 12	937 ± 68	283 ± 9	1159 ± 34

* γ/ml solution.

Enzymes of glycolysis

Although it is possible to demonstrate glycolysis in homogenates of the various tissues, for our purposes it is also desirable to know if it proceeds by the same path that occurs in animal tissues. To learn this it is necessary to determine if the intermediate steps in the breakdown of glucose to lactic acid that occur in animal tissues also are present in fish tissues.

Only a limited amount of this information has been obtained up to this time.

Hexokinase. It has been shown that the inability of all the homogenates tested except heart muscle, to convert glucose to lactic acid, is due to a deficiency of hexokinase. A similar observation has been made with animal tissues. In all of these tissues "in vivo", the breakdown of glucose is believed to take place via this pathway.

Although glycolysis of glucose cannot be demonstrated "in vitro" in most animal tissues, these same tissues can be shown in almost all cases to have some hexokinase activity. The order of activity of the hexokinase is very low, however, and accounts for the fact that measurable glycolysis cannot be demonstrated. Why this is so has never been satisfactorily explained. Preliminary evidence suggests that a similar situation prevails in fish tissues.

Phosphoglucomutase. A very active phosphoglucomutase has been found to be present in liver, kidney, skeletal muscle and heart muscle homogenates of the steelhead trout.

SUMMARY NO. 7

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

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

Last year evidence had been obtained that succinic dehydrogenase, fumarase, malic dehydrogenase and the condensing enzyme occurred in cell-free extracts of marine bacterium B-16 and the response of the first three enzymes to Na^+ and K^+ was reported (Summary No. 49 of this Station's Annual Report for 1957-58). During this past year, all of the other enzymes of the tricarboxylic acid cycle have been demonstrated and the effect of inorganic ions on their activity determined.

Aconitase. This enzyme was measured spectrophotometrically by following the optical density change at 240 mμ due to the formation of cis aconitic acid from either citrate or isocitrate. The enzyme was found to be almost inactive in media of low ionic strength. The ion requirement for maximum activity proved to be very non-specific. Chloride or sulfate salts of Na^+, K^+ and tris hydroxymethyl amino methane were found to be capable of providing the required ionic atmosphere. The activity of aconitase toward isocitrate was found to be 2.4 times its activity toward citrate under optimum conditions, a ratio in remarkably good agreement with the figure of 2.1 found by other workers for pig heart extracts.

Isocitric dehydrogenase. Great difficulty was at first experienced in demonstrating isocitric dehydrogenase activity in this organism. Since the response of all enzymes to Na^+ and K^+ was desired, cells were washed with a $MgCl_2$ solution prior to sonicing to provide an extract containing as little contaminating Na^+ and K^+ as possible. Such a procedure has been used when testing for all of the other tricarboxylic acid cycle enzymes and had proven to be satisfactory. When no isocitric dehydrogenase activity could be demonstrated in such preparations, a K_2SO_4 solution of higher ionic strength than the $MgCl_2$ solution was used to wash the cells. An active isocitric dehydrogenase could then be detected. It was at first felt that this activity was probably due to the fact that the K_2SO_4 solution was of higher ionic strength. When, however, the ionic strength of the K_2SO_4 solution was reduced to that of the $MgCl_2$ solution, the preparation from cells washed with K_2SO_4 was still active and furthermore an extract obtained from cells washed with $MgSO_4$ was also active. These findings suggested then either that Cl^- was inhibitory or that $SO_4^=$ was required by the enzyme.

It was found, however, that Cl^- did not inhibit the enzyme once an active preparation had been obtained and that $PO_4^{\equiv}$ could replace $SO_4^{=}$ in the wash solution.

The ionic strength of the wash solution even when K_2SO_4 was used in its preparation was found to be very important. Optimum activity was achieved when the ionic strength of the wash solution was about .3. At suboptimal ionic strength a chloride salt could contribute to the activity of the resulting isocitric dehydrogenase activity of the extract so long as a SO_4 salt was present during sonic treatment. The same or higher concentration of a chloride salt alone in the wash solution produced an extract which was not only inactive as a source of isocitric dehydrogenase but could not be activated by the further addition of K_2SO_4 after sonic treatment. Bromide and iodide salts used alone in the preparation of wash solutions for the cells, like chlorides produced extracts with no demonstrable isocitric dehydrogenase activity. These ionic effects were not peculiar to extracts prepared by sonic treatment but were found also to apply when extracts were prepared by alumina grinding. Since it has not been possible to demonstrate a positive effect of the halide ions on the enzyme, one is forced to postulate a negative effect to explain the results. It is possible that $SO_4^{=}$ and $PO_4^{\equiv}$ in some way protect the enzyme from physical damage during sonic treatment or alumina grinding of the cells while halide ions have no capacity to provide this protection.

Quite apart from the ionic atmosphere required for the preparation of an extract from whole cells containing an active isocitric dehydrogenase, the activity of the enzyme itself was found to be a function of the ionic strength of the medium. A non-specific response was observed to increasing salt concentration in the medium. Optimum activity was obtained when the ionic strength reached 0.2 to .3. Chloride salts were as active as sulfate salts in the system.

As in animal tissues, Mg^{++} was found to be required for isocitric dehydrogenase activity. The enzyme was active with TPN and only 15% as active with a DPN preparation.

The product of the oxidation of isocitric acid is oxalosuccinic acid. The latter compound decarboxylates under the influence of oxalosuccinic decarboxylase to a α-ketoglutaric acid. Since oxalosuccinic acid is very unstable and its decarboxylation proceeds spontaneously even in the absence of the enzyme, it is considered sufficient evidence of the course of the reaction to be able to demonstrate α-ketoglutaric acid formation with isocitrate as a substrate. This product was in fact demonstrated by identification by paper chromatography of the 2,4 dinitrophenylhydrazone derivative of the keto acid.

<u>α-Ketoglutaric hydrogenase</u>. This is the enzyme which in most bacterial systems has been found to be the hardest to demonstrate because of its instability. Our initial attempts to find the enzyme in extracts of the organism were unsuccessful. By substituting a growth medium containing only glutamic acid, alanine and aspartic acid for the complex nutrient broth yeast extract medium used previously, by adding 10^{-2}M cysteine to the wash solution used in preparing the cell suspension and by reducing the time of sonic treatment from 5 min to 3 min an active α-ketoglutaric dehydrogenase could be demonstrated. Enzyme activity was followed by measuring DPN reduction in the presence of α-ketoglutaric acid. TPN was not active in the system. CoA but not GTP was found to be a necessary additive to the reaction system.

Salts did not activate α-ketoglutaric dehydrogenase. The addition of Na^+ and K^+ salts decreased rather than increased the activity of the enzyme.

The enzymes between α-ketoglutaric dehydrogenase and aconitase were demonstrated last year. All of the steps of the tricarboxylic acid cycle thus have been

shown to be present in this marine bacterium. Although it has now been shown that in extracts of the organism all of the enzymes are present and active, it remains to be demonstrated that they function as a cycle in the intact organism.

The presence of all of these enzymes, however, does show the close relation between marine bacterial cells and other forms of life. Although marine bacteria probably appeared early in the evolutionary scale, it is evident that their biochemical systems were very highly developed.

Isocitritase. Although this enzyme catalyzes what is essentially a side reaction in the tricarboxylic acid cycle, it has been postulated to be of considerable importance when two carbon fragments serve as the sole source of carbon and energy for growth. Isocitritase cleaves isocitrate to succinate and glyoxylate. The glyoxalate then combines with acetate under the influence of malate synthetase to form malate. Thus, a supply of four carbon fragments required for the operation of the tricarboxylic acid cycle are made available to permit the metabolism of the two carbon compound serving as a source of carbon and energy.

We had attempted previously to demonstrate isocitritase in extracts of our marine bacterium but had had no success (Summary No. 49 of this Station's Annual Report for 1957-58). When a medium containing acetate as the sole source of carbon was substituted for the more complex nutrient broth yeast extract medium previously used, glyoxalate formation from isocitrate could be demonstrated. This would indicate that in our organism isocitritase is an adaptive enzyme, forming in response to a need for the enzyme, rather than as is usually the case, to the presence of the specific substrate in the medium. This observation also lends support to the postulated function of isocitritase as aiding in the utilization of two carbon fragments by the cell.

Localization of Enzymes in the Microbial Cell

All of the enzymes discussed above were found to be present in sonic extracts of the cells clarified by centrifuging at 5,000xg for 30 minutes. Such an extract was crystal clear and was used in this state to avoid any possibility that enzyme activity might be due to undisrupted cells in the extract rather than to cell-free enzymes. Since many enzymes in most cells are associated with particulate components which would be sedimented by this force of gravity, it was of interest to know what proportion of the total enzymatic activity was present in the clear extract. Accordingly, the activity of each enzyme in the TCA cycle was tested in a sonic extract before and after centrifuging. None of the enzymes had a higher specific activity in the uncentrifuged than in the centrifuged preparation. This does not mean that in the living cells of this organism certain of the enzymes are not associated with particulate matter. Sonic treatment is known to disrupt many particulate cell components and in the case of this marine bacterium, the total activity could be quantitatively recovered in a clear supernatant extract.

Dialysis. Although none of the enzymes of the TCA cycle could be shown to require Na^+ or K^+ for activity except malic drhydrogenase, the possibility still remained that the small amounts of these ions present in the reaction mixture could satisfy the requirements for these ions. As the principal source of contaminating traces of Na^+ and K^+ was the added enzyme extract, attempts to lower its content of these ions by dialysis were made. Dialysis was carried out in the cold, using as a dialyzing medium a solution containing cysteine and Tris buffer dissolved in freshly boiled and cooled distilled water. All of the enzymes were less active after only 2 hours dialysis and very much less active after 18 hours. The activity could not be restored by adding back Na^+ and K^+ alone or together, the ash of the extract or a concentrate of the dialysate. It was thus impossible to prove whether or not traces of Na^+ or K^+ might be required for the activity of these enzymes. It was

evident, however, that levels required for the metabolism of the whole cells had no specific positive effect upon any of the enzymes in the extract except malic dehydrogenase.

SUMMARY NO. 8

OXIDATIVE METABOLISM OF A MARINE BACTERIUM
II STUDIES ON THE MECHANISM OF PROPIONATE OXIDATION — C.A. Claridge

Studies reported last year on this topic (Summary No. 48 of this Station's Annual Report for 1957-58) have been continued and extended.

It is known that marine bacterium B16 will oxidize propionate completely but succinate only partially, using O_2 uptake as a basis of measurement. This has been interpreted as evidence for the possible existence of an alternate system to the conventional tricarboxylic acid cycle for the complete oxidation of propionate.

Much early work in establishing pathways of oxidation in bacteria depended upon experiments wherein an isotopic substrate was permitted to be metabolized in the presence of a non-isotopic compound suspected of being an intermediate. The degree of incorporation of isotope from the substrate presumably indicated whether or not the non-isotopic compound was an intermediate. However, if an intermediate existed within the cell in an active form, then it would not necessarily equilibrate with the added carrier compound. Therefore, by employing large amounts of enzyme preparations and micro-isolation procedures, it may be possible to isolate metabolically formed intermediates without the addition of carrier compounds.

Large-scale experiments have been conducted with B16 using C^{14}-labelled succinic and propionic acids so that some of the intermediates can be isolated and identified.

Each experiment with labelled substrate was fractionated as reported in the accompanying table. This table records the counts per minute (using an end-window counter) obtained in each fraction. It can be seen that a large amount of the radioactivity from succinate-1-C^{14} appears in the CO_2. This would be expected if succinate is being oxidized *via* the tricarboxylic acid cycle. However, with propionate-1-C^{14}, and to a lesser degree with propionate-2-C^{14}, a relatively small amount of labelling appears in the CO_2.

If the metabolism of propionate involves an initial carboxylation to form succinate or isosuccinate, which has been demonstrated to occur in some animal tissues and in certain bacteria, then more labelling in CO_2 would be expected.

From each experiment, four of the fractions ((1) supernatant direct distillate; (2) supernatant steam distillate; (3) supernatant ether solubles, and (4) cells ether soluble) were passed through celite columns to separate the acids (Swim and Utter - Methods in Enzymology IV, p. 584, Academic Press, New York, 1957). Five ml-aliquots were collected in an automatic fraction collector and titrated with 0.006M NaOH. Then each tube was taken to dryness and made to a 1.0 ml volume for radioactivity determinations.

The curves showing titratable acidity and radioactivity for the four fractions listed above are given in Figures 1 to 4.

In Fig 1, the large peak around tubes 50 to 80 contains succinate, α-ketoglutarate and possibly other acids. As would be expected from succinate-1-C^{14},

there is considerable radioactivity in this peak, but none from propionate-1-C_{14} and propionate-2-C^{14}. These data rule out a carboxylation of propionate to succinate as the first step in its metabolism. The relatively large peak at tube 120 to 130 is malic acid and likewise contains considerable activity from succinate-1-C^{14} but none from propionate-1- or 2-C^{14}. As can be seen in Fig. 1, there are several prominent radioactive peaks in the region of tubes 10 to 50 which, as of yet, are unidentified. Particularly prominent are two radioactive peaks near tube 30 from the propionate-2-C^{14} experiment.

Except in the case of succinate-1-C^{14}, the ether solubles fractions from the cells as shown in Fig 2 contained relatively little activity. The majority of the activity is centred in the succinate peak in that case, whereas from propionate-1 and 2-C^{14} the activity appears in still unidentified peaks near the beginning of the column.

The volatile and steam volatile fractions in Fig 3 and 4 all show two major peaks, followed by a third smaller peak, near the beginning of the column. The first one, in the region of tube 10, is radioactive in the case of propionate and only from the succinate in the volatile fraction. The second peak is only appreciably radioactive in the volatile fraction from propionate-2-C^{14} and not in any of the steam distillate fractions. The large radioactive peak at tube 60 from succinate-1-C^{14} is residual substrate. Although succinic acid is not considered to be volatile, control experiments have shown that a sufficient amount is distillable to appear in these fractions.

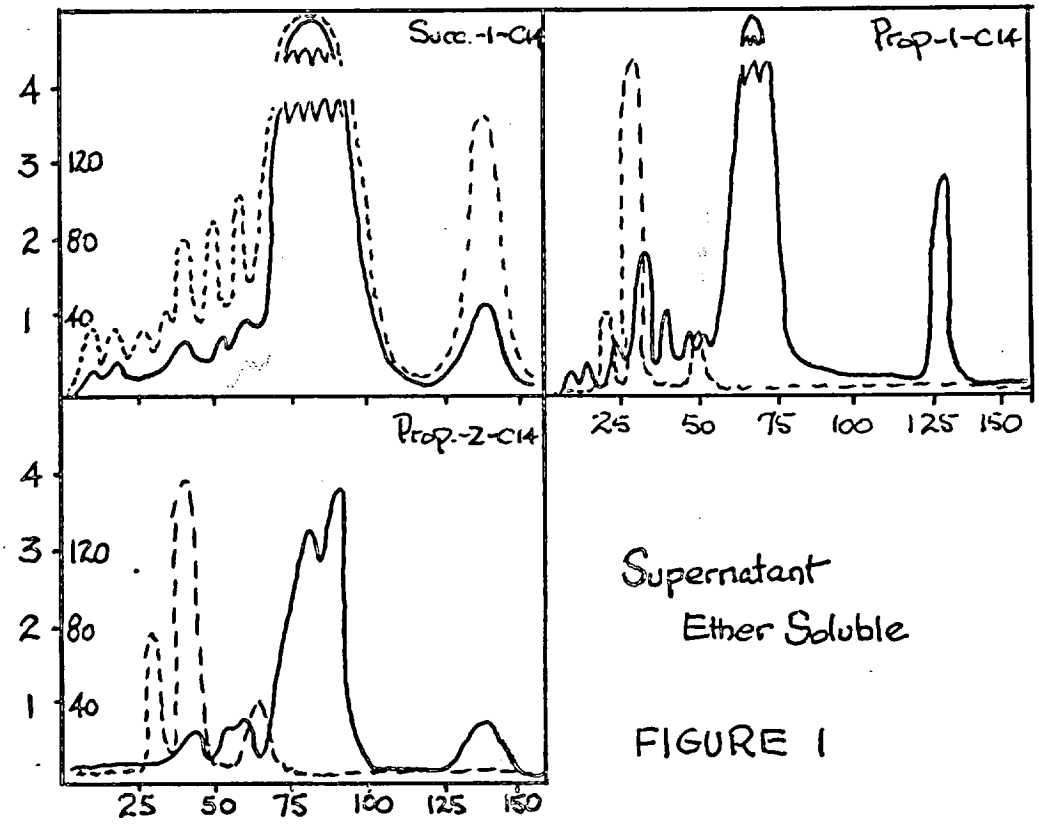
Other workers have proposed as intermediates in propionate oxidation acrylate, β-hydroxypropionate, methylmalonate and lactate. Positions of elution of these acids from the celite columns have been determined and they have been shown to overlap with other acids of the TCA cycle. Acrylate is eluted from the column in the region of propionate and acetate near the beginning, whereas β-hydroxypropionate, methyl malonate and lactate fall in the region of succinate and α-ketoglutarate and malonate.

The identification of the unknown peaks and the positions of the proposed intermediates in propionate metabolism are being investigated.

TABLE I - Counts per minute in fractions from labelling experiments.

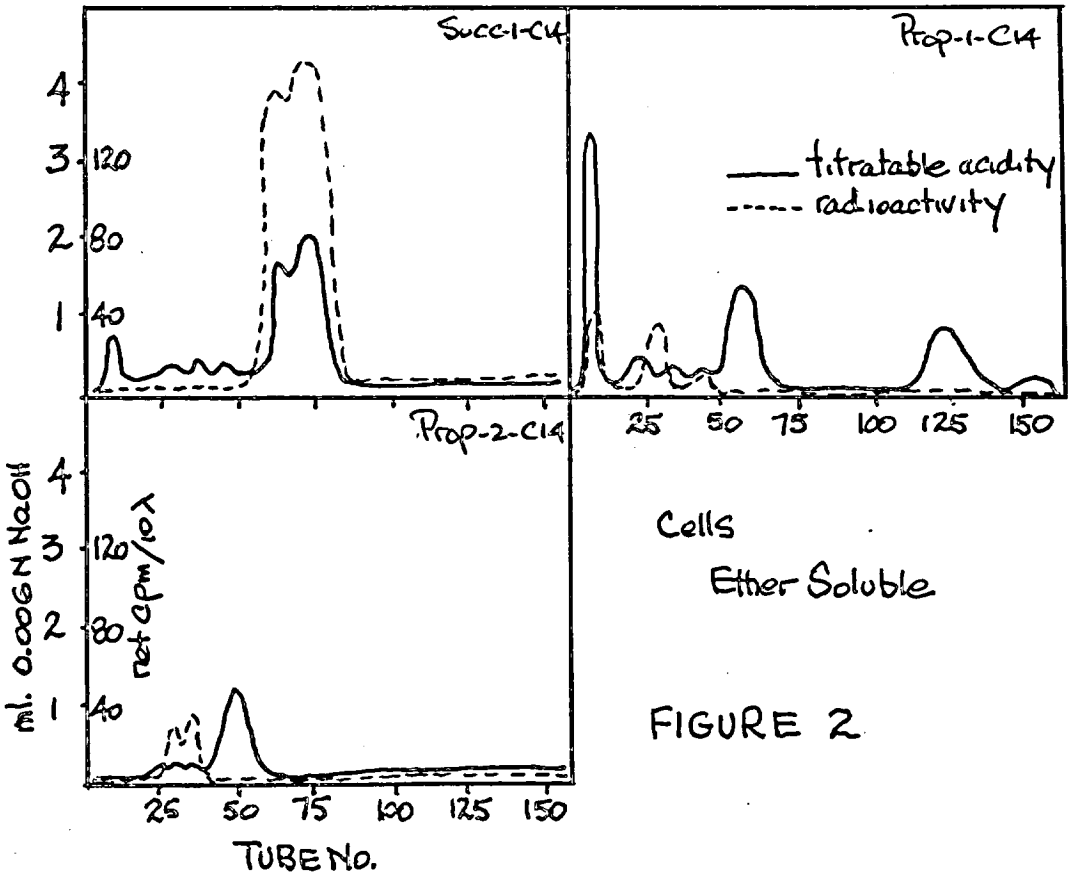
	Prop-1-C^{14}	Prop-2-C^{14}	Succ-1-C^{14}
Initial	18,680,000 cpm		18,927,000 cpm
Supernatant			
Direct dist	15,690,000	8,017,000	991,000
Steam dist	304,800	179,900	372,900
Ether sol	66,460	126,600	15,530,000
H_2O sol	29,960	28,800	577,000
Cells			
Ether sol	5,960	15,600	318,000
H_2O sol	0	3,300	17,800
H_2O insol	64,000	22,400	25,800
CO_2	108,300	36,250	1,280,000

Each experiment contained: 22 mM potassium phosphate buffer, pH 6.4; 4000 µm substrate (labelled and unlabelled); 200 ml of cells in seawater (approx 80 gm wet wt); distilled water to 500 ml.



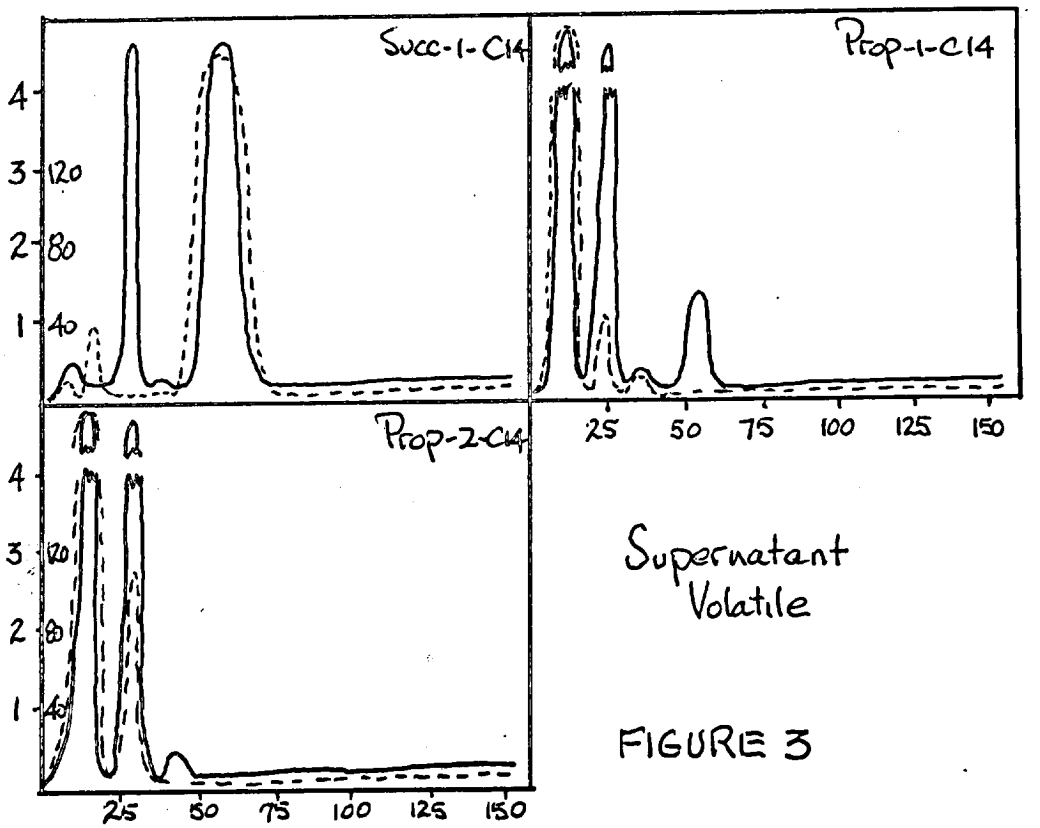
Supernatant
Ether Soluble

FIGURE 1



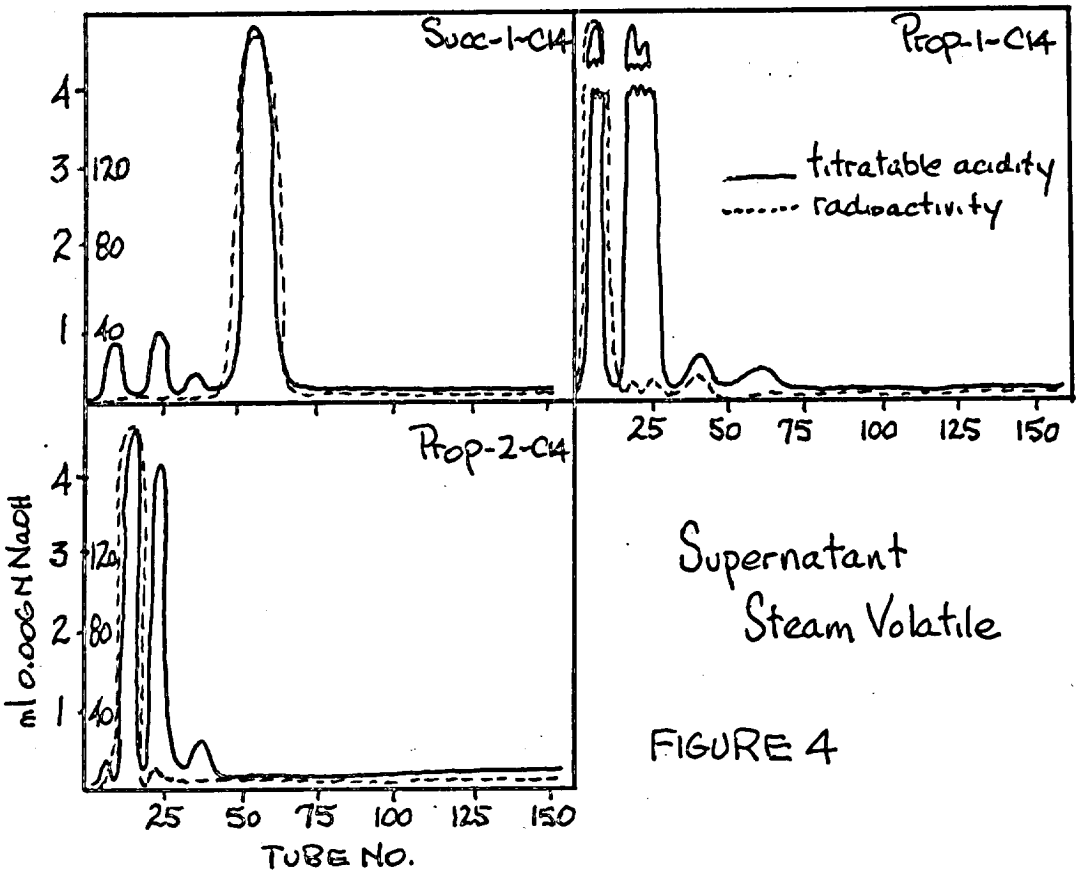
Cells
Ether Soluble

FIGURE 2



Supernatant Volatile

FIGURE 3



Supernatant Steam Volatile

FIGURE 4

SUMMARY NO. 9

Na^+, K^+ AND WEIGHT CHANGES IN FISH HELD IN REFRIGERATED-SEA-WATER AND OTHER SOLUTIONS

R.E.E. Jonas
R.A. MacLeod

Previous reports have shown that when fish are stored in refrigerated-sea-water, Na^+, K^+ and weight changes occur in the flesh (Summaries Nos. 5 and 23 of this Station's Annual Report for 1955-56). Efforts have been made to devise solutions which would minimize these effects (Summary No. 25 of this Station's Annual Report for 1956-57).

The present study was designed to extend the observations to a wider range of species of commercially important fish and to improve on the methods of preventing water uptake by, and Na^+ and K^+ changes in, the flesh.

The earlier observations with coho salmon (Oncorhynchus kisutch) were confirmed and extended using sockeye salmon (Oncorhynchus nerka). Artificial rather than natural sea-water was employed as the immersing solution to provide more readily reproducible salt concentrations. Since sea-water along the coastline is frequently diluted with fresh water, particularly near points where rivers discharge into the sea, the artificial sea-water was tested at both full and half strength. As fish are frequently eviscerated immediately after capture, a comparison was made of the extent of the changes occurring in whole and eviscerated fish. Weight changes in the fish were also determined. The results are recorded in Table I. For this experiment, sockeye salmon freshly caught in a gill net at the mouth of the Fraser River were quickly transported to the laboratory and immediately immersed in previously prepared solutions. Less than 4 to 6 hours elapsed between the time of capture of the fish and the beginning of the experiment.

The results show, Table I, that half-strength sea-water raised the Na^+ level of the flesh of the fish approximately half as high as full strength sea-water but produced little difference in the K^+ concentration. Though the eviscerated fish presented a greater surface exposed to the salt solution than did the whole fish, surprisingly little difference was observed in the Na^+ and K^+ concentrations of the flesh from the two groups. Fish held in full strength sea-water tended to gain more weight than those in the half-strength solution, while the increase observed in eviscerated fish exceeded that in whole ones.

In the experiment just described the first analyses were made after immersion of the fish for one week in the sea-water solutions. In previous experiments with coho salmon in which fish were immersed for short intervals of time in sea-water, it was noted that the natural levels of Na^+ and K^+ were maintained in the fish for a period of approximately 24 hours, after which time a rather abrupt rise in Na^+ and fall in K^+ occurred.

To determine whether a similar phenomenon could be observed in another species, an experiment was conducted using sockeye salmon. In an effort to cast further light on the nature of the phenomenon, the flesh in a one-inch slice taken through the center section of the fish was divided into inner and outer layers. The inner layer represented the flesh from the backbone to a point midway between the backbone and the outer surface of the fish, while the outer layer was made up of the flesh from the midpoint to the outer surface. The layers were rendered skin and bone free and then homogenized. The flesh from a second one-inch slice through the fish taken at a point adjacent to the first was pooled in the usual manner for analysis. For this experiment sockeye salmon were caught in gill nets at the mouth of the Fraser River and transported immediately to the laboratory. Approximately four hours elapsed from the time of capture of the fish until the whole fish were immersed in the experimental sea-water tank. At each six-hour interval three fish were removed from the tank for sampling and analysis. The results obtained are recorded in

Fig 1. The outer layer of flesh showed a slow rise in Na^+ concentration and fall in K^+ which was evident from the earliest analyses. After approximately 72 hours these changes began to take place much more rapidly. No change occurred in the ion levels of the inner layer of flesh during the first 48-hour period. As can be seen, the increase in the rate of change of the ion concentrations in the outer layer occurred after changes in Na^+ and K^+ levels had begun to take place in the inner layer of flesh. As might be expected, values obtained from a flesh sample representing a composite of the inner and outer layers, were approximately the average of the two sets of values shown.

The initial slow rise in Na^+ and concomitant fall in K^+ in the outer layer of the flesh (Fig 1) may well have been due to equilibration of the immersing solution with the extracellular fluid of the tissue, while the more rapid changes occurring subsequently could represent equilibration with intracellular fluid. Studies with much smaller sections of tissue would be required to test this hypothesis further. (See Fig 1, page 35.)

Information on ion and weight changes in fish immersed in refrigerated sea-water has so far been restricted to studies on various species of salmon. As a step toward extending these observations to include other commercially important species of fish, an experiment was conducted with halibut (Hippoglossus stenolepsis). For this purpose, halibut ranging in weight from 10 to 20 lb. were caught at sea and packed in ice for transport to the laboratory. The experiment was begun within 24 hours of the time of capture of the fish. In this study a slice one-inch wide was taken from the center section of the fish and divided into inner and outer layers.

TABLE I - Na^+, K^+ and weight changes in sockeye salmon held whole and eviscerated in refrigerated sea-water.

Sea-water concentration	Immersion time	Na^+	K^+	Wt increase**
		mg/100 gm*		%
Control (Not immersed)	-	33.5 ± 1.1	411 ± 15	-
		Whole fish		
Full strength	1 week	277 ± 13	276 ± 5	2.98 ± .79
	2 "	422 ± 18	198 ± 6	4.33 ± 1.25
Half strength	1 "	149 ± 12	265 ± 9	2.38 ± .58
	2 "	221 ± 20	201 ± 17	3.45 ± .65
		Eviscerated Fish		
Full strength	1 week	297 ± 38	272 ± 19	4.63 ± 1.39
	2 "	487 ± 26	193 ± 19	8.16 ± 1.69
Half strength	1 "	178 ± 29	257 ± 34	3.89 ± .99
	2 "	250 ± 21	178 ± 25	5.60 ± 1.00

* Based on the wet weight of the muscle tissue. Each value recorded represents the average and the average deviation of four fish.

** Of the entire fish.

As in the previous salmon experiment, the inner layer represented the flesh from the backbone to a point midway between the backbone and the outer surface while the outer layer was composed of flesh from this midpoint to the outer surface. The layers were freed of skin and bone and then homogenized. The results (Table II), show that after one week in half-strength sea-water, the inner layer of flesh of the halibut was almost unchanged with respect to Na^+ and K^+ concentration, while the values for the outer layer had changed very appreciably. After two weeks the two layers had still not equilibrated. The weight increases recorded after one and two weeks are slightly higher than for sockeye salmon held under comparable conditions.

TABLE II - Na^+ and K^+ changes in the inner and outer layers of the muscle tissue of halibut and weight increases in the fish when preserved in half-strength refrigerated-sea-water.

Time	Portion of tissue analyzed	Na^+	K^+	Wt increase†
(weeks)		mg/100 gm*		
0	Inner layer	45.6 ± 6.0	510 ± 9	
	Outer layer	47.7 ± 5.1	493 ± 15	
	Average	46.7 ± 5.6	501 ± 12	
1	Inner	67.4 ± 5.1	501 ± 15	3.7 ± .7
	Outer	149.3 ± 22.9	389 ± 17	
	Average	108.4 ± 14	445 ± 16	
2	Inner	137.4 ± 19.1	413 ± 22	5.1 ± .9
	Outer	210.8 ± 19.1	302 ± 23	
	Average	174.1 ± 19.1	358 ± 23	

* Based on the wet weight of the muscle tissue. Each value recorded represents the average and the average deviation of four fish.

† Of the entire fish.

It is clear from previous findings (Summary No. 25 of this Station's Annual Report for 1956-57) that little difficulty is experienced in preparing solutions which will maintain the level of Na^+ and K^+ in the flesh at or near their natural values. Fish immersed in such solutions, however, tended to gain more weight than when stored in sea-water. Since no significant net uptake of salt was involved when these solutions were used, the weight increase could only be due to uptake of water by the flesh. Water uptake would be expected to occur if the osmotic pressure of the immersing solution was lower than that of the tissue fluids of the fish. It was therefore desirable to find an agent which would be sufficiently soluble to provide the required osmotic pressure in the solution but be impermeable to the tissue membranes of the fish. Starch and casein had proven not to be particularly effective as such agents.

It seemed possible that the polyoxyethylene sorbitan macromolecules obtainable by saponifying a commercial Tween might have the necessary solubility and high molecular weight required to prevent water uptake by the fish. Accordingly, a sample of Tween 40, a palmitate derivative, was saponified and the fatty acid removed. The water soluble fraction was dialyzed free of salt and small molecules and concentrated. When added to an immersing solution at a level of 5% (Table III), this agent reduced the weight increase in brill (Eopsetta jordani) by 66%. A comparable level of Tween 40 was much less effective.

TABLE III - Changes in the Na^+ and K^+ content of the muscle tissue and in the weight of brill maintained in refrigerated-sea-water and other salt solutions for one week.

Sol'n. No.	Composition of solution*	Na^+	K^+	Wt. change
		mg/100 gm		%
-	Control	59.8 ± 3.4	406 ± 6	-
1	Sea-water (full strength)	527 ± 33	204 ± 23	8.9 ± 1.3
2	" (half ")	283 ± 0.3	193 ± 9	7.8 ± 1.4
3	Na^+ and K^+ to maintain flesh level constant	100 ± 5	462 ± 4	8.6 ± 2.2
4	Sol'n. (3) + 5% Tween 40	111 ± 3	466 ± 9	5.1 ± 1.3
5	" + 5% polyoxyethylene sorbitan	116 ± 5	456 ± 9	2.9 ± .2

* Solution (3) contained 2.86 gm NaCl and 9.59 gm KCl per 1.

Since the results obtained with the polyoxyethylene sorbitan fraction of the Tween were encouraging when brill were the test fish, an experiment using a wider range of concentrations was conducted with lemon sole (Parophrys vetulus). The results (Table IV) show that a level of the macromolecule prevented water uptake almost completely from a solution containing Na^+ and K^+ at concentrations designed to maintain flesh levels constant, while higher levels actually caused the fish to lose weight. As can be seen, when 15% of the polyoxyethylene sorbitan was added, the loss in weight of the fish amounted to 10%. The results also show that the addition of the macromolecule prevented water uptake from both full- and half-strength sea-water. The NaCl and KCl levels used in Solution 1 (Table IV) were modified from those employed in previous experiments (Table V) in an effort to obtain a solution better able to maintain the flesh level of the lemon sole constant.

TABLE IV - The effect of adding polyoxyethylene sorbitan macromolecules to various immersing solution on the extent of weight changes in lemon sole after immersion for one week.

Sol'n. No.	Salt composition of immersing solution*	Macro-molecule addition	Na^+	K^+	Wt. change
		%	mg/100 gm**		%
-	Control (not immersed)	-	75 ± 2.9	407 ± 4	-
1	Na^+ and K^+ to maintain flesh level constant	0	99.0 ± 3.1	390 ± 0	+ 4.0 ± 1.4
2	Same as solution 1	5	98 ± 3.1	411 ± 9	+ 0.3 ± 1.7
3	" " " "	10	101.5 ± 2.7	434 ± 0	- 5.7 ± 1.1
4	" " " "	15	108.7 ± 1.8	444 ± 5.3	-10.5 ± 2.2
5	Sea-water (half strength)	0	348 ± 11	141 ± 9	+ 5.5 ± 1.9
6	" " " "	10	373 ± 4	158 ± 12	- 8.1 ± 1.8
7	" " (full strength)	0	563 ± 14	184 ± 19	+ 6.4 ± 1.5
8	" " " "	10	613 ± 19	177 ± 22	- 8.8 ± 1.2

* Solution (1) contained 2.29 gm NaCl and 8.31 gm KCl per 1.

** Based on the wet weight of the muscle tissue. Each value recorded represents the average and the average deviation of 10 fish.

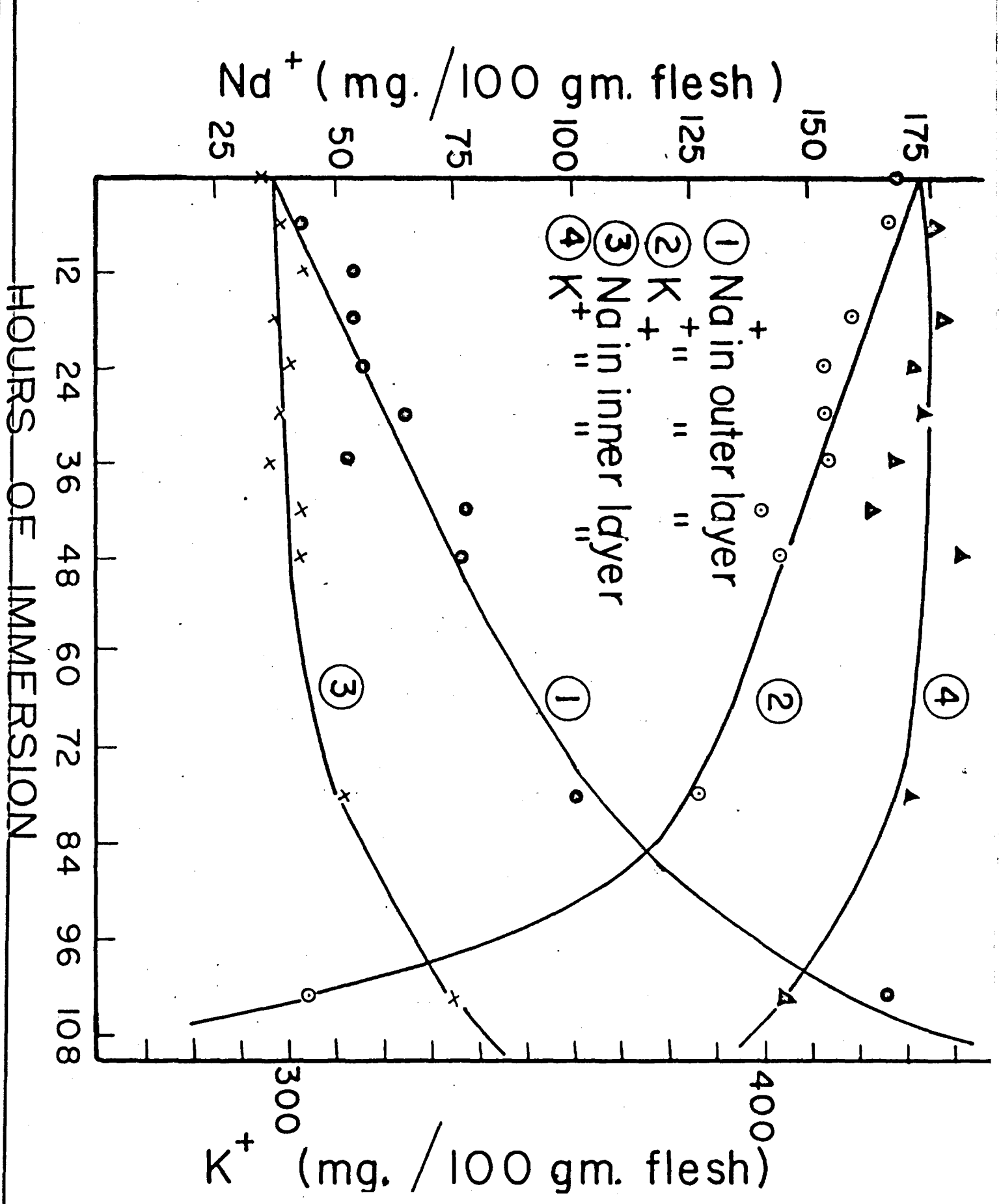


Figure 1. Na^+ and K^+ changes in the inner and outer layers of the muscle tissue of sockeye salmon at various intervals after immersion of the fish in full strength refrigerated-sea-water. Each recorded point represents the average value for three fish.

SUMMARY NO. 10

DOGFISH

R. Moyer
B.A. Southcott
E.G. Baker

Through the co-operation of the Nanaimo Station, approximately 100 dogfish were obtained live from the Investigator I. The fish were immediately eviscerated and cleaned; half of the fish were frozen and glazed for frozen storage studies while the remainder were divided into four groups and held under the following conditions: (a) in ice; (b) in ice, after previous dip in 50 ppm CTC; (c) in refrigerated-sea-water; (d) in refrigerated-sea-water + 10 ppm CTC.

The fish were sampled periodically throughout a 3-week interval and were examined for bacterial numbers, pH, total volatile base and trimethylamine nitrogen. The results of these tests are shown in Tables I and II.

Volatile base and trimethylamine determinations were carried out on alcohol extracts of the muscle samples. Viable bacterial counts, using sea-water agar at pH 6.5, and pH values were made on muscle samples which had been held overnight at 8°C.

The results in Tables I and II indicate that the spoilage of dogfish under all conditions tested did not occur to any extent during the first 18 days of storage. At 21 days, the fish could still be considered acceptable.

The pattern of spoilage found in this trial differs considerably from that found elsewhere to be typical of shark spoilage, but the conditions of storage in these experiments are probably better than those used heretofore by other investigators. Typically, in advanced spoilage stages the pH values and total volatile bases are said to rise, while the trimethylamine content should remain fairly low. Viable bacterial counts should reach a peak and then subside. Table I shows that the pH and volatile bases remained comparatively low while the bacterial counts were just beginning to rise sharply at 21 days. This seems to indicate that the experiment was not carried on for a long enough period of time. The whole experiment is therefore being repeated, with allowance being made for a 28-day holding period.

TABLE I - Bacterial counts and pH values of dogfish muscle held in ice and refrigerated-sea-water.

	Ice		Ice (CTC dip)		Sea-water		Sea-water + 10 ppm CTC	
Days Storage	pH	Bact count/gm musclex10^6	pH	Bact count/gm musclex10^6	pH	Bact count/gm musclex10^6	pH	Bact count/gm musclex10^6
2	6.4	0.006	6.7	0.002	6.2	0.002	6.2	0.0007
6	6.3	0.013	6.5	-	6.2	0.247	6.4	0.042
7	6.3	0.480	6.2	0.004	6.6	1.180	6.1	0.038
8	6.2	1.130	6.1	0.002	6.3	2.700	6.4	0.186
9	6.3	0.910	6.2	0.013	6.3	1.430	6.6	0.127
10	6.2	3.500	7.0	0.008	6.2	1.480	6.1	0.650
11	6.4	3.800	6.1	0.048	6.3	0.750	6.1	0.620
12	6.6	15.400	6.4	0.453	6.3	2.500	6.1	3.450
13	6.4	8.400	6.3	8.750	6.3	8.500	6.1	5.750
16	-	-	6.8	15.100	6.5	6.550	6.5	6.200
18	6.8	74.000	6.7	35.000	6.7	4.100	6.6	4.800
21	7.4	151.500	6.6	64.500	6.8	19.000	6.7	30.500

TABLE II - Total volatile base and trimethylamine nitrogen (mg/100 gm) in dogfish muscle held in ice and refrigerated-sea-water.

Days storage	Ice		Ice (CTC dip)		Sea-water		Sea-water + 10 ppm CTC	
	TVB	TMA	TVB	TMA	TVB	TMA	TVB	TMA
2	15	5.0	15.0	5.0	7.5	-	13.0	-
6	10	-	10.0	-	5.0	-	6.0	-
7	8.5	2.5	9.5	3.5	7.5	2.5	5.5	3.5
8	7.5	-	14.5	2.5	6.0	-	7.5	-
9	12.5	2.5	8.5	2.5	9.5	3.5	6.0	-
10	8.5	2.5	5.0	-	7.5	-	8.5	2.5
11	9.5	3.5	9.5	3.5	11.0	5.0	7.5	-
12	13.5	3.5	13.5	3.5	12.5	5.0	13.5	3.5
13	12.5	5.0	9.5	3.5	11.0	6.0	12.5	5.0
16	-	-	13.5	6.0	24.5	17.5	13.5	3.5
18	17.0	-	10.0	-	18.0	5.0	12.5	2.5
21	18.5	-	13.0	-	18.0	13.0	20.0	10.0

SUMMARY NO. 11

ANTIFUNGAL ANTIBIOTICS

B.A. Southcott
E.G. Baker

In a continuation of work described in Summary No. 29 of this Station's Annual Report for 1957-58, several antifungal antibiotics have been tested for their ability to control mold growth on salted, smoked fish.

Sorbic acid is presently allowed in certain food products at a level of 0.1%; since about 10% of the dip concentration is taken up by fish flesh, 1% and 0.5% dips of potassium sorbate were used. It is anticipated that antifungals may eventually be used in foods as have certain antibacterial antibiotics, at a level of 5 ppm. For this reason, dips of 50 ppm (.005%) and 100 ppm (.01%) were used. In order to eliminate bacterial contamination during storage of the fish, 10 ppm of CTC was included in all dip solutions.

Pieces of fresh lingcod 3"x1"x1" were soaked 1 hour in 20% NaCl solutions to which the antifungal antibiotics, dissolved or suspended in ethyl alcohol or formamide, were added. Five samples were used in each solution. The samples were drained 1 hour on wire racks, then smoked 6 hours and cooled in the smoking tunnel overnight. Each piece of fish was stored in a separate plastic bag at 4°C.

The amount of solvent required to dissolve or suspend the antifungal antibiotics was up to 2% of the total volume of brine prepared; in making up solvent control solutions, a 2% level of solvent was used.

The results of these tests, unlike those previously reported, show that formamide alone possessed marked antifungal activity. This effect has recently been found by other investigators. Inconsistencies such as this are bound to occur in work of this kind since in each experiment different fungi or yeasts may be concerned due to variations in natural contamination.

	Test solution	Solvent	No of samples out of 5 showing growth Days stored								
			12	21	29	32	40	46	53	57	64
1	Brine control		0	0	5						
2	" + CTC control		0	0	5						
3	" + formamide control	formamide	0	0	0	0	0	0	0	0	0
4	" + ethyl alcohol "	ETOH	0	0	5						
5	" + CTC + formamide control	formamide	0	0	0	0	1	2	3	3	
6	" + " + ethyl alcohol control	ETOH	0	0	4	4	5				
7	Potassium sorbate 1.0%		0	0	3	3	4	4			
8	" " 0.5%		0	0	5						
9	Phytostreptin (Pabst) 100 ppm (.01%)	ETOH	0	0	5						
10	Phytostreptin (Pabst) 50 ppm	"	0	0	5						
11	Phytoactin (Pabst) 100 ppm	"	0	0	3	5					
12	Phytoactin " 50 ppm	"	0	0	0	2	5				
13	Filipin (Upjohn) 100 ppm	"	0	0	1	4	4	5			
14	Filipin " 50 ppm	"	0	0	2	5					
15	Ayfactin (Bristol) 100 ppm	formamide	0	0	0	0	1	1	2	2	
16	Ayfactin " 50 ppm	"	0	0	0	4	4	4	4		
17	Amphotericin A (Squibb) 100 ppm	"	0	0	0	2	3	3	3	4	
18	Amphotericin 50 ppm	"	0	0	0	2	4	4			
19	A5283 (Lederle) 100 ppm	"	0	0	0	0	0	0	0	0	1
20	" 50 "	"	0	0	0	0	0	0	3	3	3

SUMMARY NO. 12

BACTERIOLOGICAL STUDIES OF FISH STORED IN REFRIGERATED BRINES

B.A. Southcott
E.G. Baker

In order to facilitate the study of the bacteriological aspects of fish preservation in refrigerated-sea-water, two tanks were constructed at the laboratory in which it was possible to hold fish under conditions more nearly approximating those in commercial installations than was possible with the methods described in Summary No. 34 of this Station's Annual Report for 1957-58. The temperature of the circulating refrigerated brine was maintained at 30-32°F. Both artificial and natural brines were used at different times.

In each experiment, the samples of fish to be examined were incubated at 10°C overnight before bacterial counts were made. Total bacterial numbers were determined in the usual manner; viable counts were made using sea-water agar prepared with modified artificial sea-water. When anaerobic plates were required, .08% sodium

thioglycollate was added to the agar just prior to pouring and the plates were incubated in an atmosphere of 95% N_2 + 5% CO_2.

In one experiment, lingcod of average weight 8 lb 4 oz were stored in complete artificial sea-water with and without 10 ppm CTC. Each fish was weighed before being placed in the tank and again when removed for sampling. The results (Table I) show that during the first week's storage the gain in weight did not exceed 0.5%, and that the average gain throughout the 20-day experiment was about 3.5%.

TABLE I - Weight increase of lingcod held in refrigerated-sea-water.

	Weight gain %
All fish, all storage times	3.5
CTC-treated fish, all storage times	3.7
Non-CTC-treated fish, all storage times	3.2
Fish examined after (7 days	0.5
(11 "	1.4
(14 "	3.8
(18 "	4.1
(20 "	4.0

Table II records the results of organoleptic tests and bacterial counts of fish and sea-water, and the amounts of CTC in the sea-water, skin, and flesh of the fish. The results show that for 11 days all fish remained "fresh" organoleptically, and that after this time fillets cut from untreated fish were slightly putrid or putrid. On the other hand, those cut from the fish held 15 days in refrigerated-sea-water containing CTC were fresh, and were only slightly stale even after the fish had been stored 20 days. The total bacterial counts were very much higher than the viable counts, and, with the single exception of the as yet unexplained results obtained after the 11-day storage period, were lower with the CTC-treated fish. Also, with one exception, the aerobic viable counts were lower with the CTC-treated fish. The very low aerobic and anaerobic viable counts which persist throughout the first half of the experiment indicate that a very large proportion of the bacteria in refrigerated-sea-water are dead, or at least will not readily grow aerobically or anaerobically under laboratory conditions. After 7 days no important difference was found between viable bacterial counts in the treated and untreated sea waters.

The CTC content of the sea-water decreased slowly. The concentrations were usually higher in the skin than in the flesh, but the flesh values were in general considerably higher than those found previously with halibut stored in refrigerated-sea-water containing between 3 and 5 ppm.

In a similar second experiment, lingcod were held in tanks of natural brine (from the Aquarium) with and without 10 ppm CTC. Tests on the brine using Brilliant Green Bile Broth and Endo Agar indicated the presence of coliform organisms. After the brine had been in the tanks for 7 days, no coliforms could be detected in the tank containing CTC, whereas in the untreated sea-water typical coliform reactions were obtained. Table III shows the results of bacterial counts in this experiment.

TABLE II - Comparative rates of spoilage of eviscerated lingcod stored in refrigerated-sea-water with and without added CTC.

Days stored	CTC	Condition of fillets When cut	Condition of fillets After 18 hr at 10°C	Bacteria millions/gm fish Total	Bacteria millions/gm fish Viable aerobic	Bacteria millions/gm fish Viable anaerobic	Bacteria, millions/ml brine Viable aerobic	Bacteria, millions/ml brine Viable anaerobic	CTC per million (parts) in Skin	CTC per million (parts) in Muscle	CTC per million (parts) in Brine
0	...	fresh	fresh	4.77	.012	...	...	...	0	0	8.6
7	-	"	"	76.43	0.19	0.011	16.3	3.07	...	...	...
	+	"	"	6.63	.002	...	0.22	0.065	0.15	0 to trace	5.9
11	-	"	"	13.25	0.27	...	23.4	...	...	...	...
	+	"	"	71.18	.065	...	19.2	...	1.85	2.25	5.4
14	-	sl putrid	sl. putrid	43.00	0.29	...	54.5	...	...	...	...
	+	fresh	fresh	5.30	0.46	...	91.0	...	0.79	0.04	4.2
18	-	sl putrid	sl putrid	24.38	11.3	2.12	105.0	18.5	...	...	...
	+	sl stale	stale	14.05	2.1	0.26	157.5	205.0	0.95	1.12	3.2
20	-	sl putrid	putrid	23.53	15.5	...	...	...	...	...	...
	+	fresh	sl stale	3.07	0.83	...	...	...	1.12	0.28	4.4

TABLE III - Bacterial counts of lingcod in refrigerated natural brine.

Days stored	CTC	Condition of fillets when cut	Fillets after 18 hrs at 10°C	Bacteria x 10^6 per gm fish Viable Total	10°C aerobic	10°C anaer	25°C aerobic	25°C anaer	Bacteria x 10^6 per ml brine Viable 10°C aerobic	10°C anaer	25°C aerobic	25°C anaer
0		fresh	fresh	0.27	.01		0.13				0.26	
2	-	fresh,firm	fresh	1.06	.08		0.10		0.60		0.62	
	+	fresh,firm	fresh	0.53	.001		<.01		0.30		0.40	
6	-	strong fish odour	sour,fairly firm	20.7								
	+	fresh	fresh fairly firm	3.7								
8	-	stale	putrid	27.6			1.1	1.08			9.2	2.5
	+	fresh	stale	6.4			.07	.004			2.2	0.1
10	-	very stale	putrid	38.2								
	+	fresh	fresh	6.4								
13	-	putrid	putrid	44.0								
	+	fairly fresh	stale,soft	8.5								

In connection with experiments planned in connection with occurrence and development of CTC-resistant bacteria, it was thought desirable to plate fish samples from the next experiment on agar with and without 5 ppm CTC; tests were necessary to determine the stability of CTC added to seawater agar and incubated up to 3 days at 25°C.

The inclusion of gum arabic was tried as a means of protecting the CTC. Gum and sea-water agar were sterilized together and the CTC added to the cooled agar just prior to pouring. Plates were incubated at 25°C; at 1 and 3 days, plugs of agar were cut from the plates with sterile cork borers and placed on the usual assay plates. The size of the agar plugs was the same as that of the assay filter discs. The results below (Table IV) show that under these conditions, gum arabic did not protect CTC from gradual destruction while plates were incubated at 25°C. It was decided to use sea-water agar at pH 6.5 since this kept the level highest up to 3 days. It appeared necessary to add 10 ppm to the agar if about 5 ppm was the desired level.

TABLE IV - Recovery of CTC added to sea-water agar plates.

			Recovery					Recovery	
pH	% gum	ppm CTC	1 day	3 days	pH	% gum	ppm CTC	1 day	3 days
6.5	0	5.0	2.8	2.7	7.5	0	5.0	2.3	1.7
"	.01	0.5	0.29	0.3	"	.01	0.5	.08	.08
"	.01	1.0	0.64	0.5	"	.01	I.0	0.32	0.19
"	.01	5.0	3.3	1.9	"	.01	5.0	1.5	1.7
"	0.1	0.5	0.27	0.21	"	0.1	0.5	0.2	0.1
"	0.1	1.0	0.39	0.36	"	0.1	1.0	0.32	0.21
"	0.1	5.0	1.8	1.5	"	0.1	5.0	1.2	1.5
"	1.0	0.5	0.28	0.18	"	1.0	0.5	0.1	0.1
"	1.0	1.0	0.62	0.29	"	1.0	1.0	0.19	0.26
"	1.0	5.0	3.7	1.7	"	1.0	5.0	0.94	1.6
"	1.0	0	0	0	"	1.0	0	0	0
"	0	0	0	0	"	0	0	0	0

A series of sea-water agar plates at pH 6.5 was prepared; 10 ppm CTC was added just before pouring. It was hoped that this would provide a level of 5 ppm in the agar since it was at this level, i.e. the level permitted in fish, that the existence of resistant forms was to be studied. To the usual standard curve of the CTC assay were added levels of 1, 5 and 10 γ per ml. Plugs of agar were assayed with the following results:

agar immediately after pouring = 4.2 ppm
agar after 2 days at 25°C = 2.8 "
agar after 6 days at 25°C = 0.52 "

In each case plugs of sea-water agar with no added CTC gave no zone of inhibition of the assay organism.

Lingcod were stored in tanks of complete artificial sea-water with and without 10 ppm CTC in the brine. The viable counts were carried out using sea-water agar at pH 6.5 with and without 10 ppm added CTC. When the fish had been stored 11 days, the tanks were drained, the remaining fish discarded and a fresh lot of artificial sea-water and fish introducted. The tanks were not cleaned or rinsed between the two lots of fish. The results of this experiment are summarized in Table V.

TABLE V - Bacterial counts of lingcod on agar ± CTC.

Days stored	CTC	Condition of fillets as cut	Condition of fillets after 18 hrs at 10°C	Bacteria x 10^6 per gram fish Total	Viable reg agar	Viable agar+ CTC	Bacteria x 10^6 per ml bring Viable reg agar	Viable agar+ CTC
0		rec'd fresh	fresh, firm		.012	.002		
3	+	fresh, firm	fresh, firm		low	low	.03	.03
	-	fresh, firm	fresh, firm	7.5	low	low	0.36	0.22
6	+	fresh, firm	fresh				0.15	0.15
	-	fresh, firm	fresh				35.65	28.08
11	+	fresh, firm	fresh,sl soft	19.2	0.12	0.10		
	-	"fishy"	stale, soft	50.8	0.51	0.14		
Tanks drained but not cleaned - fresh sea-water and fish placed in tanks.								
0		rec'd fresh	fresh, firm		.003	.0002		
1	+	fresh					.06	.05
	-	fresh					12.5	10.5
2	+	fresh	fresh, firm		.006	.003	0.13	0.11
	-	"Fresh fish"	"fresh fish"		0.14	.07	20.1	11.8
7	+	fresh, firm	fresh, firm	27.9	0.40	0.28	1.4	1.6
	-	stale	very stale	115.3	0.14	0.15	24.8	22.7
9	+	fresh, firm	fresh, firm		0.21	0.13		
	-	putrid	soft, putrid		0.16	.07		

In order to determine the numbers of organisms initially present in the slime of fresh fish which would be resistant to high levels of CTC, samples of slime scraped from the skin and gut of a fresh grey cod were plated on sea-water agar containing 100 ppm CTC. Duplicate sets of plates were incubated at 25°C for 3 days and at 4°C for 10 days. The colonies which developed on the CTC plates were minute and could not be counted accurately; approximate results are listed in Table VI.

TABLE VI - Bacterial counts of skin and gut scrapings of grey cod.

	Bacteria x 10^6 per gram Skin		Gut	
Temp	agar +CTC	reg agar	agar +CTC	reg agar
25°C	0.25	117	.08	18.5
4°C	<.002	44	<.001	9.5

SUMMARY NO. 13

BACTERIOLOGICAL ANALYSES OF FROZEN SHRIMP MEAT — B.A. Southcott

At the request of a fishing company, total bacterial counts were made on a series of ten shrimp meat samples. Five of the samples were received in the frozen state and five were received after having been thawed at 30-40°F for 41 hours.

The samples were brought to temperature equilibrium and incubated at 38°F; all had stale, sour or "cold storage" odours. Direct total counts were made by the usual method after 18 hours and 40 hours.

Sample		Bacteria per gram x 10^6 Incubated 18 hrs at 38°F	Incubated 40 hrs at 38°F
Samples rec'd thawed			
sample	1	80	349
	2	223	530
	3	530	530
	4	530	530
	5	230	530
Samples rec'd frozen			
	6	45	58
	7	81	270
	8	72	530
	9	56	435
	10	12	31

Subsequently, two lots of shrimp meat were received from the same company. One sample had been processed the previous day, then frozen; one sample was received fresh on the day of processing. The samples were brought to temperature equilibrium, then incubated overnight at 10°C. Total bacterial counts were made in the usual manner.

	Bacteria x 10^6 per gm
fresh	29.7
frozen	5.3

A sample of frozen shrimp meat with an extremely strong odour was received from a different source. The shrimp meat was thawed overnight at 10°C, then total and viable bacterial counts were done.

Total - 237 x 10^6 per gram
Viable - 0.91 x 10^6 per gram.

SUMMARY NO. 14

THE STABILITY OF ANTIOXIDANT-TREATED HERRING MEALS IN STORAGE A THIOBARBITURIC ACID TESTS FOR OIL OXIDATION — N.A.W. LePage, F.F. Claggett

The results of 2-thiobarbituric acid (TBA) tests made on seven herring meals (Summary No. 20 of this Station's Annual Report for 1957-58) are presented graphically in Fig 1.

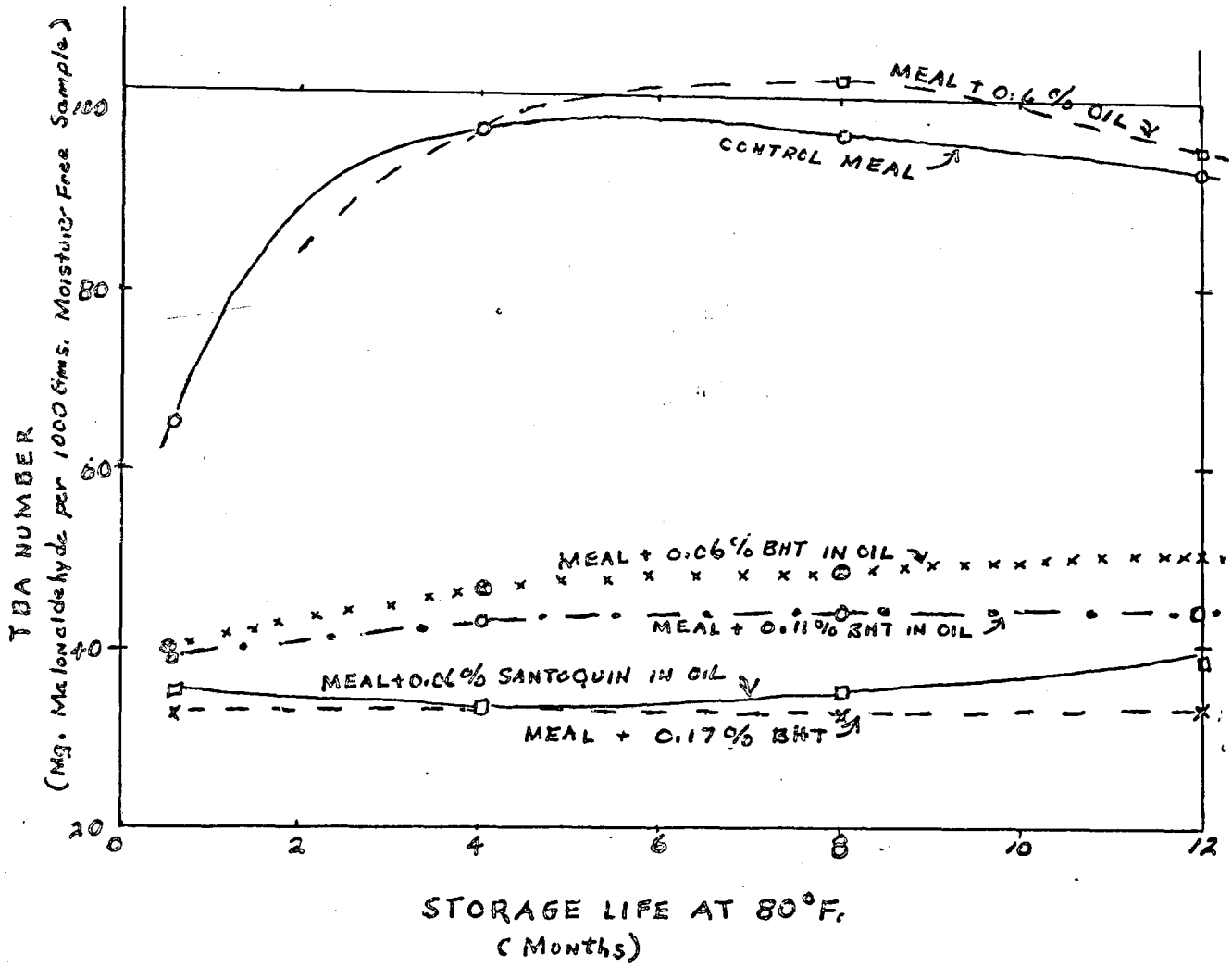


Figure 1.

All of the treated meals showed little increase in TBA number after the first three weeks. The untreated meals, however, showed increasing TBA numbers up to and after four months. Meals treated with 0.17% butylated-hydroxytoluene (BHT) and 0.06% Santoquin showed the best protection by this test. The smaller dosages of BHT gave poorer, but still adequate protection.

The treated meals all appeared to retain a darker colour close to that of the fresh meal. The controls were turned to a light orange colour by the end of 12 months and had developed the definite "acrid" smell characteristic of stored meals. The treated meals were much fresher smelling, although the first traces of acrid odour were becoming apparent at the end of 12 months' storage.

B CHICK NUTRITION TESTS

J. Biely, Mrs. B.E. March
(Dept. Poultry Science, U.B.C.)
N.A.W. LePage
F.G. Claggett

Concurrently with the TBA tests performed in Part A, chick nutrition tests were performed using the seven herring meals. The composition of these meals is set out in Table I.

TABLE I - Composition of herring meals.

Description	Moisture	Fat	Protein	Ash
Meal #1, control, no addition	5.9	8.6	78.2	11.3
#2, " 0.6% herring oil	5.5	9.9	72.7	11.0
#1, 0.17% BHT (dry)	5.9	9.1	74.0	11.7
#2, 0.17% BHT (dry)	4.7	9.1	74.4	11.5
#3, 0.11% BHT in 0.6% herring oil	5.2	9.5	74.4	11.5
#4, 0.06% BHT in 0.6% herring oil	5.8	10.7	72.7	10.9
#5, 0.06% Santoquin	5.7	10.3	73.5	11.3

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

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

Herring meal supplement	Average weights at 4 weeks (grams) Storage interval		
	Start*	4 months	8 months
Control #1	326	264	284
" #2	321	267	273
Treated #1	332	240	280
" #2	342	244	275
" #3	328	252	283
" #4	324	256	278
" #5	321	255	297

* 32 days feeding.

The chick nutrition test results for 12 months' storage were not available at the time of writing this report.

It may be seen from Tables II and III that the results of this test bear out the results obtained in the 1957-58 series, namely, that the incorporation of antioxidants into herring meal has no significant effect on the protein quality or folic acid content.

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

Herring meal supplement	Average weight at 4 weeks (grams) Storage interval		
	Start*	4 months	8 months
Control #1	320	232	196
" #2	317	232	186
Treated #1	301	240	205
" #2	316	230	174
" #3	327	252	188
" #4	303	244	184
" #5	332	235	195

* 32 days feeding.

C FAT STABILITY STUDIES

F.G. Claggett
J. Biely, Mrs. B.E. March
(Dept. Poultry Science, U.B.C.)

Since it has been shown fairly conclusively that the incorporation of antioxidants into herring meal has little or no effect on the protein quality or folic acid content, an experiment has been started to see if the digestibility of the fat present in herring meals is affected by this addition.

In early December of 1958, six bags of herring meal were treated for this series. Two bags were taken fresh, quick frozen, and the fat extracted immediately by a chloroform-methanol mixture using the facilities of a local company which produces D vitamins. Of the rest, two bags were treated at a level of 0.15% BHT and stored at room temperature along with the remaining two. These meals will be extracted again at the end of six months and the fat used to compare the ability of chicks to utilize these fats.

At the same time another set of meals was taken and treated as follows:

1. Normal meal	100 lb stored in a freezer. 100 lb stored at room temp.
2. Normal meal treated with BHT at a level of 0.15% one week after production	100 lb stored at room temp.
3. Herring meal treated with BHT at a level of 0.15% at time of production	100 lb stored at room temp.
4. Herring meal quick-frozen after production	100 lb stored in freezer 100 lb stored at room temp.

Biological tests are being made using the herring meal (a) as a protein supplement to a diet which contains adequate levels of the vitamin B complex, and (b) as protein supplement to a diet which is deficient in the B complex vitamins.

The completion of this series should give an overall picture of the value of the addition of antioxidants to herring meals.

The writer gratefully acknowledges the assistance of Mr. David Barbour of the British Columbia Packers, Ltd., who arranged to make available the facilities of one of their herring reduction plants.

SUMMARY NO. 15

DISTRIBUTION OF CTC IN ICE MADE FROM HARD WATER

R. Moyer
B.A. Southcott

The investigation mentioned in Summary No. 37 of this Station's Annual Report for 1957-58 has continued. Previous work (Summary No. 29 of this Station's Annual Report for 1955-56 and Summary No. 6 of this Station's Annual Report for 1956-57) had shown that chlortetracycline (CTC) could be effectively dispersed throughout ice blocks with the aid of the hydrophilic colloids, carrageen (Irish moss extractive) and carboxymethylcellulose (CMC). Recent tests with purified sodium and potassium alginates have indicated that these are almost as effective for this purpose. Though these colloids provide good dispersion in relatively "soft" waters, they are not nearly so satisfactory when applied to "hard" waters. Reasonably good dispersion may be obtained with all colloids tested in ices from hard water if the liquid is kept nearly neutral (pH 6.0-8.0), but under these conditions a gelatinous precipitate and foamy scum are found on melting the ice. The precipitate and scum are probably double salts of calcium or magnesium with the hydrocolloid and CTC. As indicated in Table I, as much as half of the antibiotic may be carried down in the form of a relatively insoluble precipitate and lost from the point of view of fish preservation, although the precipitate still contains antibiotic activity.

It is apparent from Table I that there is less inactivation of CTC if the dispersant is CMC rather than carrageen. Experiments have indicated that CMC in the same concentration is a slightly poorer dispersant than carrageen but still provides satisfactory distribution. It was also noted that a preparation of hard water containing carrageen and CTC retained most of its activity and formed very little precipitate if left unfrozen at 0°C for one week. As shown in Table I, a solution of antibiotic alone in hard water (no hydrocolloid added) retained most of its activity on freezing. Since the physical action of freezing in the presence of hydrocolloid seemed to be responsible for the formation of the precipitate, preparations of hard and soft waters with hydrocolloid and CTC were lyophilized and the residues reconstituted with distilled water. The residue from the soft water preparation was slowly soluble while that from the hard water was largely insoluble. This leads to the suggestion that the precipitate is the result of a change in the state of hydration of the hydrocolloid during freezing. In the case of the hard water, this change (if such occurs) appears to be irreversible accounting for the insolubility of the double salt after melting.

Ices prepared from faintly acid (pH 3.0-5.0) solutions containing a hydrocolloid plus CTC retained a greater percentage of their activity than did those nearer the neutral point (pH 6.0-8.0) but the distribution of CTC was extremely poor. A number of attempts to devise methods for good distribution under acid conditions have failed, as have attempts to remove the calcium and magnesium from hard waters by the addition of sequestering agents. Conditioning of the hard water by passing through a cation exchange resin (Amberlite IR-120) in the sodium form provides a water from which good CTC distribution can be obtained and in which stability is fairly good, as shown in Table I.

TABLE I - Loss of antibiotic activity in ice blocks prepared from "hard" and "soft" waters.

Composition of solution prior to freezing (plus 10 ppm CTC)	Concentration of CTC in filtrate of melted ice
Hard water + 0.01% carrageen	5.3
Hard water + 0.01% CMC	7.7
"conditioned" water + 0.01% carrageen	7.8
Hard water	8.3
Demineralized (soft) water + 0.01% carrageen	8.7
Demineralized (soft) water + 0.01% CMC	9.3
Demineralized (soft) water	9.8

The artificially prepared hard waters contained 66.9 parts per million (PPM) of calcium and 31.4 ppm magnesium, both added as the carbonate. The pH of the solution was 6.2. Conditioning was by passing through a column of Amberlite IR-120 in the sodium form after which the pH was 7.8. Hercules CMC No 70 LL was used in this experiment but higher viscosity types have been used with similar results. The carrageen was "Seakem Type No. 2".

SUMMARY NO. 16

COMMERCIAL APPLICATION OF SODIUM ISOASCORBATE — R.H. Moyer

At the request of a local company, an experiment was initiated to test the feasibility of using sodium isoascorbate for protection of frozen sole fillets under commercial conditions. Preliminary measurements of dip concentrations during operation and isoascorbate residues after freezing indicated that a concentration of 0.90% to 1.0% was necessary in the dipping solution to obtain a residual of approximately 0.05% on the frozen fillets. Under these conditions, the rate of depletion of isoascorbate was approximately 1 lb per 1000 lb fillets. Since only 0.5 lb per 1000 lb could be accounted for as residue, the remainder was either lost or had already fulfilled its function as antioxidant.

Under current operating and marketing conditions it is said that treatment at these concentrations would not be economically feasible. Good protection at half the cost would apparently be necessary to warrant the commercial use of the antioxidant application.

In order to determine the degree of protection of both high and economical levels of isoascorbate, a long-term storage experiment was arranged. Skinless sole fillets from a commercial operation were dipped in 25° brine, 0.8% and 1.0% isoascorbate. These, along with controls, were taken off the line after packaging, appropriately labelled, and returned to the line to be frozen and stored as in commercial practice.

Sampling in duplicate is at two-month intervals. One set is evaluated immediately by taste panel and thiobarbituric acid determination while the other is placed at 16°F for two weeks before similar evaluation. The purpose of the additional two weeks storage at an elevated temperature is to simulate the poorest possible handling conditions between storage and the consumer. After two months there was no significant differences between treatments or between treated and control samples. Tests are continuing.

SUMMARY NO. 17

VACUUM AND CONTROLLED ATMOSPHERE PACKAGING — R. Moyer

The investigations described in Summary No. 40 of this Station's Annual Report for 1956-57 have been continued. Troll-caught red spring salmon from one general location were minced and vacuum packaged in pouches made from polyethylene laminates with mylar, saran-coated mylar, cellophane, and light extrusions of saran on cellophane using the "Flex-vac" model 6-8 2 R.G. In addition, the polyethylene laminates of mylar, cellophane, and extrusions of saran on cellophane were used for inert atmosphere (nitrogen) packaging. Saran-sprayed polyethylene was tried but a satisfactory seal could not be obtained using the "Flex-vac" machine and this material. The packaged samples were frozen and stored at 0°F.

Quality deterioration is assessed at two-month intervals using the tasting technique outlined in Summary No. 38 of this Station's Annual Report for 1956-57 as well as by colour measurements, peroxide value and thiobarbituric acid determinations.

Results to date indicate that the close cling of a vacuum package is more protective than a loose wrap; that saran-coated mylar is an improvement over mylar alone, and that extrusions are generally preferable to laminates for low-temperature storage. The tests are still proceeding.

SUMMARY NO. 18

RIBONUCLEIC ACID (RNA) AND DESOXYRIBONUCLEIC ACID (DNA) IN MIGRATING SOCKEYE SALMON — Vera M. Creelman

This work is a continuation of that described in Summary No. 7 of this Station's Annual Report for 1957-58.

The methods described in last year's report were further refined and a procedure that enabled measurement of the separate nucleotides from the RNA of various salmon tissues was developed. The most suitable means of measuring DNA was found to be by determination of DNA phosphorus following separation of DNA from RNA. The complete procedure was as follows:

A 5-gm sample of tissue was homogenized at ca 1°C with 7.5 ml distilled water. To the homogenate 25 ml of ice-cold N $HClO_4$ was added. The mixture was centrifuged 15 min at 10,300 x G and the supernatant solution was discarded. The residue was washed twice more with 35 ml portions of N $HClO_4$, the supernatant solutions being discarded. The residue was washed in turn with 35 ml 80% ethanol and 35 ml 95% ethanol at 4°C. The washings were discarded and the residue was boiled 3 minutes in 20 ml of ethanol-ethyl ether (3/1, v/v). The ethanol-ether was discarded and the process repeated.

The residue was taken up in 15 ml of 0.3 N KOH (pH 13 to 13.5) and allowed to digest (with constant stirring) at 36°C for 18 hours.

The digest was made 1 N in $HClO_4$ by addition of 70% $HClO_4$ and allowed to stand for 35 minutes at 1°C. The mixture was centrifuged 10 min 4°C and 10,300 x G. The supernatant solution was removed and retained and the precipitate was twice washed with 10 ml portions of N $HClO_4$ (cold). The washings were combined with the first supernatant solution. The combined solutions contained the digested RNA and will be referred to as the RNA fraction.

To the precipitate, containing DNA, 5 ml of 0.5 N $HClO_4$ was added and the mixture was heated 20 min at 70°C. The supernatant solution was removed and retained and the process repeated with the residue, the second supernatant solution being combined with the first. The combined solutions will be referred to as the DNA fraction.

The RNA fraction was adjusted to pH 3.5 to 4.5 and the $KClO_4$ precipitated was separated by centrifugation. The precipitate was washed twice with ice-cold distilled water and these washings were combined with the first separated solution. The precipitate was discarded.

A flash evaporator was used to reduce the volume of the RNA fraction to about 1.5 ml. For this purpose a special flask fitted with a thick-walled capillary tubing tip, calibrated in 0.1 ml graduations, was used for the final step in the evaporation. This flask enabled both the volume of the final concentrate and of the small amount of $KClO_4$ precipitated at this stage to be measured. Both values were recorded and in the subsequent calculations corrections were made for the space occupied by the precipitate.

Suitable aliquots, in duplicate, of the solution were subjected to electrophoresis on Whatman #3 paper in ammonium formate buffer pH 3.5, 0.25 M, at 25 volts/cm and 8°C for 2 to 2½ hours. Spots containing nucleotides were located by means of an ultra-violet lamp and eluted for 1½ hours at 30°C in 3 ml of 0.5 M sodium phosphate buffer, pH 7.0. The optical density of the solutions were determined with a Beckman DU spectrophotometer at appropriate wavelengths in the U-V. Reference blanks were prepared from pieces of paper of the same size as the spot-containing area, but cut from an area adjacent to the spot free from nucleotide. The quantity of the nucleotide was calculated by means of the absorption data of Elson, Gustafson, and Chargaff (J.B.C., 209, 285, 1954). By combining the values found for the four nucleotides, the total amount of RNA phosphorus in the tissue was determined.

For determination of DNA suitable aliquots of the DNA fraction (made up to 3 ml when necessary with 0.5 N $HClO_4$) were digested with 1 ml 70% $HClO_4$. The volume was made up to 10 ml and phosphorus determinations were made on suitable aliquots by the method of King (B.J., 26, 292, 1932).

The results of the work are tabulated in Tables I to III. The salmon examined were from the Stuart Lake run of 1957 (see Summary No. 1 of this Station's Annual Report for 1957-58). The same pooled samples were used in the present work. With the exception of heart and spleen, the analyses were made on the tissues from only one of the groups of fish of each sex taken from each of the three points along the route of migration. For the heart and spleen, due to the limited quantity of these tissues available, the analyses were made on a sample (for each sex) obtained by combining 1 gm amounts from each of the four groups. Preliminary work indicated that results of determinations from different groups of fish obtained at the same station were in agreement with each other within ±6%.

The data (Table I) indicate the following changes in RNA in the various tissues:

Flesh and alimentary tract. There is a marked loss in total RNA in both sexes in each of these tissues during migration. The concentration of RNA also decreases during this time.

Heart, liver, spleen and kidney. The total RNA in each of these tissues does not appear to change greatly during migration. An interesting point here is the greater concentration and total amount of RNA in the female liver compared to that of the male.

Gonads. The quantity of RNA in the roe nearly doubles during migration, while in the milt there is a decrease in total RNA.

Head, skin, tail, bones, etc. (combined). There is an increase in RNA in these combined tissues during migration.

TABLE I - The ribonucleic acid content of migrating sockeye salmon.

Tissue	Sex	Point in migration: Lummi Island µM/g[1]	Lummi Island Total mM[2]	Lillooet µM/g	Lillooet Total mM	Forfar Creek µM/g	Forfar Creek Total mM
Flesh	M	2.05	3.01	1.51	1.97	1.50	1.95
	F	2.27	3.03	1.69	1.91	1.63	1.52
Alimentary Tract	M	10.57	0.96	7.77	0.23	5.63	0.09
	F	11.90	0.93	8.47	0.22	6.61	0.08
Heart	M	3.25	0.02	3.41	0.02	3.64	0.02
	F	3.68	0.02	3.42	0.02	3.61	0.02
Liver	M	26.36	0.83	24.42	0.62	24.31	0.91
	F	34.42	1.38	37.44	1.67	39.13	1.58
Spleen	M	12.01	0.03	10.79	0.02	8.36	0.03
	F	11.43	0.02	12.16	0.02	11.49	0.025
Kidney	M	9.57	0.25	10.90	0.25	10.12	0.28
	F	8.87	0.20	10.89	0.22	10.76	0.21
Gonads	M	14.02	0.79	11.13	0.91	5.60	0.48
	F	1.27	0.10	1.15	0.16	0.63	0.19
Head, skin, bones, tail, etc	M	2.14	1.88	2.05	1.78	2.78	2.93
	F	1.97	1.54	1.75	1.34	2.26	1.89
Total for whole fish	M		7.77		5.80		6.68
	F		7.22		5.56		5.51

[1] The concentration of ribonucleic acid in the tissue on a wet weight basis expressed as µmoles of mononucleotides or of RNA phosphorus/gm of tissue.

[2] The total RNA in each tissue expressed as mmoles of mononucleotide or of RNA phosphorus. The weight of each tissue used in the calculation was taken from Tables II and III of Summary No. 1 of this Station's Annual Report for 1957-58.

The changes in DNA indicated (Table II) are as follows:

Alimentary tract. For both sexes there is a marked loss in total DNA in this tissue. It appears that the DNA is lost more slowly than is the tissue as a whole. This leads to an apparent increase in the DNA concentration of this tissue.

Gonads. There is an increase in total DNA in both milt and roe.

Head, skin, bones, tail, etc. (combined). In these combined tissues there is a marked increase in total DNA during migration.

All other tissues. DNA changes in these tissues are relatively small and irregular.

TABLE II - The desoxyribonucleic acid content of migrating sockeye salmon.

Tissue	Sex	Point in migration: Lummi Island µM/g[1]	Lummi Island Total mM[2]	Lillooet µM/g	Lillooet Total mM	Forfar Creek µM/g	Forfar Creek Total mM
Flesh	M	0.87	1.28	0.79	1.03	0.77	1.00
	F	0.68	0.91	0.68	0.77	1.06	0.99
Alimentary Tract	M	5.97	0.54	8.56	0.26	10.43	0.17
	F	6.41	0.50	9.46	0.25	9.24	0.12
Heart	M	6.45	0.04	6.53	0.04	5.00	0.03
	F	6.58	0.03	5.53	0.03	5.33	0.02
Liver	M	13.16	0.42	15.10	0.38	9.94	0.37
	F	12.00	0.48	9.68	0.43	13.81	0.56
Spleen	M	30.35	0.09	32.58	0.06	28.43	0.10
	F	26.95	0.05	29.55	0.04	30.71	0.07
Kidney	M	19.10	0.50	21.68	0.50	19.10	0.54
	F	22.79	0.49	19.87	0.39	21.68	0.43
Gonads	M	76.13	4.30	86.45	7.09	72.90	6.20
	F	0.93	0.07	0.84	0.12	0.68	0.20
Head, skin, bones, tail, etc	M	0.93	0.82	1.36	1.18	1.43	1.51
	F	0.87	0.68	1.45	1.11	1.74	1.45
Total for whole fish	M		7.99		10.54		9.92
	F		3.21		3.14		3.84

[1,2] Expressed in a similar manner to Table I, but as DNA phosphorus.

For both males and females, when the whole fish is considered, it will be seen that there is a net loss in RNA and a net gain in DNA during migration.

In Table III are recorded the ratios of the nucleotides found in the alkaline hydrolysate of the RNA of the various tissues. With certain exceptions, the ratios for RNA's from different tissues were remarkably similar to each other. There was a tendency for the RNA of the spleen and kidney to possess a higher content of uridylic acid than did the RNA of other tissues. Similarly the RNA of roe and of the combined head, skin, bones and tail, etc., had a higher content of guanylic acid. The RNA of the milt of fish taken at Lummi Island and at Lillooet was lower in guanylic acid than was that of the fish taken at Forfar Creek. It will be noted that there was a large decrease in the total RNA of the milt between Lillooet and Forfar Creek. The RNA of the spleen, particularly of female fish, was much lower in guanylic acid at Lillooet than at the other two points in migration. No satisfactory explanation of this irregularity can be advanced at present, but it should be pointed out that the spleens of both sexes were notably lighter in weight at Lillooet than at the other two stations (Summary No. 1 of this Station's Annual Report for 1957-58).

TABLE III - The ratios of ribonucleotides in the RNA of migrating sockeye salmon.

Tissue	Sex	Point in migration: Lummi				Lillooet				Forfar Creek			
		C[1]	A	G	U	C	A	G	U	C	A	G	U
Flesh	M	1.19	1.00	1.46	0.81	1.22	1.00	1.48	0.88	1.27	1.00	1.38	0.68
	F	1.26	1.00	1.44	0.91	1.15	1.00	1.39	0.76	1.32	1.00	1.46	0.89
Alimentary tract	M	1.14	1.00	1.24	1.03	1.26	1.00	1.48	0.99	1.20	1.00	1.40	0.96
	F	1.05	1.00	1.11	0.84	1.19	1.00	1.35	0.98	1.20	1.00	1.38	0.93
Heart	M	1.20	1.00	1.38	0.95	1.20	1.00	1.25	0.92	1.18	1.00	1.47	0.93
	F	1.39	1.00	1.38	0.99	1.18	1.00	1.21	0.82	1.19	1.00	1.41	0.91
Liver	M	1.21	1.00	1.41	0.91	1.16	1.00	1.49	0.98	1.09	1.00	1.50	0.95
	F	1.18	1.00	1.42	0.93	1.25	1.00	1.39	0.96	1.22	1.00	1.47	1.01
Spleen	M	1.28	1.00	1.36	1.08	1.26	1.00	1.20	1.18	1.23	1.00	1.47	1.04
	F	1.24	1.00	1.48	1.06	1.11	1.00	0.94	0.95	1.17	1.00	1.47	1.01
Kidney	M	1.23	1.00	1.33	1.05	1.21	1.00	1.44	0.85	1.11	1.00	1.60	1.14
	F	1.21	1.00	1.00	1.03	1.24	1.00	1.26	1.03	1.21	1.00	1.46	1.22
Gonads	M	1.17	1.00	0.93	0.93	1.16	1.00	0.93	0.86	1.18	1.00	1.32	0.93
	F	1.34	1.00	1.45	0.79	1.14	1.00	1.69	0.78	1.24	1.00	1.59	0.85
Head, skin, bones, tail, etc.	M	1.25	1.00	1.56	0.83	1.22	1.00	1.67	0.68	1.31	1.00	1.66	0.92
	F	1.34	1.00	1.76	0.70	1.20	1.00	1.58	0.77	1.16	1.00	1.53	0.95

[1] Abbreviations: C Cytidylic acid; A Adenylic acid; G Guanylic acid; U Uridylic acid.

SUMMARY NO. 19

LINGCOD MUSCLE NUCLEASE AND PHOSPHODIESTERASES — N. Tomlinson

The study of enzymes concerned with the hydrolysis of ribonucleic acid and some phosphodiesters (Summary No. 24 of this Station's Annual Report for 1957-58) has been continued.

A chromatographic procedure for separation and purification of these enzymes has been developed. With the purified preparations further detailed information regarding the substrate specificity of the enzymes has been obtained. The enzyme referred to in last year's report (vide supra) as a "ribonuclease" is called a "nuclease" in the present report as a result of some of these findings.

The following abbreviations will be used:

DEAE	Diethylaminoethyl cellulose.
Tris-HCl	Tris(hydroxymethyl)aminomethane. HCl.
RNA	Ribonucleic acid.
DNA	Desoxyribonucleic acid.
2':3'-AMP	Adenosine 2':3'-cyclic phosphate.
2':3'-UMP	Uridine 2':3'-cyclic phosphate.

Materials and Methods

Where it is stated in this report that certain procedures were carried out as "previously described", the reference is to last year's annual report (vide supra).

The procedure for preparation of muscle extracts of lingcod (Ophiodon elongatus) suitable for use in chromatography was as previously described, except that purification by isoelectric precipitation at pH 4.5 was omitted, and heating at pH 7.0 for 10 minutes at 58°C was substituted for heating at pH 9.0.

Protein and light absorption measurements were made as previously described. Temperature of incubation in all enzymatic assays was 25°C.

Nuclease and phosphodiesterase assays of fractions from chromatographic columns were performed by methods similar to those previously described, but modified to provide greater sensitivity. Thus for assay of nuclease, the reaction mixture contained sodium succinate buffer pH 6.4, 0.025M, RNA (95 µg RNA-P) and 0.5 ml of the fraction assayed, in a total volume of 1 ml. Aliquots (0.4 ml) of the reaction mixture were removed immediately after the addition of the enzyme, and after a 2-hour incubation period. Each aliquot was mixed with 0.5 ml of MacFadyen's uranyl acetate-trichloracetic acid (UTCA) reagent and 0.1 ml of water in a 75 mm x 10 mm test tube. The mixture was kept at room temperature for ½ hour and was then centrifuged at low speed for 10 min. An 0.4 ml aliquot of the supernatant solution was carefully removed with a pipette and added to 0.8 ml of distilled water. The optical density of this solution at 260 mµ was determined, the reference blank being a suitable dilution of the UTCA reagent. Digestion blanks with enzyme omitted were used. Results are reported simply as net increases in O.D. at 260 mµ. Units of activity were not used in this work owing to the impracticability of making corrections to compensate for the effects that different buffer concentrations in the various fractions to be assayed had on the enzyme's activity. Phosphodiesterase activity was assayed with bis(p-nitrophenyl)phosphate as substrate. The digestion mixture contained sodium succinate buffer pH 6.0, 0.025M, substrate 0.001M, and 0.2 ml of fraction assayed, in a total volume of 0.33 ml. Incubation period was 4 hours. At the end of this time the p-nitrophenol released was determined as previously described.

Assays for activity with p-nitrophenyl phosphate, thymidine 3'-or thymidine 5'-p-nitrophenyl phosphate were carried out as for the phosphodiesterase activity with the same substrate concentrations. The time of incubation was reduced to 2 hours with certain exceptionally active fractions.

Examination of the products of digestion of RNA by the purified nuclease were made by means of two-dimensional paper chromatography and paper electrophoresis as previously described, with the exception that spots were eluted in 0.05M sodium phosphate pH 7.0, rather than in 0.01 N HCl. The same procedure for quantitative measurement of nucleotides separated by chromatography or electrophoresis was used with the benzyl esters of the adenylic acid isomers and the cyclic ribomononucleotides (vide infra). For the latter the absorption data of Brown, Magrath and Todd were used.

The ability of the nuclease to hydrolyse 2':3'-AMP and 2':3'-UMP, and the effects of these two compounds on the hydrolysis of RNA by the enzyme were investigated. The digestion mixture contained either of the cyclic mononucleotides alone (0.006M), or RNA (206 µg P/ml) with or without a cyclic mononucleotide (0.006M), together with sodium succinate buffer pH 6.4, 0.025M, Aureomycin 5 ppm, and enzyme. The Aureomycin does not inhibit either nuclease or phosphodiesterase activity at this concentration and was used to inhibit bacterial growth in extended incubations. The total volume of the digestion mixture was 80 µl, and incubation was carried out in small, tightly stoppered test tubes. Controls without enzyme were used.

Aliquots of 25 µl were removed at the beginning of the experiment and after 10 and 20 hours of incubation. When the mononucleotides alone were the substrate, the aliquots removed were subjected to electrophoresis in 0.2M sodium phosphate buffer, pH 7.4 according to the method of Davis and Allen. When RNA with or without the mononucleotide was the substrate the aliquots were subjected to two-dimensional paper chromatography using iso-propanol-water (70/30, v/v) with 0.35 ml ammonia solution (0.88 sp gr) for each litre of gas space in the tank as the first solvent system, and with iso-butyric acid 0.5N ammonium hydroxide pH 3.7, as the second solvent.

The ability of the enzyme to attack the benzyl esters of adenosine 2'-, 3'-, and 5'-monophosphates was investigated in an identical manner to that used with the cyclic mononucleotides, including examination of the results by paper electrophoresis, except that the aliquots were taken for examination at 4 hours and 24 hours.

Column chromatography of proteins was carried out with jacketed columns refrigerated at 1°C and with the tubes in the fraction collector held in a bath of refrigerant at the same temperature.

DEAE Type 20, capacity 0.85 meq/gm was obtained from the Brown Co., Berlin, N.H. 2':3'-AMP and 2':3'-UMP, as the barium salts, were obtained from Schwarz Laboratories, Inc., Mount Vernon, N.Y. These two compounds were converted to their sodium salts by treatment with Dowex 50 (x 8) in the sodium form before use. Adenosine 2'-, 3'-, and 5'-benzyl phosphates were the gift of Dr. G.M. Tener; thymidine 3'- and 5'-p-nitrophenyl phosphates were the gift of Dr. W. Razzell and bis(p-nitrophenyl)phosphate was the gift of Dr. J.G. Moffatt, all of whom are with the B.C. Research Council Laboratory at the University of B.C.

Results

The results of the chromatography of lingcod muscle extracts prepared as described above are shown in Figure 1 and 2.

The DEAE used in the chromatogram of Fig. 1 was equilibrated with tris-HCl 0.02M pH 6.9 before the protein was added. Gradient elution was used to develop the chromatogram, the gradient being obtained by means of a single reservoir and 125 ml mixing flask. The mixing flask was filled initially with tris-HCl 0.02M pH 6.9 and the reservoir contained tris-HCl 0.5M pH 5.8. With this procedure, although three or four fractions containing nuclease were obtained free from phosphodiesterase activity - at least within the limits of sensitivity of the phosphodiesterase assay - separation of the nuclease from the phosphodiesterases was not good. The use of tris-HCl 0.25M pH 5.8 or pH 7.0 in the reservoir did not provide improved separation. In addition, it was observed that the buffered DEAE had a surprisingly low capacity for the protein contained in these preparations, for when 95 mg of protein rather than 61 mg as in Figure 1 was applied to a column of the same dimensions a much larger proportion of the total protein and a large part of the nuclease was not retained by the DEAE, indicating overloading. The capacity of DEAE in the free-base form for the protein was examined and was found to be very much greater than that of the buffered form. In view of the known moderate stability of the nuclease and phosphodiesterases under alkaline conditions, chromatography, using DEAE in the free-base form and stepwise elution with increasing concentrations of tris-HCl pH 7.0, was investigated. Figure 2 presents the results. It is obvious that much better separation of the nuclease from the phosphodiesterases and greater purification of the nuclease with respect to protein were obtained. The three chromatograms show the influence of different degrees of loading of the DEAE with protein. In Figure 2A the DEAE was very lightly loaded, in 2B it was slightly overloaded, and in 2C it was quite heavily overloaded. Moderate overloading can be used to advantage with the nuclease in order to obtain greater concentration of this activity in the effluent.

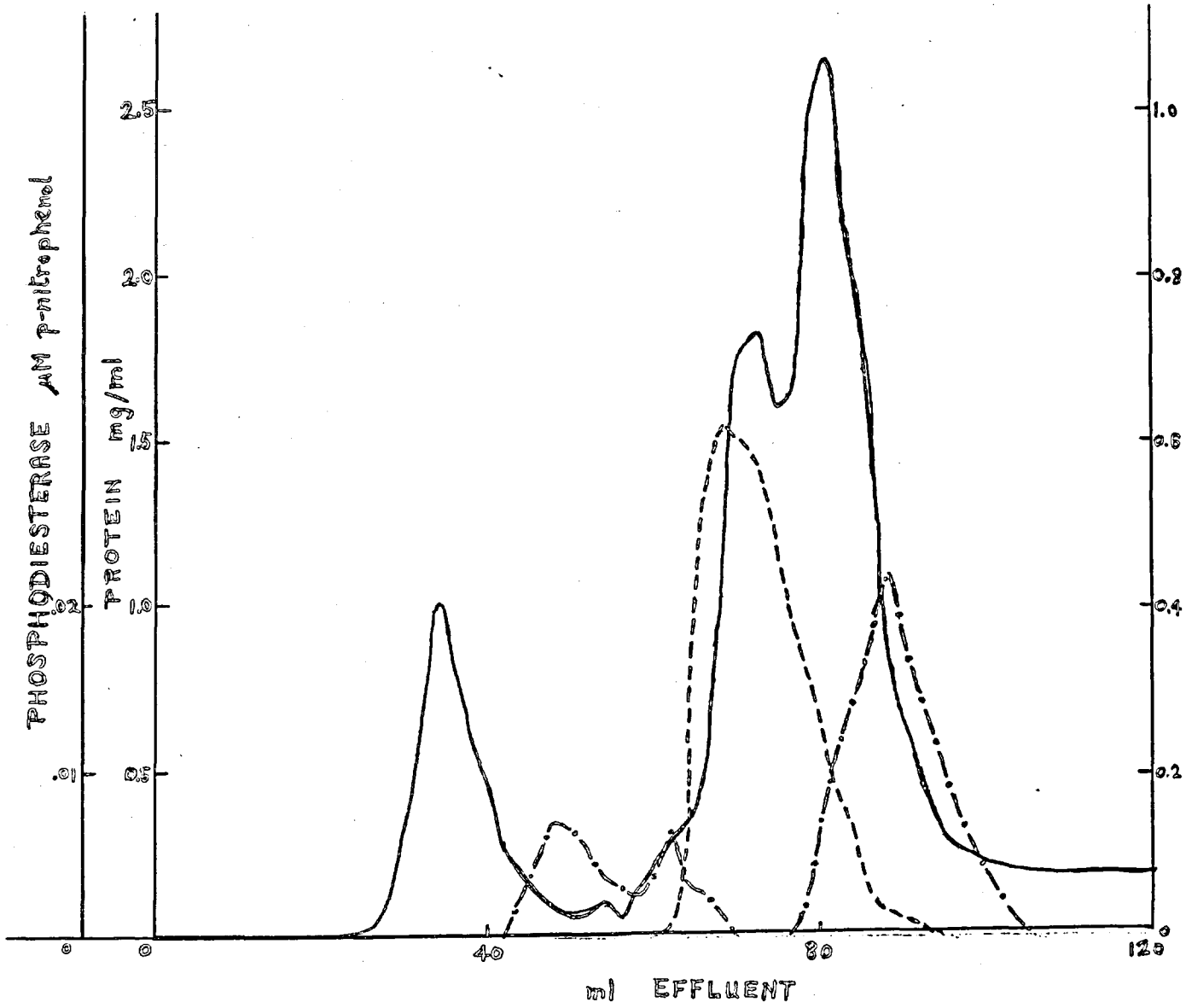


Figure 1. Effluent diagram of protein preparation from lingcod muscle. 61 mg protein applied on 12 g DEAE-cellulose previously equilibrated with tris-HCl 0.02M pH 6.9. Column 29 cm long x 18 mm diameter. Gradient elution with tris-HCl (see text). Flow rate, 0.25 ml/min. Fractions, 2 ml. First fraction collected after addition of protein. Hold-up volume about 38 ml.
Protein ——. Nuclease -----. Phosphodiesterase —. —. —.

The nuclease activity accompanying the phosphodiesterase activity eluted with M tris-HCl, and the small peaks of nuclease activity preceding the main nuclease peak in the overloaded column are regarded as artif actitious. It is possible for this condition to arise when stepwise elution is used. It is certain the nuclease and phosphodiesterase eluted together are not identical, for a portion of each was obtained free from the other by chromatography on buffered DEAE (Figure 1), and a preliminary experiment has indicated the two can be separated on the free-base form by suitable intermediate steps in tris-HCl concentration. It will be noted also that the quantity of nuclease activity eluted with the phosphodiesterase does not increase with increased protein added to the column as does the phosphodiesterase, presumably due to the displacement of the nuclease by more firmly bound material.

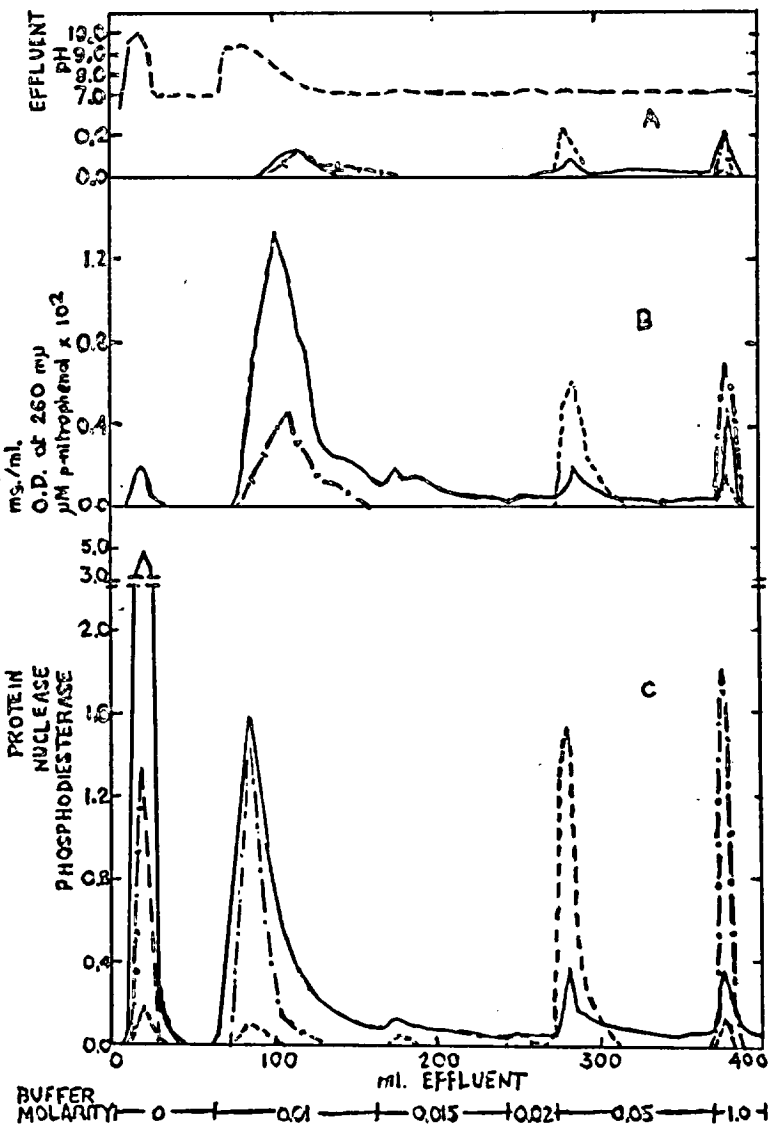


Figure 2. Effluent diagrams of protein preparation from lingcod muscle. Each column contained 0.7 g DEAE in the free-base form. Protein applied: A, 14 mg; B, 75 mg; C, 120 mg. Column 2.5 cm long x 18 mm diameter. Stepwise elution, following distilled water wash, with tris-HCl pH 7.0 at concentrations indicated. Flow rate, 1 ml/min. First fraction collected during addition of protein. Hold-up volume ca 6 ml. Fractions, 5 ml. Effluent pH for A _ _ _ . Protein ———. Nuclease ------. Phosphodiesterase —·—·—.

The effluent pH for the chromatogram of Figure 2A is shown. For the chromatograms of 2B and 2C the changes in effluent pH are not shown, but were similar to those of 2A, reaching slightly higher values during addition of the protein and slightly lower values during the first passage of tris-HCl through the column, with the peak value being displaced slightly to the left. The alkaline fractions were neutralized with cold N HCl immediately after they were collected.

The ability of the nuclease, following purification by either chromatographic procedure, to hydrolyse RNA, DNA, several simple esters of ribo- and desoxyribomononucleotides, and bis(p-nitrophenyl)phosphate has been examined.

The nuclease hydrolyses RNA with the release of nucleoside 3'-phosphates as previously reported, and hydrolyses adenosine 3'-benzyl phosphate with the release of adenosine 3'-phosphate. However, using the assay conditions described above, with sufficient enzyme to provide over 90% hydrolysis of adenosine 3'-benzyl phosphate and hydrolysis of RNA to the extent that 70% of the RNA-P could be recovered as the nucleoside 3'-phosphates after 20 hours incubation, no hydrolysis whatever of adenosine 2'- or 5'-benzyl phosphates, of 2':3'-AMP or of 2':3'-UMP could be detected. When either of the cyclic nucleotides was used together with RNA as substrate, 60 to 70% inhibition of the release of nucleoside 3'-phosphates was observed with no detectable change occurring in the amount of the cyclic nucleotide. With adenosine 3'-benzyl phosphate as substrate no formation of the cyclic nucleotide could be detected, although this would have been readily found by means of the electrophoretic technique used if any appreciable amount had accumulated. These findings indicate that the hydrolysis of RNA or the simple esters of ribomononucleotides to the nucleoside 3'-phosphates does not proceed via cyclic mononucleotides as is the case with certain ribonucleases from animal and plant tissues.

No hydrolysis of DNA by this enzyme, as measured by release of acid-soluble, ultra-violet absorbing material, could be demonstrated, but the enzyme hydrolysed thymidine 3'- and thymidine 5'-p(nitrophenyl)phosphate, with the release of p-nitrophenol. The 5'- isomer was hydrolysed at less than 2% of the rate of the 3'- isomer. It thus appears that the nuclease does not possess absolute specificity for phosphodiester linkages involving ribose, but can also hydrolyse those involving desoxyribose. The very slow hydrolysis of thymidine 5'-phosphate seems to be an exception to the apparent specificity of the enzyme for phosphodiester linkages in which the linkage not hydrolysed must be to the 3'- position of ribose or desoxyribose. However, it may be that the p-nitro group influences the reaction in such a fashion as to modify the specificity of the enzyme. The possibility that this activity is due to a contaminating enzyme has been considered, but is unlikely. It cannot be due to contamination by the phosphodiesterases from which the nuclease appears to be separated on the chromatogram, for the one preceding it from the column does not hydrolyse thymidine 5'-p-nitrophenyl phosphate, while the one following it from the column does hydrolyse this compound, but is not inhibited by 0.005M monoiodoacetate, whereas the activity of the nuclease is inhibited nearly completely. The fact that the nuclease purified by either chromatographic procedure hydrolyses the same compounds and that 0.005M monoiodoacetate inhibits the hydrolysis of all of them, strongly suggests that one enzyme is probably concerned. No hydrolysis of bis(p-nitrophenyl)phosphate by the nuclease has been detected. The sensitivity of the assay was such that if it does occur, it must be at a rate less than 0.5% of that of thymidine 3'-p-nitrophenyl-phosphate.

Of all the enzymes so far described from animal and plant sources that are able to degrade ribonucleic acid, the fish muscle nuclease most closely resembles one that has been found in animal spleen.

In addition to the compounds that have been previously investigated as possible inhibitors or activators of the nuclease, it has been found that heparin, a very powerful inhibitor of certain ribonucleases, is without effect.

During the course of this work some information regarding the substrate specificity of the phosphodiesterases has been obtained. The only substrate yet found for the two phosphodiesterases that precede the nuclease from the chromatogram in Figure 1 is bis(p-nitrophenyl)phosphate. The phosphodiesterase that follows the nuclease from the column in both Figure 1 and 2 hydrolyses thymidine 5'-p-nitrophenylphosphate, bis(p-nitrophenyl)phosphate, and adenosine 5'-benzylphosphate. No hydrolysis of adenosine 2'- or 3'-benzyl phosphates could be detected. The first of the substrates was hydrolysed at about 5 times the rate of the second which in turn was hydrolysed at about 20 times the rate of the third. This enzyme resembles a phosphodiesterase found in rattlesnake venom in so far as these simple substrates are concerned, but it has not yet been possible to demonstrate any attack on RNA or DNA by the enzyme, whereas each of these is hydrolysed by the snake enzyme.

The crude aqueous extracts of lingcod muscle have been found to contain an enzyme capable of hydrolysing DNA. This enzyme is not retained on the buffered DEAE column and therefore comes through immediately after the holdup volume and is thus separated from the nuclease. Beyond the fact that this enzyme can degrade DNA with the release of acid-soluble, ultra-violet absorbing material, nothing is yet known of its specificity of action.

SUMMARY NO. 20

LINGCOD MUSCLE ACID PHOSPHOMONOESTERASES A WITH p-NITROPHENYL PHOSPHATE AS SUBSTRATE

N. Tomlinson

This is a continuation of work described in Summary No. 25 of this Station's Annual Report for 1957-58.

Material and Methods

Procedures of assays have been as described in last year's report (vide supra). Chromatography using diethylamino ethyl cellulose (DEAE) was carried out as described in Summary No.1of this Annual Report (Figure 1).

Results

In last year's report evidence for the presence of two zinc activated phosphomonoesterases in aqueous lingcod muscle extracts was presented. This was obtained by means of apparent differential destruction by heat and differences in pH optima.

By means of chromatography on buffered DEAE with gradient elution, separation of these two activities has now been accomplished. In a chromatogram like that described in Figure 1 of the preceding Summary, one of these enzymes was removed from the column with the first protein peak (at about 30 ml of effluent). This enzyme had a pH optimum between pH 6.1 and 6.3 and was inactivated by heating at pH 7.0, 58°C 10 min. The second of these enzymes was removed from the column between 116 and 136 ml of effluent. It had its pH optimum between 5.7 and 5.9 and was quite stable to heating at 58°C in neutral solution. The more labile enzyme removed first from the column is inactive at pH 5.0, whereas the other retains about 50% of its maximum activity at this pH. Each enzyme is activated by Zn or Mn ions, the activation by Zn (0.005M) being slightly greater than that by Mn. Mg activates both enzymes to about 10% of the activation by Zn. Ca, Cd, K and Na do not

cause any activation. In the presence of 0.005M Zn as activator, 0.005M $CuSO_4$, 0.025M ethylenediamine-tetra-acetate or 0.17M formaldehyde are very strong inhibitors of either enzyme. 0.005M sodium tartrate and 0.0005 sodium fluoride are not inhibitory.

In addition to the two phosphomonoesterases described above, a third phosphomonoesterase was obtained from the DEAE column between about 70 and 90 ml of effluent. This enzyme appears to be the one previously described (this Station's last year's Annual Report, vide supra) as being present in the "pH 4.5 purified" preparation. This enzyme is inhibited about 50% by 0.005M $ZnSO_4$, and 100% by 0.005M sodium tartrate.

It has been observed that aqueous muscle extracts contain an alkaline phosphomonoesterase with a pH optimum near 8.0.

B WITH VARIOUS PHOSPHORYLATED COMPOUNDS AS SUBSTRATES

K.G. Reid
N. Tomlinson

While a good deal of information has been obtained regarding certain acid phosphomonoesterases of lingcod muscle (part A above), very little is known of their ability to hydrolyze naturally occurring phosphorylated compounds. The present work was undertaken with a view to attempting to obtain this information and also to investigating the possibility of the existence of other phosphomonoesterases in fish muscle.

Materials and methods

Enzyme extracts were obtained from lingcod muscle in the same manner as for the phosphomonoesterases described previously (Summary No. 25, this Station's Annual Report for 1957-58). The extracts were assayed for ability to hydrolyse various compounds by setting up, in 75 mm x 10 mm test tubes, a digestion mixture containing sodium succinate buffer pH 5.5, 0.025M, substrate to provide 20 µg P, and the extract assayed, all in a total volume of 0.5 ml. The mixture was incubated 90 min at 25°C and was then deproteinized with an equal volume of ice-cold 10% trichloroacetic acid. The inorganic phosphate released by the enzyme was determined by the method of Bruemmer and O'Dell (J.B.C., 219, 283, 1956). Digestion blanks with substrate or with enzyme omitted were used.

Results

The results are tabulated in Tables I and II.

The crude aqueous extract contains enzymes capable of hydrolysing all of the substrates tested, and most of these activities are also found in the preparation following its partial purification by ammonium sulphate precipitation.

The data of Table II indicate clearly that hydrolysis of the compounds is brought about by a very complex mixture of enzymes. Phosphoethanolamine and phosphocholine appear to be the only substrates possibly hydrolysed exclusively by the same enzyme(s). It seems that each of the other substrates is hydrolysed either by a separate, distinct enzyme, or by a different combination of enzymes from that hydrolysing the other substrates.

TABLE I - Hydrolysis of various phosphorylated compounds by enzyme preparations from lingcod muscle.

Substrate	Inorganic phosphate released by Crude enzyme[1] µg	$(NH_4)_2SO_4$ ppt'd enzyme[2] µg
Ribose-5'-phosphate	5.2	5.3
Adenosine 5'-phosphate	5.6	6.4
Adenosine 3'-phosphate	5.4	5.6
Adenosine 2'-phosphate	5.2	4.8
d(+) 2-phosphoglyceric acid	6.4	2.4
D(-) 3-phosphoglyceric acid	3.2	1.4
β-glycerol phosphate	3.0	1.8
Phosphothreonine	1.8	0
Phosphoserine	8.6	0
Phosphoethanolamine	4.2	0
Phosphocholine	1.4	0
Glucose-6-phosphate	8.4	2.2
Fructose-1:6-diphosphate	3.8	0
Inositol phosphate	4.2	0.6
6-phosphogluconic acid	3.0	0.4

[1] Crude enzyme. Aqueous muscle extract, lyophilized and dialysed. 2 mg protein N/tube.

[2] Ammonium sulphate precipitated enzyme. Crude preparation precipitated with 80% saturated $(NH_4)_2SO_4$, the precipitate dialysed and lyophilized. 0.84 mg protein N/tube.

TABLE II - The influence of magnesium, zinc and manganese ions on the phosphatase activity of lingcod muscle extract.[1]

Substrate	% activation (+) or inhibition (-) by $MgSO_4$[2]	$ZnSO_4$[2]	$MnSO_4$[2]
Ribose-5-phosphate	+33	-82	+26
Adenosine 5'-phosphate	+22	-53	+118
Adenosine 3'-phosphate	+11	-17	0
Adenosine 2'-phosphate	+10	-64	+7
d(+) 2-phosphoglyceric acid	+320	+136	+185
D(-) 3-phosphoglyceric acid	+46	+46	+8
β-glycerol phosphate	-82	-75	-12
Phosphothreonine	+37	-37	+12
Phosphoserine	+195	+155	-100
Phosphoethanolamine	+60	-100	0
Phosphocholine	+78	-100	0
Glucose-6-phosphate	+18	-9	+18
Fructose-1:6-diphosphate	-100	-17	-47
Inositol phosphate	+125	+50	+125
6-phosphogluconic acid	+260	+100	+260
p-nitrophenyl phosphate	0	+49	+33

[1] Same crude enzyme as in Table I.

[2] 0.005M in digestion mixture.

SUMMARY NO. 21

LINGCOD MUSCLE PHOSPHORIBOISOMERASE AND RIBULOSE 5-PHOSPHATE 3 EPIMERASE

H.L.A. Tarr

The importance of ribose and certain of its phosphate esters in browning reactions in fish muscles has been brought out in previous reports (Summaries Nos. 26 and 27 of this Station's Annual Report for 1957-58). Studies have now demonstrated some of the complex enzyme actions which lead to post-mortem formation of such pentose phosphate esters as ribose 1-phosphate, ribose 5-phosphate, ribose 1,5-diphosphate and the corresponding deoxyribose derivatives. It was also shown that ketopentose sugars were formed, and some preliminary work was carried out concerning their formation. This report deals in detail with these sugars which, apart from their possible post-mortem role in occasioning browning reactions, have very considerable importance in biochemical processes of living cells.

Materials Employed

In this work certain of the chemical compounds used are unobtainable from usual sources and must be specially prepared, usually by known procedures. The following outlines the proportion and certain of the properties of some of the compounds used.

O-Nitrophenylhydrazine used in preparation of ribulose o-nitrophenylhydrazone was made from o-nitroaniline. D-ribulose o-nitrophenylhydrazone was prepared by the method of Glattharr and Reichstein and recrystallized from absolute methanol. The melting point and specific rotation agreed well with those obtained by other investigators: $[\alpha]_D^{21}$ + 51.8° in methanol (C, 1.35); m.p. 165.5 - 166°C (tube); 163 - 164°C (block). D-xylulose p-bromophenylhydrazone was prepared as described by Schmidt and Treiber. This compound (mp 128 - 128.5°C) exhibited the pronounced mutarotation noted by the above workers: $[\alpha]_D^{21}$ + 19.9° - 20.6° 15-20 minutes after dissolving in anhydrous pyridine (C, 2.094) and $[\alpha]_D^{21}$ - 34.2° to -35.6° after 7 - 8 days at this temperature. These compounds were used directly as standards in the cysteine carbazole reaction and were decomposed as described by Schmidt and Treiber to obtain the free pentuloses for chromatographic standards.

Barium R5P was prepared from chromatographically pure 5' adenylic acid (Schwarz) and was dried in high vacuum over P_2O_5. This compound was practically free from orthophosphate, possessed about the theoretical organic phosphorus and pentose content (based on MW 365.5), and gave the specific rotation characteristic of R5P of high purity; $[\alpha]_D^{23}$ + 23.2° in 0.2 N HCl (C, 1.909) based on the free acid, MW 230. The RU5P content was 2 - 3% as determined by the cysteine carbazole reaction and the presence of alkali labile pentose. The enzymic preparation of RU5P is described later.

DEAE cellulose Type 20 was obtained from the Brown Co., Berlin, New Hampshire. Dowex 1 x 10 and Dowex 50 x 8 (200 - 400 mesh, analytical grades) were obtained from the California Foundation for Biochemical Research, and Amberlite IR 45 (analytical grade) from the Fisher Scientific Co.

Methods

Aldopentose and aldopentose phosphates were determined by the orcinol procedure with a 40-minute heating period and arabinose as standard. The optical density at 670 mμ with pentulose phosphates in this method was multiplied by 1.76 in order to determine the concentration present, since these compounds give about 57% of the value of an identical amounts of arabinose on the basis of the pentulose content. With this method the average ratios of absorbency at $\frac{540}{670}$ mμ were:

D(-)ribose, 0.21; D(-) and L(+) arabinose, 0.19; D(-)ribulose, 0.85; D(-)xylulose, 0.49 and D-RU5P, 0.33.

Alkali labile pentulose was determined by adjusting the sample to 1.0 N with 2 N NaOH, holding 20 minutes at 25°, and then carrying out the orcinol reaction. Under these conditions pentuloses appear to be completely inactivated while R5P is very resistant. Tests indicated that approximately 4% of R5P is inactivated during this treatment, and this was taken into consideration when the method was used.

Pentuloses and pentulose phosphates were also determined directly by the cysteine carbazole reaction after 15 minutes for ribulose and 120 minutes at 30° for xylulose. The proportion of ribulose and xylulose in mixtures containing between 10 and 30 µg of the pentuloses was determined by calculating the ratio of the optical density at 540 mµ in the cysteine carbazole reaction after 15 minutes and after 120 minutes. In this test the ratio with ribulose alone was 1.04 and with xylulose alone 0.55. This method required prior hydrolysis of the pentulose phosphates with acid phosphatase (see below) but proved more sensitive than that of Ashwell and Hickman, in which the proportion of pentulose phosphates is determined directly using a modified cysteine carbazole reaction. Pentulose phosphates were determined directly using the cysteine carbazole reaction with a 2-hour heating period at 37°. Under these conditions experience showed that the amount of pentulose found multiplied by the factor 1.85 gave the concentration of pentulose in pentulose phosphates.

Protein was determined by the quantitative buiret method or by that of Lowry et al, the latter proving exceedingly useful where only small amounts of protein were measured. The method of Lowry and Lopez with the modification of Bruemmer and O'Dell, and also that of Gomori were used to determine orthophosphate, after appropriate hydrolysis procedures where necessary.

Acid phosphatase was prepared from "Polidase" (Schwarz), using a recent modification of the original procedure of Hochster. Its activity (226 µM of R5P hydrolysed by 1.0 ml in 30 minutes at 37°) was similar to that reported by the above investigators.

Barium ions were removed from Ba R5P and Ba RU5P by passage through small columns of Dowex 50 H+ resin, adjusting the effluent and washings to pH 7 - 7.5 with NaOH or KOH as required, and standardizing the solutions by the appropriate methods.

5-Phosphoribonic acid was prepared by oxidation of Ba R5P with alkaline periodate to yield the tricalcium salt $(C_5H_8O_9P)_2\ Ca_3$ (P calc 10.23; found 9.77).

All enzyme reactions were carried out at 37°C. Chromatographic separation of the R5P and RU5P was carried out using 16-hour descending development on Whatman No. 1 paper with n-propanol: 28% NH_4OH : H_2O (6:3:1). Separation of the free pentuloses was effected by 16-hour ascending development, using 90% phenol as solvent in presence of KCN crystals. The usual spray reagents were employed. The enzyme reactions were carried out in 160 x 15 mm tubes, the appropriate colorimetric reaction being carried out in the same tube.

Preparation of Enzymes

In this work one unit of enzyme is arbitrarily defined as that amount which caused turnover of one micromole of substrate in 1 minute at 37°C where conditions were such that the reaction was approximately linear with respect to time. The specific activity is recorded as units per mg of protein.

Satisfactory preparations were obtained from fresh or frozen lingcod, and all procedures were carried out at 0 - 3°C. In a typical case 1.5 kg of the muscle of a fish which had been frozen and stored about 3 months at -20°C was thawed until it could be cut readily, and was then blended for about 1 minute with 3 litres of water and sufficient 1 N HCl to adjust the suspension to pH 6.0. The suspension was centrifuged 15 minutes at 14,000 G. The supernatant liquid was filtered through glass wool to yield 1460 ml of fairly clear liquid. This was saturated with $(NH_4)_2SO_4$ (2.46 kg) at pH 7.0, about 35 ml of 2 N NaOH being required to adjust the reaction. The precipitated protein was collected by filtering for 16 hours at 0°C through Whatman No. 1 paper. The moist filter cake (about 250 g) wad dialyzed 2 hours against running tap water (7°C) and then for 24 hours against several changes of demineralized water to yield 870 ml of crude enzyme preparation (16.8 mg of protein/ml) (see Table).

Purification of PRI

Fraction	Ml	Mg protein per ml	Total protein (mg)	Specific activity	Total units
Saturated $(NH_4)_2SO_4$	870	16.8	14,700	0.175	2565
Heated to 55°C and lyophilized	170	39.4	6,700	0.284	1900
Eluate from DEAE column	43,500	0.015	650	20.4	1330

170 ml of the crude enzyme, immersed in a stainless steel tube held in a 65°C water bath, was stirred rapidly until the temperature attained 55°C, and was then promptly chilled to 0°C. The coagulated protein was removed by centrifuging 5 minutes at 10,000 G. The clear supernatant liquid was lyophilized and the residue suspended in water, insoluble protein being removed by centrifuging. The pH, which was about 5.5, was cautiously adjusted to pH 7.0 with a little 1 N NaOH to yield 35 ml of a solution containing 39.4 mg protein /ml.

A jacketed column containing 13 g dry weight of DEAE (34 x 1.8 (diam) cm) was conditioned with 0.02 M tris-HCl buffer, and 6 ml (236 mg protein) of the lyophilized enzyme preparation in 0.02 M tris was run on to the column, which was maintained at 1°C. The column was eluted at 1.3 ml/min under 5 lb/sq in pressure, using a gradient in which 0.25 M tris-HCl buffer was run into 250 ml of 0.02 M tris-HCl, 5 ml fractions being collected. The chilled fractions were tested for protein and for PRI and EPIM activity. These eluates were conveniently buffered at pH 7 - 7.3 so could be assayed directly.

The position of PRI was determined by incubating 0.1 ml of the column eluates directly with 0.02 ml of a 10 µM/ml solution of Na R5P, and determining the RU concentration by the 2-hour cysteine carbazole procedure. The position of EPIM was determined by three different procedures as follows: 0.04 ml of eluate was incubated with 0.02 ml of Na RU5P solution (0.2 µM) at pH about 7.2 for 30 minutes at 37°C. The solutions were promptly chilled to 0°; 0.01 ml of 0.004% methyl red indicator, 0.01 ml of 0.01 M $MgCl_2$, and sufficient 0.1 M CH_3COOH to adjust the pH to about 5.0 (0.01 - 0.015 ml) was then added. After adding 0.005 ml of acid phosphatase to each, the tubes were again incubated as above and the proportions of xylulose and ribulose determined by the cysteine carbazole method. 0.1 Ml each of the column eluate and a 10 µM/ml solution of NaRU5P were incubated 10 minutes at 37°C and a modified cysteine carbazole reaction carried out directly, the proportions of RU5P and XU5P being

determined by the change in optical density due to XU5P formation. 0.1 Ml each of eluate and a NaRU5P solution (2.5 µM) were incubated 1 hour at 37°C. Acid phosphatase (0.1 ml), 0.1 M $MgCl_2$ (0.01 ml), and 0.1 M acetic acid to adjust the pH to about 5.0 with methyl red indicator were added and the solutions incubated 1 hour at 37°C. They were concentrated to about 0.1 ml in vacuo over P_2O_5, a little Dowex 50 H^+ resin added to remove cations, and the solution chromatographed using 90% phenol. After drying the chromatograms and extracting residual phenol with diethyl ether RU (Rf 0.65) and XU (Rf 0.55) were detected by the trichloracetic acid-orcinol spray, followed by aniline phosphate or aniline hydrogen phathalate sprays.

The results obtained in this experiment are shown in Figure 1. With PRI the results obtained with a second identical column are also shown to indicate the close reproducibility obtained. No significant PRI or EPIM activity was found in the three major protein peaks emerging up to fraction 35, and these fractions accounted for 204 mg, or 86%, of the protein applied to the column.

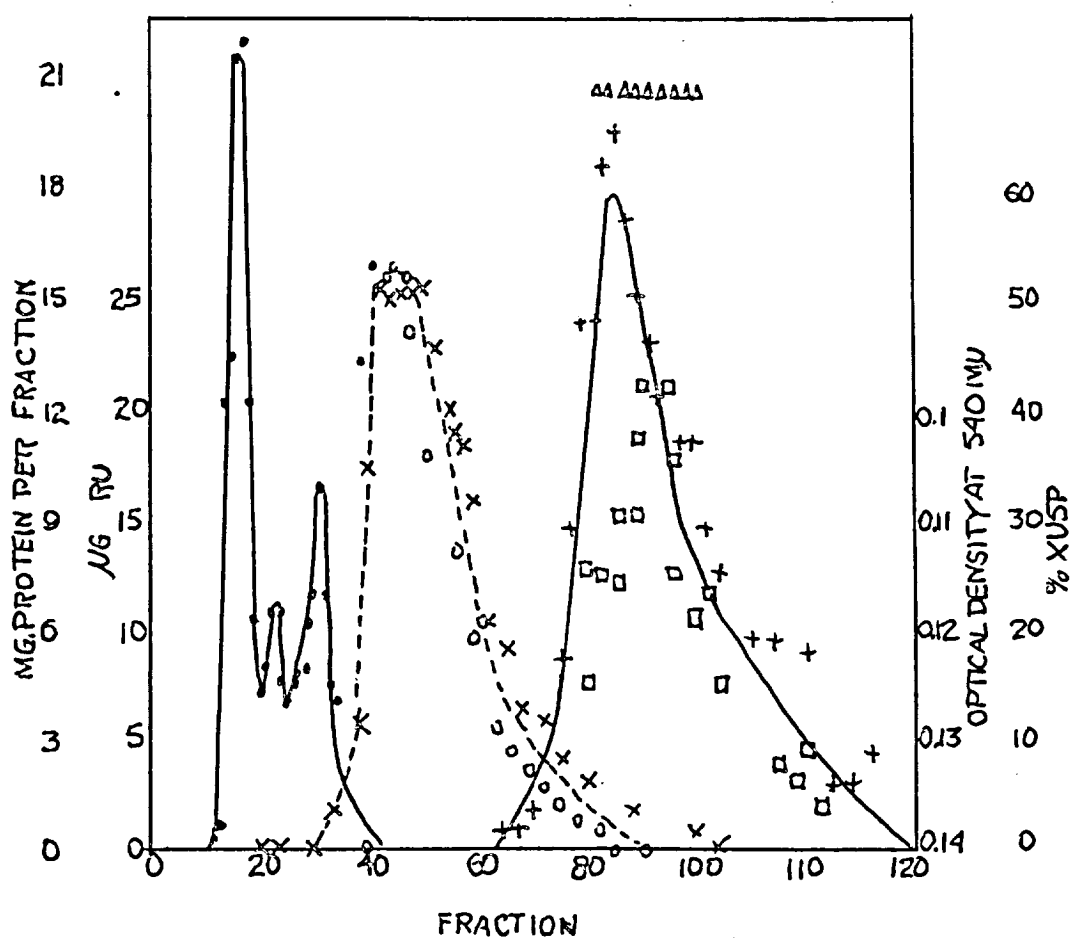


Figure 1. Separation of PRI and EPIM using a DEAE column. Protein, . —— .; PRI as determined by RU concentration (two different columns), X - - - X and O - - - O; EPIM as determined by % XU5P formed, + —— +, by decrease in OD at 540 mµ after 30 minutes at 37°C in the modified cysteine-carbazole reaction and recalculated on the basis of 0.1 µM of RU5P giving OD of 0.15 and 0.1 µM of XU5P OD of 0.05 under these conditions, □——□, and by paper chromatography method as indicator of XU, ΔΔΔ.

PRI was obtained by pooling fractions 40-70 inclusive (146 ml). The preparation had 15 µg of protein per ml and a specific activity of 20.4 (see Table). If it is assumed that the original muscle (1.5 kg) had 2560 units of PRI, and the muscle protein content was 18%, then the specific activity would be roughly 0.01. On the basis of the original muscle the maximum purification attained in the procedures as outlined in the preceding Table, would then be about 2,000 times.

Fractions 78-110 inclusive (Figure 1) were pooled to yield 156 ml of EPIM preparation containing 5 µg of protein per ml. Since the purified enzyme is rather labile, all tests were carried out within 1 week at 0°C. A number of tubes containing 0.05 ml of EPIM plus 2 µM of NaRU5P in 0.02 ml were incubated at 37°C and 4 tubes removed at intervals of 5, 10, 15 and 20 minutes for determination of XU5P formation by the cysteine carbazole reaction after hydrolysis by acid phosphatase. The results showed that under these conditions 0.25 µg of protein (per sample) formed 0.0054 µM of XU5P in 1 minute. Thus this preparation had a specific activity of 21.6. The recovery from 1.5 kg of muscle was 233 units as compared with 1330 units of PRI. While PRI proved very stable when frozen, EPIM preparations lost all measurable activity within 3 months at about -20°C.

Alternative Method for Preparation of PRI

The above DEAE column method provided a fairly satisfactory separation of PRI and EPIM. An alternative rapid procedure for preparing PRI free from EPIM is as follows:

In a typical experiment 43 ml of crude ammonium sulphate fraction containing 18.8 mg of protein per ml was heated to 55° and chilled as described above and centrifuged. The supernatant liquid was fractionated with $(NH_4)_2SO_4$ at pH 7.0, and the fraction precipitating between 0.6 and 0.75 saturation collected and dialyzed against frequent changes of demineralized water for 24 hours at 0°C to yield 8 ml of a preparation containing 42.5 mg/ml of protein. This preparation possessed a specific activity of 1.27, but the actual recovery was poor since much of the PRI remained in the fraction precipitating below 0.6 saturation with $(NH_4)_2SO_4$. This preparation was free from EPIM as determined by incubating samples with RU5P, dephosphorylating with phosphatase and carrying out chromatography for RU and XU as described above. Only RU was found.

Preparation of RU5P

The barium was removed from BaR5P (2.6 mM) as described under Methods, and a solution of the potassium salt was obtained and incubated at pH 7.2 with 6.0 ml (7.6 units) of the PRI described in the preceding paragraph. The amount of RU5P formed was determined at intervals using the cysteine carbazole reaction and the 1.85 factor. After 2.5 hours about 33% of the R5P was isomerized and, after a further 2 hours the reaction was complete with 43% RU5P formation. The solution was chilled to 0°C, deproteinized with 3 ml of 60% perchloric acid and centrifuged 5 minutes at 10,000 G. The clear supernatant liquid was adjusted to pH 6.0 with 1 N KOH and the solution evaporated to dryness at 40° using a rotary evaporator. The dry residue was extracted at 0°C with water, the potassium perchlorate being removed by filtration. The clear filtrate and washings (35 ml) were treated with bromine water to remove aldopentose phosphate.

The clear colourless filtrate was run slowly on to a 12 x 2.8 (diam) cm Dowex 1 x 10 formate column, washed with 100 ml of H_2O and eluted with 0.1 M sodium formate in 0.1 M formic acid at 15 ml per minute collecting 14 ml fractions. Fractions 41-56 inclusive, which contained practically all the cysteine carbazole positive material were pooled. The solution was adjusted to pH 5.0 with 1 N KOH, and then to pH 6.2 with warm, freshly filtered barium hydroxide solution. The BaRU5P was precipitated

by adding 4 volumes of absolute ethanol and the flocculent precipitate collected by centrifuging. The precipitate was dissolved in 50 ml of H_2O, a small amount of insoluble material removed by centrifuging, and the BaRU5P reprecipitated and dried in high vacuum over P_2O_5 at 0°. The yield was 250 mg of approximately 78% pure BaRU5P as judged by the cysteine carbazole and orcinol reactions.

The product was dissolved in 3 ml of H_2O and run slowly through a 3 x 2 cm Dowex 50 x 8 H+ column at about 3°C. The eluate and washings, after removing insoluble material by centrifuging, were promptly adjusted to pH 6.2 with warm barium hydroxide solution, the BaRU5P precipitated and collected, and washed with 80% and absolute ethanol before drying in high vacuum in P_2O_5 at 0° (yield 170 mg). The product gave 55.5 to 59% of the absorption of arabinose at 670 mμ in the orcinol reaction, 51% of the absorption of free RU after 2 hours at 37°C in the cysteine carbazole reaction, was free from orthophosphate and had 97% of the theoretical organic P content, (assuming MW 365.5). It contained 7.0% alkali stable pentose phosphate, the ratio of optical density $\frac{540}{670}$ mμ was 0.33 in the orcinol reaction, and the specific rotation was $[\alpha]_D^{22}$ -20.2° degrees (C=1.85 in 0.2 N HCl) based on the free acid (MW 230). This rotation compares favourably with that given by a fairly pure sample of L RU5P; $[\alpha]_D^{20}$ + 20°. Two zones, one characteristic of RU5P (2.5 - 3.0 cm from origin) and the other of R5P (6.0 cm from origin) resulted when the free acid was chromatographed. On the basis of these data the product contained about 90% BaRU5P and 7.0% R5P. This, or products of similar purity, were used in the following studies.

pH - Activity of PRI

Portions of a solution containing 10 μM/ml of NaRU5P were either used without addition or were made 0.05 M with respect to glycine or sodium acetate. These were then adjusted to different pH values by cautious addition of traces of 1 N HCl or 10 N NaOH (Figure 2). Duplicate 0.1 ml portions of these solutions were incubated 10 minutes with 0.02 ml of purified PRI (1 μg of protein) and the amount of RU5P formed determined by the 2-hour cysteine carbazole reaction and expressed as μg of RU. The results (Figure 2) show that the enzyme had an optimum activity between about pH 7 and 8; lost its activity markedly between about pH 7 and 4, but had considerable activity at pH values between 8 and 11. A rather similar pH-activity pattern was observed for rabbit muscle PRI.

pH - Activity of EPIM

Purified EPIM containing 5 μg/ml of protein, and which was buffered with approximately 0.2 M tris-HCl pH 7.3 as eluted from a DEAE column was adjusted to lower pH values with very small amounts of 0.1 M acetic acid and to higher pH values with 1 N NaOH. Tubes containing 0.05 ml of buffered enzyme and 0.02 ml (0.19 μM) of NaRU5P solution were incubated at 37° for 30 minutes. They were promptly chilled to 0°C and subjected to hydrolysis with acid phosphatase for 30 minutes at 37° under similar conditions to those described in the section dealing with isolation of the enzyme. The concentration of XU5P formed was determined by the cysteine carbazole reaction (Method 1). The results, shown in Figure 2, indicate that the pH activity curve of this enzyme is quite similar to that of PRI.

Equilibrium with Crude Preparations Containing both PRI and EPIM

Two preparations were studied, namely, the supernatant liquid from a crude enzyme which had been heated to 55°C and centrifuged (10.4 mg per ml of protein) and a similar preparation which had been lyophilized (35 mg per ml of protein). Duplicate portions of these preparations (0.01, 0.02 and 0.04 ml) were incubated at pH 7.2 for 10 minutes at 37° with 0.02 ml of a solution containing 0.18 μM of NaR5P. The reaction was promptly stopped by immersing the tubes in a solid CO_2-acetone mixture, and

the samples were subjected to analysis for total pentulose phosphate and XU5P content by the cysteine-carbazole method after prior hydrolysis with acid phosphatase. Very similar results were obtained with the two enzyme preparations used and with the three concentrations. The total pentulose phosphate concentration varied between 50% and 58.5% (average 53.5% of the initial amount of R5P used). The XU5P concentration varied between 52% and 59% (average 54%) of the total pentulose phosphate, and that of the RU5P between 41% and 48% (average 46%). The average R5P content (by difference) was 46.5%. The proportion of phosphate esters in this experiment, therefore, was about R5P, 46%; XU5P, 29%; RU5P, 25%.

A very similar experiment was carried out using only 0.01 and 0.02 ml of the same two enzyme preparations and half the amount of R5P (0.095 µM). In this instance the concentration of alkali labile pentulose phosphate was determined. On completion of the reaction the samples contained from 54.5% to 56.6% (average 55.7%) of alkali; labile pentulose phosphate. In this experiment the ratio was therefore: total pentulose phosphates, 55.7% : R5P, 44.3%, which is very similar to that found in the preceding test.

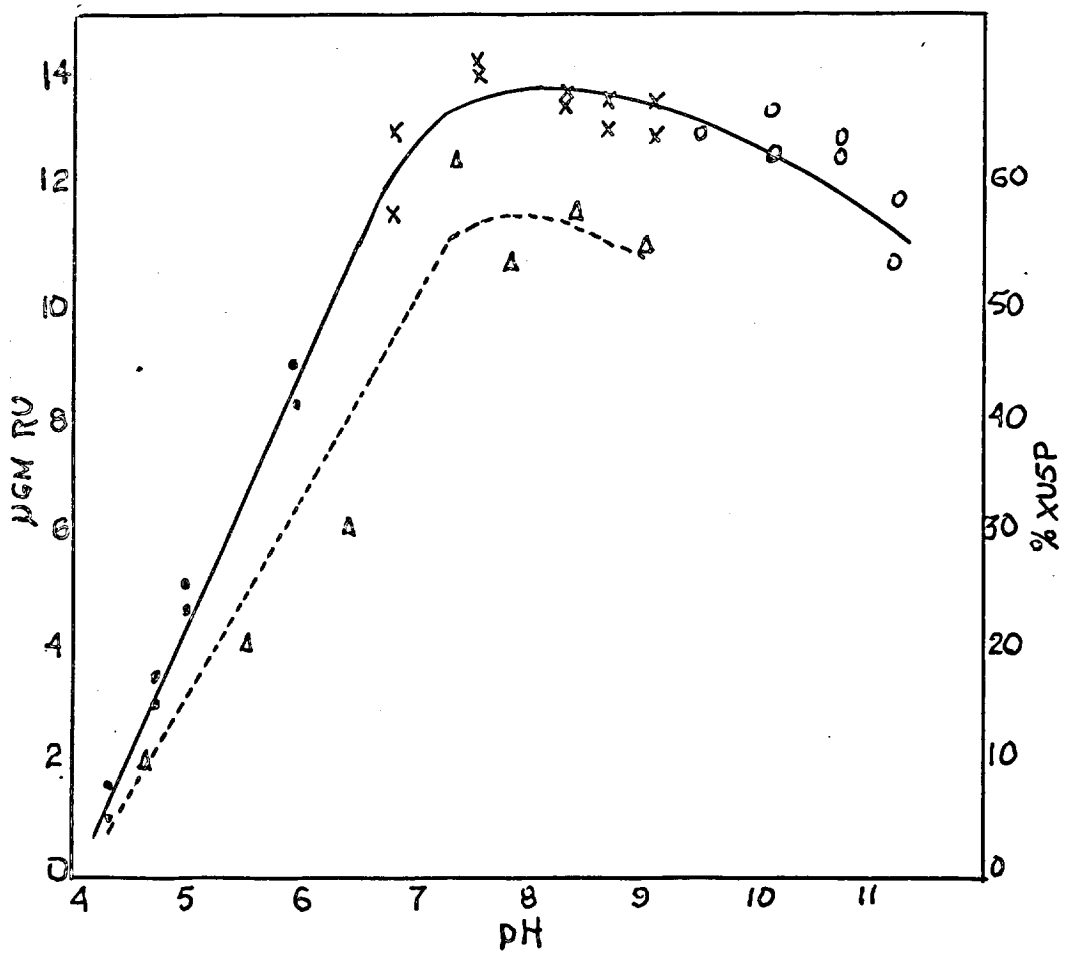


Figure 2. pH-Activity curves for PRI and EPIM. PRI with acetate, c——o, tris, X —— X and glycine-NaOH O —— O buffers. EPIM Δ - - - Δ (see text for method used in adjusting pH). (See page 67 re Figure 2.)

Equilibrium with a Mixture of Purified PRI and EPIM

A mixture containing 19 μM of NaR5P, 2 ml of purified PRI (0.03 units) and 6 ml of EPIM (1.4 units) in a 10 ml volume was incubated at pH 7.2 and 37°C, duplicate 0.5 ml samples being removed at 10-, 30-, 45-, 60- and 90-minute intervals for cysteine carbazole determinations after dephosphorylation with acid phosphatase in the usual manner. The results showed that the total pentulose phosphate concentration increased up to 45 minutes, and that thereafter it remained approximately constant. The XU5P : RU5P ratio did not alter appreciably throughout the experiment, and the results obtained after 60 and 90 minutes showed that this ratio was 57% XU5P : 43% RU5P, and that the total pentulose recovered was 1440 μg.

Alkali stable pentose phosphate was also determined, using duplicate 0.15 and 0.2 ml aliquots of the reaction mixture after the 90-minute period. It was found that between 1080 and 1150 μg (avg. 1110 μg) of alkali stable pentose remained. Thus, the total recovery was 2560 μg of pentose plus pentulose, or 90% of the pentose of the R5P used. The ratio calculated from the above data was 43.0% R5P : 32.5% XU5P : 24.5% RU5P, which agrees well with that obtained with the crude mixed enzyme preparation in the preceding experiment.

Equilibrium with Purified PRI

Tubes containing 0.095 μM of NaRU5P and 0.05 ml of a purified PRI preparation (1.2 μg of protein) were incubated at 37°C and the decrease in RU5P content measured at intervals in one series by the cysteine-carbazole reaction and in another series by decrease in alkali labile pentulose phosphate. A similar experiment was made except that NaR5P was used instead of RU5P. The results (Figure 3) show that equilibrium was attained with 43 - 45% RU5P : 55 - 57% R5P.

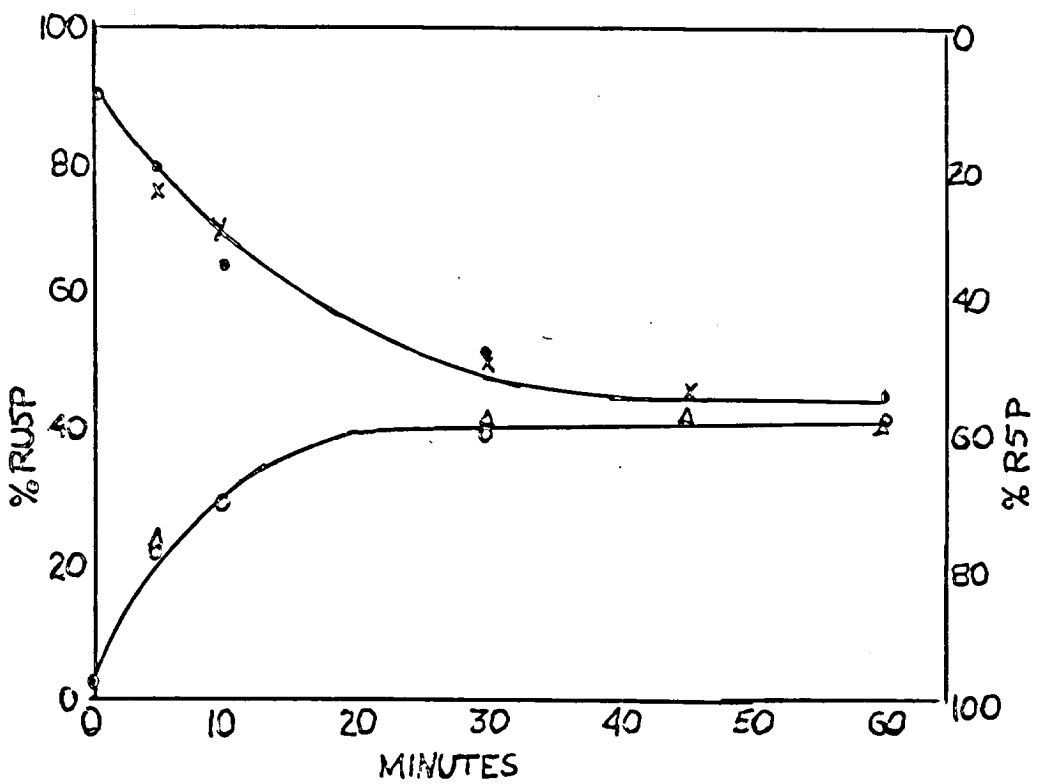


Figure 3. Equilibrium with purified PRI. Enzyme plus RU5P, R5P formation determined by the cysteine-carbazole reaction. ——— and by increase in alkali stable pentose X——X. Enzyme plus R5P, RU5P formation determined by the cysteine-carbazole reaction O——O and by decrease in alkali stable pentose Δ —— Δ.

In a second experiment 0.5 mM of NaR5P were incubated at 37° and pH 7.4 with 85 ml of PRI (2 mg protein = 41 units) in a total volume of 100 ml. To ensure equilibrium the solution was incubated 40 minutes. Analyses then showed 43.6% RU5P by measurement of alkali labile pentulose, and 42.1% by the cysteine-carbazole reaction. The total pentose content by the orcinol reaction indicated a decrease of 22.5% at 670 mμ. This would account for the formation of 40% RU5P which gives about 57% of the optical density of R5P in this reaction. The average of these determinations indicated that the solution contained 42% RU5P and 58% R5P.

The solution was deproteinized at 5°C with 2 ml of 60% perchloric acid, centrifuged 5 minutes at 10,000 G, and the clear supernatant liquid adjusted to pH 5.5 with 1 N KOH. The solution was evaporated to dryness using a rotary evaporator at 37°C. The potassium perchlorate was largely removed from the crystalline residue by several extractions with water (1°C) followed by filtration. After adjusting the solution to pH 7.2 it was run on to a jacketed Dowex 1 x 10 formate column 35 x 1.8 (diam) cm, and was eluted at 1°C at the rate of 3 ml/minute, first with 0.15 M ammonium formate containing 0.0005 M sodium borate, and then with 0.5 M sodium formate. The RU5P was removed with the first eluant (fractions 72-107) and the R5P (fractions 113-128) with the second eluant. The RU5P fraction (460 ml with washings) accounted for 34.4% of the R5P used, as determined by the cysteine-carbazole reaction using the 1.85 factor, and 35.4% as determined by optical density at 670 mμ in the orcinol reaction using the 1.76 factor. The second fraction (205 ml with washings) contained 49% of the R5P used as determined by the orcinol reaction. The recovery was therefore 84% of the R5P used and the ratio of RU5P : R5P was 1 : 1.5. Since further elution of the column yielded no material which gave positive cysteine-carbazole or orcinol reactions, it would appear that no significant amount of 3 ketopentulose 5 phosphate or xylose had formed.

A portion of each of the fractions was evaporated to a small volume at low temperature using a rotary evaporator, and the concentrates, after treatment with a little Dowex 50 H+ resin to remove cations were chromatographed, using the n-propanol: ammonium hydroxide:water system referred to previously. Only single zones, characteristic of R5P and RU5P were found. The ratio of optical density at $\frac{540}{670}$ mμ of the RU5P fraction in the orcinol reaction averaged 0.334, and that of the R5P fraction 0.19.

Equilibrium in a Crude Enzyme Preparation and Separation of Free XU and RU

3 mM of NaR5P were incubated with 15 ml of a lyophilized crude enzyme preparation (59 mg protein) in 250 ml total volume at pH 7.2. Analyses of duplicate 0.05 ml aliquots by the cysteine-carbazole method showed that the reaction neared completion in 20 minutes and was complete in 30 minutes with formation of 1.56 mM of pentulose phosphates (52% of the ribose in R5P used). The solution was deproteinized with perchloric acid and potassium perchlorate removed as in the preceding experiment, the R5P being removed by bromine treatment.

The clear solution (8.7 ml) was adjusted to pH 5.2 with 1 N KOH, and 0.8 ml of 1 M $MgCl_2$ and 5 ml of acid phosphatase were added. Testing of 0.02 ml aliquots at intervals showed that formation of orthophosphate became constant after 2.5 hours, but that only 84% of the pentulose phosphates had apparently been hydrolyzed by this time. The total pentulose content of the solution was only 160 mg, or 81% of the orthophosphate liberated. At present no explanation can be offered for this discrepancy. Chromatography by ascending development in 90% phenol showed that the solution contained only ribulose (Rf 0.66) and xylulose (0.55), no other sugar being detected by the spray reagents used.

The solution was demineralized by passage through a mixed column of Dowex 50 H+ and IR 45 OH' resins, the eluate made 0.01M with $Na_4B_2O_7$, and chromatographed on a 20 x 2.8 (diam) on Dowex 1 x 10 borate column under the conditions described by workers elsewhere. The fractions containing XU were pooled (1500 ml). Analyses showed that this fraction contained 58 mg of XU, the ratio of optical density $\frac{540}{670}$ mμ in the orcinol reaction being 0.46, which agrees well with literature values. The xylulose solution was freed from cations, the boric acid removed, the solution decolorized with charcoal and evaporated to a syrup. The xylulose was extracted with n-propanol to free it from insoluble material which could not be removed by mixed bed ion exchange resin treatment. The specific rotation of the syrup, based on the xylulose content as determined by the cysteine-carbazole reaction was $[\alpha]_D^{23}$ -21.2° in water (C, 1.82), as compared with $[\alpha]_D^{18}$ -33.2° and $[\alpha]_D^{25}$ -36.8° reported for pure D-xylulose. Other workers reported a similar discrepancy with xylulose purified from Dowex 1 borate columns. Chromatography with 90% phenol revealed a single zone (Rf 0.67) characteristic of XU. The ratio of colour development in 15'/120' in the cysteine-carbazole reaction was 0.56, which is characteristic of xylulose. This evidence would indicate that the product was D-XU.

The fractions containing the RU were pooled to yield 1100 ml of solution which, as determined by the cysteine-carbazole reaction contained 38 mg of ribulose. In the cysteine-carbazole reaction colour development was complete within 15 minutes, and the absorbence ratio $\frac{540}{670}$ mμ in the orcinol reaction was 0.86. Chromatography of the concentrated solution showed one component (Rf 0.56) which gave reactions typical of RU. These values are similar to those reported for RU by other investigators.

The results of this experiment show a ratio of RU:XU of 1:1.5.

Inhibition of PRI by Phosphoribonic Acid

The sensitivity of spinach PRI to phosphoribonic acid was first observed by Axelrod and Jang, who noted 50% inhibition by a 1×10^{-4} concentration. In the present work purified PRI was also inhibited by phosphoribonic acid, the inhibition being about 45% by a 5×10^{-3}M, and 28% by 5×10^{-4}M concentrations.

Equilibrium with Purified EPIM

A number of tubes containing 0.05 ml of purified EPIM (0.25 μg protein = 0.0055 units) were incubated with 0.02 ml (0.2 μM) of NaRU5P at 37°C, 4 tubes being withdrawn at intervals to determine the XU5P concentration by the cysteine-carbazole method. The results (Figure 4) show that equilibrium is attained when the ratio of XU5P:RU5P is approximately 1.5:1.0.

The equilibrium in the PRI system has been found by most investigations to be from 70-80% in favour of R5P formation and 20-30% in favour of RU5P formation. On the other hand, the equilibrium with PRI prepared from erythrocytes was 41.5% RU5P:58.5% R5P, and from rabbit muscle 36-47% RU5P:53-64% R5P. In the present work the equilibrium with a fairly highly purified PRI from fish muscle has been found in different experiments to be 40-45% RU5P:55-60% R5P.

There has usually been quite close agreement in the results that different investigators have obtained in studying equilibria with EPIM preparations from different sources, namely 40-45% RU5P:55-60% XU5P. On the other hand other investigators have reported ratios of 24-26% RU5P:74-76% XU5P for EPIM preparations from spinach and muscle. In the present work with a fairly highly purified fish muscle EPIM the ratio was about 40% RU5P:60% XU5P.

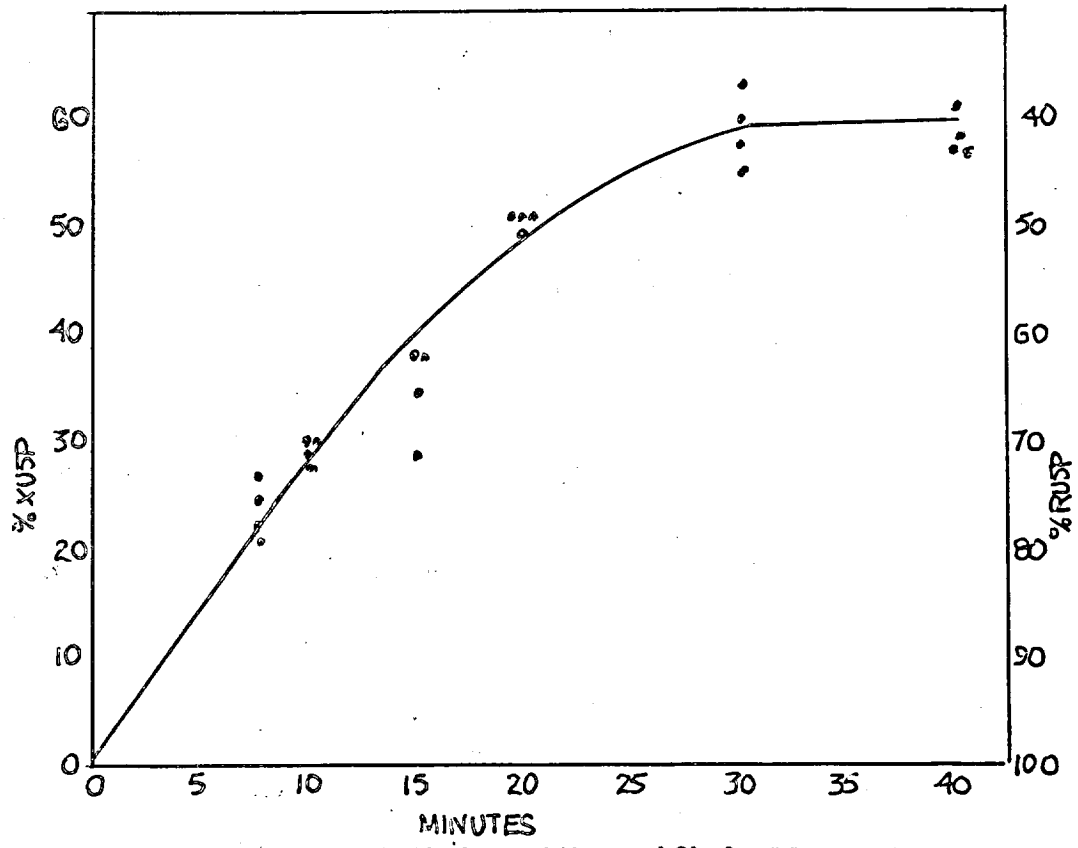


Figure 4. Equilibrium with purified EPIM; XU5P formation determined by ratio of rate of colour development after 15 and 120 minutes in the cysteine-carbazole reaction (see text).

The equilibria in systems containing both PRI and EPIM have been reported as 35% R5P:28% RU5P:37% XU5P and 41% R5P:14% RU5P:45% XU5P. The present work has shown that the equilibria obtained with a crude fish muscle preparation containing both PRI and EPIM, and with a mixture of the fairly highly purified enzymes are similar; namely, 43.5-46% R5P:26.5-29% RU5P and 25-30.5% XU5P respectively. These results indicate somewhat higher proportions of both R5P and RU5P and smaller proportions of XU5P than those previously recorded for this system. However, the ratio of XU5P to R5P found in the present work with both mixed purified enzymes and the crude enzyme mixture (pentulose phosphates 53.5-55.7%:R5P 40.5%-44.3%) is very similar to the average values reported for mixed rabbit muscle PRI and EPIM, namely 58.5% mixed pentulose phosphates:41.5% R5P.

The possible reasons for the apparent discrepancies in the ratios of reactants in the above systems have been discussed but so far there has been no really satisfactory explanation for them.

SUMMARY NO. 22

LINGCOD MUSCLE LIPASE — J.D. Wood

Frozen fish deteriorates gradually during storage. One aspect of the deterioration is the off-flavour produced by fat spoilage. The most well-known type of fat spoilage is oxidative rancidity. There is, however, a second type, namely, hydrolysis of the fat to produce free fatty acids. This report deals with an

investigation, begun in the current year, of an enzyme which was isolated from lingcod muscle and which was capable of hydrolysing lipids.

Isolation and Purification

The enzyme was isolated from lingcod muscle by homogenizing the tissue in 3 volumes of water and centrifuging at 20,000 g for 20 minutes. The clear supernatant fluid contained the lipase. The enzyme was purified by precipitation with saturated $MgSO_4$. The purification obtained was seven-fold.

Measurement of Activity

The lipase activity was measured either by titration of the released fatty acids or by measuring the CO_2 evolved from bicarbonate buffer by the action of the acids. The latter method, which employs manometric estimations by the standard Warburg procedure, was used in the majority of the experiments.

Properties of the Enzyme

The enzyme preparation was found to be somewhat unstable in aqueous solution. There was a significant loss in activity when the preparation was dialysed overnight at 0°C. Table I shows the effects of dialysis on the ability of the enzyme to hydrolyse triacetin. The loss in activity with dialysis was reduced but not eliminated by dialysing against 0.01M $MgCl_2$. The addition of $MgCl_2$ to the enzyme preparation which had been dialysed against water did not restore the loss in activity. The presence of cysteine during dialysis failed to prevent destruction of the enzyme. The preparation was stable in the frozen state and could be kept at -20°C for 4 weeks without any loss of activity.

The lipolytic activity of the enzyme preparation was studied at a series of pH's from 6.34 to 9.0. The rate of hydrolysis increased with pH to an optimum at 8.2. Above this pH the activity decreased rapidly.

The hydrolysis of triacetin by the enzyme was studied at temperatures from 5°C to 45°C. Activity was observed at all temperatures although the rate of reaction was slow at 5°C. At temperatures above 25°C destruction of the enzyme began to occur.

TABLE I - Stability of lingcod muscle lipase.

Preparation	µl CO_2 evolved/flask/hour Exp 1	Exp 2	Exp 3	Exp 4	Exp 5
Non dialysed	83	119	69	76	155
Dialysed against 0.01 M $MgCl_2$	71	34		74	
Dialysed against H_2O	42	14	47	45	75
Dialysed against H_2O, $MgCl_2$ added to give concentration of 0.01M			43	47	
Dialysed against 0.01 M cysteine					75

Substrate was 8.4×10^{-2} M triacetin. Dialysis overnight except Exp 2 which was for 72 hours.

The effect of the lingcod muscle lipase on different substrates was studied, (Table II). Both monoglycerides and triglycerides were attacked, but methyl acetate and ethyl acetate were hydrolysed extremely slowly, if at all. The triglycerides containing longer chain fatty acids such as trioctanoin were not attacked. Olive oil, which is often used as a substrate in lipase studies, was not attacked by the fish muscle enzyme even when the oil was present as a very fine emulsion. However, the enzyme was capable of hydrolysing long-chain fatty acids as is shown by its action on Tween 20 which is the water soluble monolaurate ester of a polyalkylene derivative of sorbitol. The results in Table II show that the rate of hydrolysis of the monoglycerides increased with the chain length of the fatty acid. The same increase in activity with chain length did not apply to the triglycerides. However, triacetin was soluble at the concentrations used, whereas tripropionin and tributyrin were not completely soluble. In spite of this relative insolubility, tripropionin proved just as reactive as Triacetin while tributyrin showed half the activity. Triglycerides appeared to be more susceptible to attack than monoglycerides because triacetin was hydrolysed more rapidly than monoacetin.

TABLE II - Relative rates of hydrolysis of various esters by lingcod muscle lipase.

Substrate	Concentration	Relative rate of hydrolysis (8.4×10^{-2}M triacetin=500)
Triacetin	8.4×10^{-2}M	500
Tripropionin	8.4×10^{-2}M	490
Tributyrin	8.4×10^{-2}M	242
Trioctanoin	8.4×10^{-2}M	2
Monoacetin	8.4×10^{-2}M	151
Monopropionin	8.4×10^{-2}M	233
Monobutyrin	8.4×10^{-2}M	295
Tween 20	5%	166
Methyl acetate	8.4×10^{-2}M	8
Ethyl acetate	8.4×10^{-2}M	13
Olive oil	10%	3
Halibut liver oil	10%	0
Lingcod " "	10%	0

The effects of some common inhibitors of enzyme action on triacetin hydrolysis are shown in Table III.

TABLE III - The effect of various compounds on the hydrolysis of triacetin by lingcod muscle lipase.

Compound	Concentration	Percent control
p-chloromercuribenzoate	1×10^{-3} M	86
Potassium ferricyanide	2×10^{-3} M	99
Sodium iodoacetate	2×10^{-3} M	98
" "	1×10^{-2} M	95
Potassium cyanide	1×10^{-5} M	97
" "	1×10^{-3} M	97
" "	1×10^{-2} M	100
Sodium azide	1×10^{-3} M	98
" "	1×10^{-2} M	103

Substrate was 8.4×10^{-2} M triacetin.

Cyanide and azide in various concentrations had little or no effect on the activity. Of the sulphydryl reagents that were tested, p-chloromercuribenzoate and possibly iodoacetate brought about inhibition of the lipase activity. However, the inhibition even with the p-chloromercuribenzoate was never very great.

The present investigation has shown that an enzyme exists in lingcod muscle which can hydrolyse lipid material. The possibility that the formation of free fatty acids during storage of fish fillets is due to enzymes in the muscle cannot therefore be ignored.

SUMMARY NO. 23

THE EFFECT OF DIETARY FISH OILS ON SERUM CHOLESTEROL LEVELS IN CHICKENS

J.D. Wood
(J. Biely)
(Dept. Poultry Science, U.B.C.)

There has been a considerable amount of work performed in recent years on the effects of diet on cholesterol levels in blood. This work was stimulated by the observation that high blood cholesterol levels in humans were associated with heart disease, although a definite correlation between the two has not been definitely proved. Diets containing vegetable oils were found to produce lower blood cholesterol levels in several species of animals than did similar diets containing animal fat. Fish oils were not thoroughly investigated but sardine oil was found to be very effective in lowering cholesterol levels. An investigation has been recently commenced in which the effect of fish oils on the serum cholesterol levels in chickens will be studied. Preliminary results showed that dogfish liver oil has not a great effect on serum cholesterol levels.

SUMMARY NO. 24

DISTRIBUTION OF HISTIDINE IN THE TISSUES OF SOCKEYE SALMON DURING SPAWNING MIGRATION

J.D. Wood
D.W. Duncan
M. Jackson

The non-protein nitrogenous constituents of the muscle of migrating sockeye salmon were reported in Summary No. 6 of this Station's Annual Report for 1957-58. Significant changes were found to occur in the amount of histidine during the migration. Further studies were carried out in the current year to ascertain whether the changes in the amount of histidine were confined to the flesh, or whether variations in the amount of the compound also occurred in other tissues.

The results of the investigation are shown in Table I which gives the concentration of histidine in various tissues during the migratory journey. The amount of the compound in the alimentary tract followed the same pattern as that observed with the flesh; namely, a striking decrease in the concentration from Lummi to Lillooet and a less noticeable decrease from Lillooet to Forfar Creek. This behaviour occurred in both male and female fish. The liver in female fish and the gonads in male fish also showed significant decreases in the histidine content from Lummi to Lillooet but the changes were not so great as for muscle and alimentary tract. Of all the tissues investigated only the kidney in males showed a significant increase in the histidine concentration during the early stages of the migration. In the latter part of the migratory journey significant decreases in histidine occurred in the liver and gonads of the males and in the blood of the females, whereas the concentration of the compound increased in the livers of females.

TABLE I - The histidine content of various tissues from sockeye salmon at different locations on the migratory route.

Tissue	Sex	Lummi Island	Lillooet	Forfar Creek
Heart	M	21.9 ± 3.1	23.6 ± 2.6	22.1 ± 10.5
	F	22.5 ± 4.1	24.6 ± 2.4	21.6 ± 1.6
Alimentary Tract	M	37.4 ± 3.6	13.0 ± 3.3**	8.2 ± 2.9
	F	47.2 ± 10.6	10.8 ± 1.9**	5.5 ± 0.9**
Spleen	M	6.6 ± 1.0	6.6 ± 1.8	7.3 ± 1.6
	F	8.1 ± 1.6	7.3 ± 1.3	5.4 ± 1.6
Liver	M	5.7 ± 0.8	5.6 ± 0.5	4.4 ± 0.8*
	F	6.3 ± 1.0	3.3 ± 0.1**	4.5 ± 0.2**
Kidney	M	10.0 ± 0.4	12.8 ± 1.5*	12.6 ± 0.8
	F	13.9 ± 1.8	12.7 ± 0.9	13.7 ± 2.8
Blood	M		3.4 ± 0.4	3.5 ± 1.5
	F		4.2 ± 1.4	2.5 ± 0.2*
Gonads	M	5.3 ± 0.7	4.1 ± 0.6*	1.8 ± 0.9**
	F	1.8 ± 0.7	1.4 ± 0.3	1.0 ± 0.2
Muscle	M	10.8 ± 1.3	2.1 ± 0.5**	1.3 ± 0.1*
	F	15.1 ± 0.8	2.7 ± 0.4**	3.1 ± 0.1

All values are given as mg histidine nitrogen per 100 g tissue.
* Difference from value at previous location is significant at P=.05.
** Difference from value at previous location is significant at P=.01.

The distribution of histidine in the tissues was similar in both male and female fish. It was present in highest concentrations in the heart and alimentary tract followed by muscle, kidney, spleen, liver, blood and gonads, in that order.

The values in Table I are concentrations and they do not give any indication of the total amount of histidine in the tissue. The latter value can be obtained by using the figures obtained by Idler and Tsuyuki (Summary No. 1 of this Station's Annual Report for 1957-58) for the average weights of the tissues at each location on the migratory route. The total amounts of histidine calculated by this method are given in Table II.

The amount of histidine in the alimentary tract and muscle are very much greater than in the other tissues. The heart contained relatively little histidine, in spite of the high concentration of the compound, because of the small size of that organ. Since most of the histidine present in the fish is situated in the muscle and the alimentary tract, the changes in the histidine content of the fish will parallel the changes observed in these two tissues; i.e. it will decrease about 5-fold between Lummi and Lillooet with a lesser decrease between Lillooet and Forfar Creek.

The reason for the loss in histidine from the fish is at present unknown. The compound may be of exogenous origin, i.e. the fish may obtain it directly from the food. If the food supply is withdrawn then the histidine content of the tissues

could quite feasibly drop. Now, it is known that the sockeye salmon do not eat during the spawning migration and this therefore may be the reason for the observed decrease in histidine during the journey up the Fraser River.

TABLE II - The total amount of histidine in the tissues.

Tissue	Sex	Lummi Island	Lillooet	Forfar Creek
Heart	M	133	139	128
	F	114	122	99
Alimentary Tract	M	3,384	391	134
	F	3,696	283	71
Spleen	M	19	13	26
	F	17	11	12
Liver	M	180	142	165
	F	253	147	182
Kidney	M	260	298	354
	F	300	253	270
Gonads	M	299	336	154
	F	144	193	298
Muscle	M	15,800	2,700	1,700
	F	20,200	3,100	2,900

All values are given as (mg histidine-N/tissue) x 10^2.

SUMMARY NO. 25

NITROGEN EXCRETION IN MARINE TELEOSTS — J.D. Wood

Trimethylamine oxide (TMO) is found extensively in fish tissues. It is broken down during spoilage of fish to form trimethylamine (TM). Baldwin, in his book "Dynamic Aspects of Biochemistry", referred to the work of Homer Smith as showing that TMO was a major component of the total nitrogenous material excreted by marine teleosts via the gills and the kidneys. However, an examination of the literature failed to produce any work by Homer Smith on the total TMO excreted by marine teleosts. A personal enquiry addressed to Smith likewise yielded a negative result. It was therefore decided to investigate the nitrogenous excreta of marine teleosts. This study was commenced in 1958 and was completed within the year.

The species employed in the experiments were the sculpin, Leptocottus armatus; the starry flounder, Platichthys stellatus; and the blue sea-perch, Taeniotoca lateralis. The fish were caught in the Straits of Georgia, British Columbia, and kept in aquaria until required. During captivity they were fed pieces of lingcod muscle.

The fish specimen under consideration was placed in a tank slightly larger than itself and sufficient sea-water was added to cover the fish. This usually required between 1 and 2 litres of water. The water was well aerated and was kept at 12°C by placing the tank in a constant flow of cold water. After 24 hours the

fish was removed from the tank and the water acidified with a small amount of HCl and taken down to dryness *in vacuo* at 45°C. The non-protein nitrogenous compounds were largely separated from the inorganic salts with four successive extractions of 80% ethanol. The total volume of ethanol used was one-seventh of the original volume of the sea-water. The ethanol extracts were combined, filtered, and taken down to dryness *in vacuo* at 45°C. The residue was redissolved in water to give a final volume of 50 ml.

This extraction procedure yielded 97% recovery when applied to known amounts of ammonia, urea, TM, TMO, and creatine dissolved in sea-water.

The total nitrogen was estimated by the micro-Kjeldahl method and ammonia, TM and TMO by the method of Ronald and Jakobsen. Urea was estimated by measuring the colour developed with diacetyl monoxime and sodium diphenylamine-p-sulphonate in acid solution. Creatine and creatinine were determined by measuring the colour developed with diacetyl and α-naphthol in alkaline solution.

An experiment was carried out to determine the proportions of the nitrogenous material excreted by the gills and by the kidneys, respectively. A sculpin was employed as the experimental fish and it was found that 77% of the total N was excreted via the gills.

The excreta from seven sculpins, two starry flounder, and a blue sea-perch were investigated and the results are shown in Table I. The total amount excreted varied considerably between fish of the same species, but in all cases the major components of the excreta were ammonia and urea. The predominance of these compounds in the excreta was observed in all three species of fish investigated.

TABLE I - Nitrogen excretion in marine teleosts.

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6*	7*	1	2	1
Weight, g	165	182	191	391	151	245	304	335	310	360
Total N	8.31	7.45	24.88	24.38	6.04	16.22	18.50	22.46	24.52	10.71
Ammonia-N	4.37	4.66	16.77	17.64	4.02	10.11	10.23	19.37	19.97	5.14
Urea-N	1.87	1.53	6.15	4.22	1.02	3.90	3.59	2.58	2.96	4.08
Trimethylamine-N	0.00	0.10	0.39	0.33	0.11	0.46	0.36	0.25	0.05	0.03
Trimethylamine oxide-N	0.18	0.00	0.33	0.93	0.05	0.00	0.04	0.12	0.00	0.04
Creatine-N + creatinine-N	0.03	-	0.23	0.17	0.03	0.18	0.26	0.06	0.30	0.00

Note: All N values in the table are given as mg.
* These fish were newly caught and had not been kept in aquaria prior to collection of excreta.

When these results are converted to percentages of the total N excreted, Table II, it is observed that the ammonia and urea contents of the excreta remained reasonably constant within any one species, although the size of the fish varied considerably. Moreover, the newly-caught fish showed the same percentage composition of ammonia and urea as did those which had lived for a considerable length of time in the aquarium. The same constancy of composition, however, did not hold from species to species. The starry flounder had a greater percentage of ammonia and less urea than the sculpin, and the blue sea-perch had less ammonia and more urea.

TABLE II - Percentage composition of excreta from marine teleosts.

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6	7	1	2	1
Ammonia-N	52.6	62.6	67.4	72.4	66.6	62.3	55.3	86.2	81.4	48.0
Urea-N	22.5	20.5	24.7	17.3	16.9	24.0	19.4	11.5	12.1	38.1
Trimethylamine-N	0.0	1.3	1.6	1.4	1.8	2.8	1.9	1.1	0.2	0.3
Trimethylamine oxide-N	2.2	0.0	1.3	3.8	0.8	0.0	0.2	0.5	0.0	0.4
Creatine-N + creatinine-N	0.4	-	0.9	0.7	0.5	1.1	1.4	0.3	0.1	0.0
Undetermined N	22.3	15.6	4.1	4.4	13.4	9.8	21.8	0.4	6.2	13.2

The average undetermined-N values in the excreta of the sculpin, starry flounder, and blue sea-perch were 13%, 3%, and 13% respectively. The undetermined N was investigated in the sculpin excreta. Tests were made for uric acid and amino acids but neither was detected.

Although generalizations about marine teleosts cannot be made on the basis of results from only three species, the results reported here suggest that ammonia and urea are the main nitrogen-containing compounds excreted by marine teleosts. Together these compounds account for 75% to 98% of the total N. Of the two compounds, ammonia is present in the greater quantities.

TMO accounts for approximately one-third of the total N in the urine of some species. However, the amount of nitrogenous material excreted in the urine is small compared with that excreted by the gills. It is not too surprising therefore to find that the proportion of TMO in the total excreta is relatively small. The low percentage of the compound in the excreta indicates that the compound is not an important non-toxic end product of protein metabolism in marine teleosts as previously suggested. The low and rather varied values are more in accordance with the theory that TMO in marine teleosts is obtained directly from the food, i.e. it is of exogenous origin.

Mr. Murray A. Newman, Curator, Vancouver Public Aquarium Association, kindly provided the fish used during the investigation, and made available the Aquarium's facilities for keeping the fish during the course of the study.

SUMMARY NO. 26

PHOSPHOGLUCOMUTASE, PHOSPHORIBOMUTASE AND PHOSPHOGLUCOISOMERASE IN LINGCOD MUSCLE

Gerard-B. Martin

It is strongly suspected that the anaerobic breakdown of glycogen in fish muscle follows the Embden-Meyerhof pathway as it occurs in mammalian muscle. So far, nobody has made a thorough investigation of this problem. It is intended to study in detail the enzymes catalyzing the different steps from glycogen to lactic acid.

The present report is concerned with the study of phosphoglucomutase, phosphoribomutase and phosphoglucoisomerase in lingcod muscle (Ophiodon elongatus). These three enzymes catalyse the following reactions:

Glucose-1-phosphate (G-1-P) $\xleftrightarrow{\text{Phosphoglucomutase}}$ Glucose-6-phosphate (G-6-P)

Ribose-1-phosphate (R-1-P) $\xleftrightarrow{\text{Phosphoribomutase}}$ Ribose-5-phosphate (R-5-P)

Glucose-6-phosphate (G-6-P) $\xleftrightarrow{\text{Phosphoglucoisomerase}}$ Fructose-6-phosphate (F-6-P).

The presence of phosphoglucomutase in lingcod was previously demonstrated at this Station by Dr. Tarr (Summary No. 2 of this Station's Annual Report for 1957-58). Both phosphoglucomutase and phosphoglucoisomerase were found to be very active in a crude water extract from a frozen lingcod. Different procedures were then tried in an attempt to get a relatively good purification of these two enzymes, heating at different pH values and different temperatures, and fractionation with ammonium sulfate. Ion-exchange chromatography, using DEAE cellulose, was found to be a very good technique for the separation of these two enzymes. This was achieved through the following procedure.

13 g of DEAE cellulose was equilibrated with Tris-HCl buffer (0.01M) at pH 7.0 and then packed in a refrigerated column (1°C), 18 mm in diameter and 30 cm in height. Crude extract (see Summary No. 21 of this Annual Report) from lingcod muscle previously heated to 55°C and lyophilized (1 cc containing 33 mg of protein) was then adsorbed on the column and eluted with gradient elution. The gradient was obtained by means of a single 250-cc mixing flask and a reservoir. Initially the mixing flask was filled with Tris-HCl buffer (0.01M pH 7.0) and the reservoir contained Tris-HCl (0.25M, pH 7.0). A slight pressure was applied to maintain a flow rate of about 0.6-1.0 cc per minute. Fractions of 6 cc were collected using an ordinary type of fraction collector. The protein content was determined on each fraction using the Folin phenol reagent.

Phosphoglucomutase and Phosphoribomutase

It was found that a crude extract could be heated to 65°C at pH 7.0 without losing any appreciable amount of phosphoglucomutase activity, while about 75-80% of the proteins were precipitated and could be centrifuged out. Using ammonium sulfate fractionation, the fraction between 0.6-0.7 saturation was found to contain most of the phosphoglucomutase activity. When fractionating by ion-exchange chromatography on DEAE, phosphoglucomutase came off in the early fractions as is shown in the accompanying figure. A maximum of 3-fold purification was obtained, compared with the crude extract that was put on the column.

The phosphoglucomutase activity was measured by measuring appearance of G-6-P as determined by disappearance of the acid-labile phosphorus of G-1-P. The enzyme reaction was run at pH 7.0 and 30°C for 45 min. The reaction was greatly activated by magnesium and cysteine.

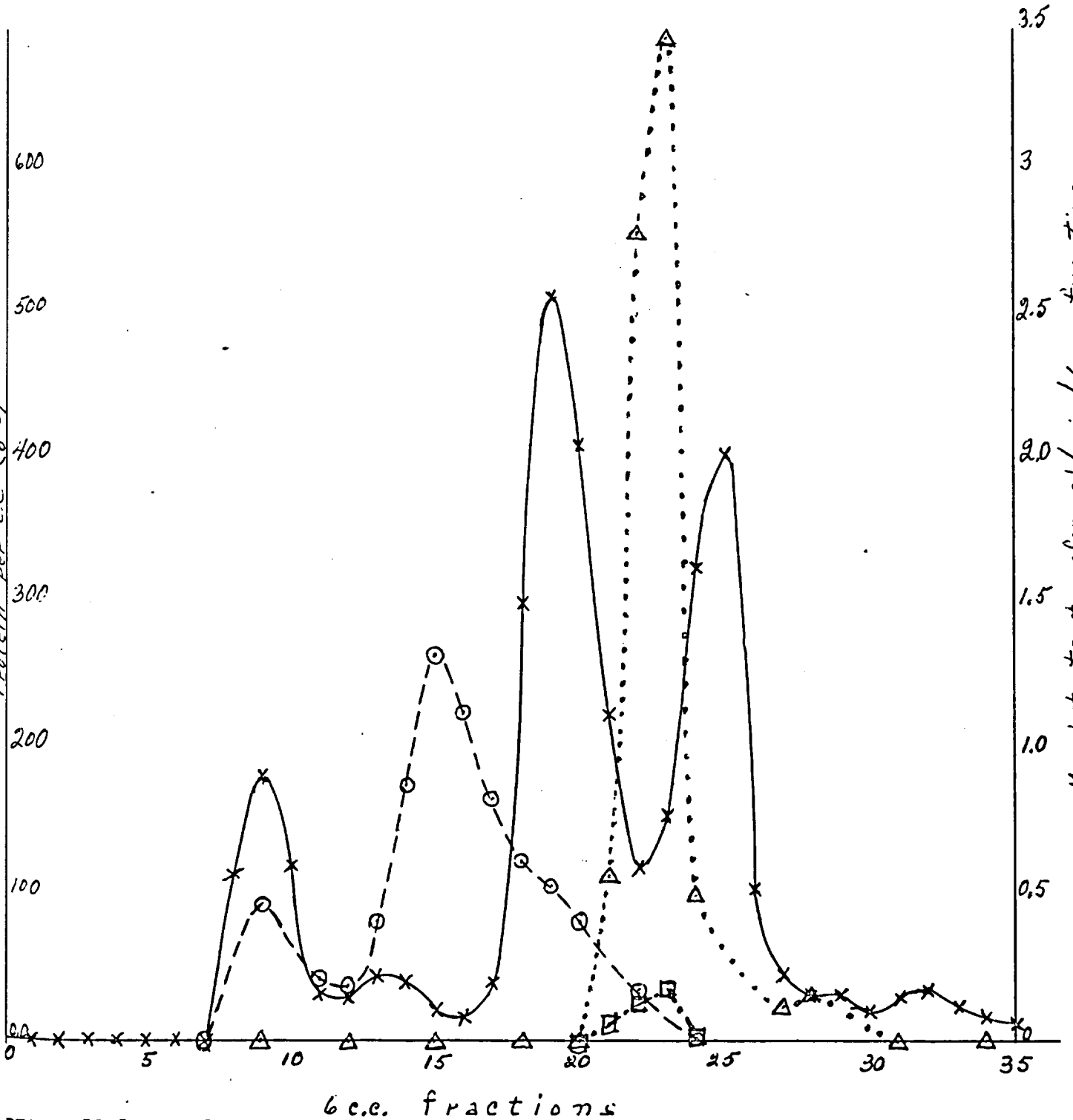
The phosphoribomutase was measured by the disappearance of acid-labile phosphorus of R-1-P. It was eluted in the same fractions as the phosphoglucomutase. This does not necessarily contradict the recent finding of Sable, who claims that these two enzymes are different. On the other hand, it does not confirm the so far general belief that they are identical.

Phosphoglucoisomerase.

The presence of phosphoglucoisomerase was measured by the formation of fructose from G-6-P as substrate, assuming that all the fructose was in the form of F-6-P. The reaction was run at pH 8.0 for 25 min at 30°C. The equilibrium was found to be approximately F-6-P:G-6-P = 4:6, which figure agrees well with previous findings on mammalian muscle. This enzyme showed activity in a very broad pH range, the optimum being around 8.0-9.0. The enzyme was destroyed if heated at 60°C. When partially purified by ion-exchange chromatography, it was found to be completely destroyed by

freezing and thawing, but it was stable at 0°C for a few weeks.

A 27-fold purification was obtained, compared with the crude extract that was put on the column.



DEAE-cellulose column - Elution diagram showing protein concentration (X——X), phosphoglucoisomerase (0----0), phosphoglucomutase (△ ···· △) and phosphoribomutase (□ -·-·- □). Mixing flask: 250 cc of Tris-HCl, 0.01M, pH 7.0. Reservoir: Tris-HCl 0.25M, pH 7.0.

SUMMARY NO. 27

STERILIZATION OF BRINES BY ULTRAVIOLET RADIATIONS

S.W. Roach
J.S.M. Harrison

Two early Progress Reports of this Station (No. 38 p 3, December 1938 and No. 39 pp 16-18, March 1939) dealt with the sterilization of brines with ultraviolet light. A review of this work, which gave promising preliminary results has lead us to further investigate the application of ultraviolet radiations as a sterilizing agent for salt solutions. We have in mind two potential applications: the treatment of refrigerated-sea-water in holding tanks, where the chief source of trouble has been an occasional build up of bacteria in the refrigerated water due to improper operating procedures, and the treatment of the washing water supplies of some fish plants. An encouraging factor is the availability of modern, inexpensive, long-life 'Sterilamps' which emit short-wave ultraviolet rays of high intensity. Approximately 95 percent of the ultraviolet radiations from 'Sterilamp' tubes are in the 2537 Angstrom unit region which is a region in the ultraviolet spectrum very near the peak in germicidal effectiveness. Research Report EL-R-6-1059-3023-1 of the Westinghouse Electric Corporation gives data on sterilizers utilizing 'Sterilamps' for the production of portable water but makes no mention of the treatment of brines which interests us. Using their data along with our own calculations we have built a small sterilizer for our experiments which has the following specifications: a single Westinghouse 'Sterilamp': type G15T8, 18 inches long, rated at 15 watts, operating current 0.3 amps, operating voltage 55 volts, starting voltage 118 volts A.C., U.V. output 3.2 watts (2537 Angstroms), ballast is a 15-watt Fluorescent type. The 'Sterilamp' is positioned inside a quartz glass jacket leaving an air space between lamp and jacket. This air space is intended to maintain the 'Sterilamp' bulb wall temperature as near as possible to its optimum temperature of 41°C (105°F). Direct contact with the brine would prohibit this and reduce the lamp efficiency to 20% of its maximum. Quartz glass is used because of its ability to transmit U.V. efficiently (about 70%). The quartz jacket is in turn enclosed within a copper tube leaving a gap of about 3 mm between the walls through which the treated brine passes. The brine enters at one end of the apparatus and leaves at the other through side openings. The tube ends are gasketted to seal them. The 3 mm gap was selected as a result of tests made using a Beckman spectrophotometer to measure the percent of 2537 Angstrom U.V. penetrating samples of sea-water contaminated with fish protein, blood and bacteria. Three millimeters proved to be about the maximum depth through which the rays would penetrate with sufficient intensity to be effective.

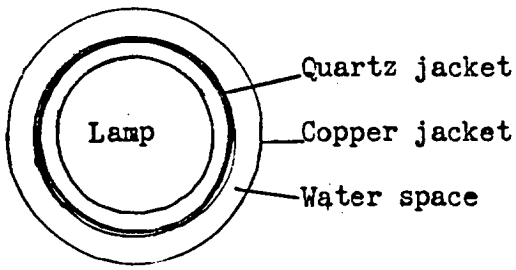
Theory:

The transmission of ultraviolet light follows the relation

$$T = \frac{I_h}{I_o} = e^{-ah}$$

Where T = transmission
I_h = intensity at distance h
I_o = " " " o
a = coefficient of transmission
h = distance from starting surface.

As representative samples of sea-water showed transmissions as low as 6% in two mm and because of the above relation, significantly greater thickness of water would only afford a bypass through the equipment.



OD of quartz jacket	32 mm
ID of copper jacket	39 mm
Thickness of water space	3.5 mm
X section area of water space	3.93 mm
Length of lamp	37.0 cm
Mean surface of water space	413 cm^2
Vol of water space	145 cm^3
Output of lamp	3.2 watts
	3.2×10^6 "
Transmission of glass	70%

In the following experiments the ultraviolet energy has been computed as follows:

Transmission for 2 mm is found by spectrophotometer. From this the coefficient of transmission α is computed:

$$\text{from } T = e^{-ah}$$
$$\log_e T = -ah$$
$$\alpha = -\frac{\log_e T}{h}$$
$$\text{since } h = 2 \text{ min}$$
$$\alpha = -\frac{\log_e T}{2}$$

The average transmission through the water is computed from:

$$T = e^{-ah}$$
$$T_{ave} = \frac{1}{h}\int_0^h e^{-ah}dh = -\frac{1}{ah}\left[e^{-ah}\right]_0^h$$
$$= \frac{1}{ah}\left[1-e^{-ah}\right]$$

since h is average thickness of annular water space

$$= 3.5 \text{ mm}$$
$$T_{ave} = \frac{1}{3.5\alpha}\left[1-e^{-3.5\alpha}\right]$$

$$\text{Time of exposure} = \frac{\text{Vol of water space}}{\text{flow rate}} = \frac{V}{Q}$$

$$\text{UV energy} = \frac{(\text{lamp output})(\text{glass transmission})(\text{water transmission})(\text{time of exposure})}{\text{mean surface area}}$$

$$= \frac{(3.2\times10^6)(.70)Tt}{413} -$$

$$= 5510Tt \quad \frac{\mu w \text{ sec}}{cm^2}$$

Experimental Results:

Experiment 1 - Treatment of sea-water with initial contamination of 8.3×10^6 bacteria/cm^3.

UV transmission in 2 mm = 32.5%

$$\alpha = \frac{-\log_e T}{2} = \frac{-\log_e .323}{2} = \frac{-(-1.124)}{2}$$

$$= .5562$$

$$T_{ave} = \frac{1}{3.5\alpha}\left[1-e^{-3.5\alpha}\right]$$

$$= \frac{1}{3.5(.5562)}\left[1-e^{3.5(.5562)}\right]$$

$$= 43\%$$

Flow rate Q cm^3/sec	µsgpm	Time of exposure sec	UV energy $\frac{\mu w\ sec}{cm^2}$	Bacteria $x10^6/cm^3$	% Kill
282	5.05	.514	1220	1.09	87
98.5	1.76	1.48	3590	1.41	84
24	.43	6.05	14560	0.34	96

Experiment 2 - Treatment of sea-water with initial contamination of 7.4×10^6 bacteria/cm^3.

UV transmission in 2 mm = 72.3%

$$\alpha = \frac{-\log_e T}{2} = \frac{-\log_e 72.3}{2} = \frac{-(-.3243)}{2}$$

$$= .1622$$

$$T_{ave} = \frac{1}{3.5\alpha}\left[1-e^{-3.5\alpha}\right]$$

$$= \frac{1}{3.5(.1622)}\left[1-e^{-3.5(.1622)}\right]$$

$$= 76.5\%$$

Flow rate Q cm^3/sec	µsgpm	Time of exposure sec	UV energy $\frac{\mu w\ sec}{cm^2}$	Bacteria $x10^6/cm^3$	% Kill
493	8.82	.294	1241	3.85	48
372	6.65	.389	1840	6.6	11
282	5.05	.514	2170	2.5	66

The outstanding result of these experiments is that significant kills have been made with very low levels of ultraviolet energy. This offsets the low UV transmission of sea-water contaminated with fish slime and indicates that the method may have practical application in bacterial control in brines.

All viable counts were made by Miss B. A. Southcott.

SUMMARY NO. 28

REFRIGERATED-SEA-WATER - "SILVER VIKING II" J.S.M. Harrison

Observations were made this year on the performance of the steel seiner-halibutter "Silver Viking II". The following was reported in the Annual Report of this Station for 1957-58:

"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."

The doubts expressed here as to the adequacy of resorcinol resin to prohibit solvent attack and the water tightness of the Fiberglas plastic proved valid. The application was a complete failure and the lining has since been dismantled. Plywood linings are now being installed, which will be Fiberglas plastic faced. In spite of these troubles the vessel brought in one-quarter of a million pounds of halibut in marketable condition in two trips.

SUMMARY NO. 29

REFRIGERATED-SEA-WATER - "RUTH G II" S.W. Roach

Summary Nos. 43 and 45 of this Station's Annual Report for 1957-58 mentioned the loss of the troller "RuthG" and the planning of a new steel vessel as a replacement. This new vessel (the "Ruth G II"), which incorporates a refrigerated-sea-water system of the latest design, was launched this year and has been described in detail in one of our reports (Progress Reports of the Pacific Coast Stations, issue No. 112, pp 3-6, December, 1958). The success of this small steel vessel in its first season, together with its attractive appearance, has generated a great deal of interest in steel vessels among fishermen in this area.

SUMMARY NO. 30

SHORE TANK INSTALLATION S.W. Roach

Salmon

The seiner "Silver Viking" (Summary No. 41 of this Station's Annual Report for 1957-58) was sold to the Fisheries Research Board and handed over to the Nanaimo Biological Station early this year. In converting the vessel to function as a research vessel, some of the refrigerated-sea-water equipment was removed. This

included four of the six sea-water tanks, one complete refrigeration unit, the chiller and its circulating pump. The remaining two (forward) tanks were left in place with their individual refrigerating systems intact. Since there were no plans for utilizing the four surplus tanks, which belong to the Industrial Development Service, we obtained permission to conduct further tests with them and arranged to have them installed at a Vancouver salmon cannery. The company management agreed to install the tanks and their auxiliary equipment on the dock outside their cannery at their expense. This arrangement is of mutual benefit since in addition to providing the company with a very useful amount of refrigerated storage space for fish at little cost, it also provided us with opportunity to observe a system of our own design in commercial use at a cannery.

The complete installation is as follows. The four tanks are mounted in line and elevated about 3 feet from the dock floor to permit easy unloading onto a conveyor. Fish are conveyed to the filling trunks on top of the tanks by means of an elevator and conveyor belt. Refrigeration is provided by one 5-H.P. Freon 12 compressor, electrically driven with water-cooled condenser. Brine cooling takes place in the chiller which is located underneath the tanks and closely connected to the $1\frac{1}{2}$" centrifugal circulating pump. The simple piping arrangement consists of lines, each containing a valve, from the bottom of the tanks connecting to a manifold leading to the pump suction. The pump discharge passes through the chiller to a single flexible hose which can be directed into the filling trunk of any of the four tanks. To obtain water of suitable salinity, since the harbour water here is too polluted for use, a mixing tank was connected to the suction side of the pump so that concentrated brine can be bled into the system until the desired salinity is obtained.

Certain difficulties were encountered in completing the installation together with the necessary fish elevators and conveyors so that the season was nearly over before it could be put into use. However, several loads of fish were held in the tanks for short periods and the equipment worked very well. A convincing demonstration of the potential value was the holding of a full load (60,000 lb) of round chum salmon for four days, after which time the fish appeared as fresh as when loaded. Most of these fish were held for later freezing as "silverbrights", and since all local freezing plants were at this time glutted with fish, the tanks proved most useful in saving labour, ice costs, and by preserving quality during a critical period.

This project has provided the following worth-while information relative to the use of refrigerated-sea-water fish-holding in canneries:

1. A simple new method has been demonstrated for dissolving salt when natural sea-water is not available.
2. It has been shown that inexpensive unloading doors of the type we have developed for the small tanks on trollers are equally suitable for these large tanks.
3. For short-term holding it is practical and desirable to use the surplus ice discharged from the fish packers to rapidly cool the fish in the tanks.
4. It has been demonstrated that a 5-HP compressor will provide enough refrigeration to cool at least 60,000 lb of fish, although additional ice for precooling is desirable.

SUMMARY NO. 31

MATERIALS TESTING PROGRAM

P.J.G. Carrothers

New materials continue to become available for use in fishing gear, and some of these have been examined during the past year. This work is a continuation of that reported in Summary No. 46 of this Station's Annual Report for 1957-58. Unfortunately, the reorganizing of fishing gear research on a broader basis has meant a gradual decrease in materials testing activity, although, as the new program develops at the Biological Station at Departure Bay, materials testing will undoubtedly again become a necessary part of the work.

The herring investigation at the Biological Station required gill nets of styles not used in the commercial fisheries. Suggestions for suitable web constructions were first based on extrapolations of test data already at hand. Samples of commercial nylon and Terylene twines, based on these suggestions, were received and were tested to determine their actual properties, and final recommendations were then based on these new test data. The twine samples were found generally to be of good quality, although the limitations of our testing equipment were apparent when evaluating the lighter twines. The severest criticism of these twines is in the use of a multiplicity of arbitrary numbers to identify the different twines. It is strongly recommended that all twine manufacturers use the same standard numbering system for identifying their twines, viz, the system described in CGSB "Specification 55-GP-1 for Nets; Fishing" and elsewhere. Considerable confusion would thereby be avoided.

Information on nylon monofilament gill nets, further to that given in last year's Annual Report, has been gained. The quality of the product has been considerably improved. The wet mesh strength has been increased by more than has the weight, although the web is still not rugged enough for our salmon fishery. The knots in the web have obviously been set, for there was no tendency for the knots to spring open on standing, nor did the knots slip during the test for mesh strength. However, it was not possible to test the knot strength of the monofilament line because unset hand-tied knots slipped undone under loads smaller than those required to break the knotted monofilament. This need to set the knots for satisfactory performance will complicate the procedure for mending the net. The mesh length increased nearly 3% when the netting was wetted; allowance should be made for this when specifying mesh length. The load-extension curves show that this monofilament has about the same low-load nodules as have the multifilament nylons , but, at higher loads, the monofilament is the stiffer. This characteristic causes the monofilament nylon to be less tough than multifilament nylon.

Canadian nylon gill nets for salmon are traditionally made of Type 300 nylon. This is a bright, high-tenacity yarn developed primarily for tire cord. An improvement over Type 300 nylon for tire cord is Type 700 nylon, which has greater strength and somewhat less stretch than its predecessor; and a modification of this new nylon, known as Type 719, has been adapted to the needs of the cordage industry. Braided nylon hanging twines in three sizes were manufactured commercially from Type 719 nylon, and these twines were examined. Test results were compared with the results of previous tests on braided Type 300 nylon hanging twines as follows: the extension at rupture of the 719 twines was about 2/3 that of the 300 twines, and the 719 twines were about 30% stronger than the 300 twines when straight and about 15% stronger when knotted. The lower extension under load of the 719 twines has two adverse effects, even though associated with greater strength. First, the strength efficiency of knotted structures is lower with 719 nylon than with 300 nylon so that some of the strength advantage of the 719 fibre is lost in fishing gear where the twine must be knotted; second, elastic stretch contributes considerably to toughness,

when the 719 twines, despite their greater strength, are only about 75% as tough as the 300 twines when knotted. Thus, although Type 719 nylon is a marked improvement over the Type 300 nylon in its more obvious properties, such as strength, there is some question as to whether or not it has overall advantages for fishing gear.

Knotless netting has been available from Japan for several years, but not until recently have samples sufficiently large for proper test been received. The samples presently considered were made of continuous filament nylon or of staple vinylon. This netting was constructed of two-ply twines in such a way that the plies of the two twines interlaced wherever the twines crossed one another to form the mesh. If an end of twine is free to rotate, then that end can be pulled quite easily out of the intersection. Therefore, cut edges should be hand-selvedged and tears should be mended as soon as possible after they occur. However, contrary to some claims, a twine will not unravel across the net if broken. The continuous filament nylon netting was definitely inferior in performance characteristics to conventional, knotted, continuous filament nylon netting, e.g. it was only about 3/4 as strong on an equal web weight basis. However, the staple vinylon knotless netting was superior to conventional cotton seine web in these same characteristics. Apparently their greater clinging capacity makes staple vinylon yarns satisfactory in knotless netting where continuous filament nylon, with its tendency to slip, is not satisfactory. A bonding agent, such as tar, could improve this situation. Because free twine ends tend to pull out of the twine intersections, knotless netting is likely to prove more satisfactory for seines and traps than for gill nets.

In 1958, a new, high-tenacity nylon became available from Japan and it was deemed advisable to determine its properties. Also, there have been manufacturers' complaints that test data for Type 66 nylon twines published in Industrial Memorandum No. 19 of this Station were too demanding and could not be met by current production. These data were the results of actual tests performed at this Station in previous years, but it seemed advisable to check these results by testing some of the present stock of twines. Consequently, comparative tests were run concurrently on samples of one brand of the new Japanese nylon, of two brands of British and of two brands of Canadian high-tenacity nylon twines. These tests established: (1) the new Japanese nylon is an improvement over the old and is very similar to its British and Canadian counterparts (there was more difference between the two Canadian brands than between the Japanese nylon and any of the British or Canadian twines), and (2) the current production of British and Canadian twines is at least equal to the averages published in Industrial Memorandum No. 19. There was a tendency for current British and Canadian twines to be better than their predecessors but differences generally were not statistically significant.

SUMMARY NO. 32

TESTING AND INFORMATION SERVICE — P.J.G. Carrothers

In some cases of dispute it is desirable to obtain an impartial opinion on the properties of goods. The Board has provided such an opinion to interested parties, on request, on fishing gear materials, even though the work does not contribute to the general program. Also, some testing has been undertaken at the request and for the information of companies and individuals, because a public testing service for many fishing gear materials is not available at the present time in Vancouver. Further, information and opinions have been forwarded, on request, to companies, government offices and individuals from data already at hand. This work is a continuation of that reported in Summary No. 47 of this Station's Annual Report for 1957-58. The following examples illustrate the type of work undertaken.

One Vancouver distributor was not satisfied that his British supplier was shipping goods of the quality ordered. He obtained samples of the nylon gill-net web and a copy of the manufacturer's test data on these materials. We tested the samples and obtained results consistently below those of the manufacturer, probably because of different test procedures. Our results were also generally below the averages of our previous tests on other samples reported in Industrial Memorandum No. 19, although they were not below the normal range. In other words, these samples were below average but up to standard. The distributor then ordered his year's requirements on this basis. During the subsequent fishing season, he received many complaints about the poor quality of his nets. Samples were obtained and tested and found generally to be sub-standard. Tests on twine removed from netting indicated a local weakening at the knots. Fortunately, from our point of view, the Board had purchased some of these nets for the Skeena and some had not been used. Tests on samples from these unused nets did not reveal a local weakening at the knots, indicating that the local damage occurred during use rather than during manufacture. However, these unused nets were sub-standard as to wet mesh strength in all but size #83, and were considerably weaker than the manufacturers' samples tested previously. This low wet mesh strength is partly caused by an abnormally large loss in strength when the netting is wetted. Tests on samples from the Board's nets which had been used revealed two major defects. First, the wet mesh strength had decreased more than is usual for the amount they had been used, corroborating the experience of commercial fishermen. Second, there was an extremely high incidence of slip knots. The netting would be useless on this latter count alone, particularly for experimental fishing, even if the strength had been normal. It has not yet been established whether the unusually large strength loss in use was caused by abnormal fishing conditions or by abnormally susceptible nylon. As a result of this experience specifications have been prepared which can be used in the purchase of nylon gill nets for salmon. It is believed that these specifications can easily be met by competent manufacturers, in fact they are met by most commercial nets now in use.

Several government agencies, both domestic and foreign, have requested information. For example, the Royal Norwegian Consulate General, on behalf of a Norwegian fishing gear manufacturer, inquired concerning the most popular types of fishing nets used in Canada. The Danish Technical Information Service wanted to know more about fish-net manufacturing techniques. The German Institute for Fishing Gear and Materials Research requested catch statistics for different types of gear. The Canadian Customs and Excise branch requested background data for a proposed "made in Canada" ruling. The St. Andrews groundfish investigation requested data on vinylon twine, netting and rope. The Nanaimo herring investigation requested comments on the coir bush rope used by North Sea herring drifters. Also, individuals and commercial firms have requested information. For example, a Deep Bay fisherman wanted technical data on herring web; Pioneer Gold Mines wanted information about the use of copper naphthenate applied to sand bags in situ to retard rot, and Guardian Chemical and Equipment Co. requested information on the desirable properties of fish-net floats.

Also allied to this testing and information service is the cooperation being extended to FAO in their very worth-while efforts to establish standard procedures for testing fishing gear and fishing gear materials.

Other activities included talks to fishery students at the Youth Training School and Fisheries Short Course, Extension Department, University of British Columbia.

SUMMARY NO. 33

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

D.R. Idler
I. Bitners

Recently, a pure race of Fraser River sockeye salmon (Oncorhynchus nerka) was sampled before the fish entered the river (Lummi Island, Wash., U.S.A.), at Lillooet 403 km up river in British Columbia, and at the end of an 1135-km spawning migration (Forfar Creek, B.C.). The data showed that the large number of fish taken (216) and the uniformity of the population permitted an accurate designation of a standard sockeye for both sexes. In effect, the realization of a standard fish permits one to obtain the data which would be obtained if a fish could be analysed, 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.

The first report in this series described the weight and/or length changes which occurred in all major organs and tissues during spawning migration and in addition discussed the changes in plasma cholesterol and electrolyte levels. Subsequent reports discussed the changes in fat, protein, free and esterified cholesterol, nitrogen compounds and water in sockeye salmon flesh. (Summary No. 1 of this Station's Annual Report for 1957-58). (See Table I for sampling and weight data.)

The present report discusses the changes in fat, protein, water and free and esterified cholesterol in the entire eviscerated fish (entire body), exclusive of the flesh, and compares and contrasts the changes with the corresponding changes which occurred in the flesh of the standard fish of either sex. The viscera of Stuart Lake sockeye have been shown to contribute very little fat and protein, 2 to 4%, as compared with the body.

The sockeye salmon were caught in reef nets, anaesthetized in ice water, and bled by severing the caudal artery. The fish were placed in polyethylene bags, stored on "dry ice" and finally were partially thawed overnight and the frozen viscera, including the kidneys, were removed. The head and tail were combined with the interiorly well-scraped skin and scraped bones, and the flesh was kept separate. The frozen samples were divided into four groups for both sexes at each location and were then blended with a silent electric cutter. Representative 1-lb (454-g) samples were heat processed in enamelled cans and used for the analyses described in this report. The calorie (Cal) is the large or kilogram-calorie, and fat is taken as equivalent to 9.3 Cal/g and protein to 4.1 Cal/g.

Changes in Percentage Composition

The water content of the combined head, skin, bones and tail (hereinafter referred to as "combined trimmings") was much smaller than for the flesh for both sexes at Lummi Island and Lillooet but the difference was much smaller at the spawning grounds. The water content of the combined trimmings from both sexes increased even more on a percentage basis than did that of the flesh during the last 750 km of the migration (Table II).

The fat content of the combined trimmings from both sexes was twice as great as that of the flesh at Lummi Island. At Lillooet the combined trimmings had 3 times the fat content of an equal weight of flesh and this decreased to 1.5 times on arrival at the spawning grounds.

The protein content of the combined trimmings from both sexes remained essentially constant throughout the river migration. By contrast, the protein content of the flesh continued to decrease as the fish moved up the river. The ratio of the

protein content of the remainder of the body to that of the flesh was similar for both sexes at each of the three locations, averaging 0.74 at Lummi Island, 0.78 at Lillooet and 0.94 at Forfar Creek.

The total cholesterol levels of the remainder of the body were more than double those of the flesh at all locations for both sexes. Similarly, the ratio of free cholesterol in the remainder of the body to that in the flesh exceeded 2 for both sexes at all locations except Forfar Creek, where the ratio was slightly less than 2.

It has previously been pointed out that the cholesterol content of the flesh of both sexes continually increased during the migration, even though the reserves of fat, protein and plasma cholesterol were depleted so extensively. It can now be seen that the entire body of the fish of both sexes appears to maintain cholesterol at a high level throughout the entire river migration.

TABLE I - Mean values of weights of body of sockeye salmon during spawning migration.

Site	Sex	No of fish	Trimmings	Flesh
			grams	grams
Lummi Island	M	23	881±33*	1467±21
Lillooet	M	40	871±10	1302±51
Forfar Creek	M	25	1057±10	1304±40
Lummi Island	F	33	785±15	1335±13
Lillooet	F	40	765±20	1135±30
Forfar Creek	F	55	834±12	934±10

* Mean ± standard error.

TABLE II - Proximate analyses and cholesterol content of the body of migrating sockeye salmon.

		Location					
		Lummi Island		Lillooet		Forfar Creek	
Components	Sex	Trimmings	(Flesh)*	Trimmings	(Flesh)	Trimmings	(Flesh)
Moisture %	M	60.2±0.7**	(67.2)	64.5±0.2	(70.7)	77.1±0.4	(78.2)
	F	59.4±0.3	(67.0)	64.8±0.5	(71.0)	76.3±0.3	(78.0)
Fat %	M	20.1±0.8	(9.34)	15.9±0.5	(5.07)	4.7±0.3	(3.23)
	F	21.7±0.5	(10.55)	15.2±0.2	(5.59)	4.3±0.3	(2.70)
Protein N %	M	2.57±.01	(3.51)	2.52±.01	(3.28)	2.55±.01	(2.68)
	F	2.60±.02	(3.51)	2.64±.02	(3.38)	2.63±.02	(2.84)
Free cholesterol mg/100 g	M	60.9±3.5	(25.7)	65.6±1.0	(30.9)	59.8±1.0	(31.1)
	F	63.9±0.6	(25.6)	64.8±0.5	(30.6)	58.3±0.3	(34.7)
Total cholesterol mg/100 g	M	71.2±3.1	(29.2)	83.5±2.8	(34.1)	78.2±0.9	(36.7)
	F	70.2±0.6	(29.0)	79.7±2.4	(32.6)	76.2±2.2	(35.9)

* Flesh values included for comparative purposes.
** Mean ± standard error.

Changes in the Standard Fish

Water

The percentage composition data shown in Table II and the weights of the combined trimmings (and of the flesh) for the standard fish (Table I) were employed to calculate the changing composition of the standard fish (Table III).

TABLE III - Fat, protein and cholesterol in the body of migrating sockeye of standard length.

Flesh component	Sex	Location: Lummi Island Trimmings	Lummi Island (Flesh)	Lillooet Trimmings	Lillooet (Flesh)	Forfar Creek Trimmings	Forfar Creek (Flesh)
		grams	grams	grams	grams	grams	grams
Moisture	M	530	(986)	562	(921)	815	(1020)
	F	466	(894)	496	(806)	636	(729)
Fat	M	177	(137)	138	(66)	50	(42)
	F	170	(141)	116	(64)	36	(25)
Protein	M	143	(322)	138	(267)	168	(219)
	F	128	(294)	126	(240)	138	(165)
Free cholesterol	M	0.537	(0.377)	0.571	(0.402)	0.632	(0.406)
	F	0.502	(0.342)	0.496	(0.347)	0.486	(0.324)
Total cholesterol	M	0.627	(0.428)	0.727	(0.444)	0.826	(0.479)
	F	0.551	(0.387)	0.609	(0.370)	0.636	(0.335)
Ratio of free to total cholesterol	M	0.86	(0.88)	0.79	(0.91)	0.76	(0.85)
	F	0.91	(0.88)	0.81	(0.94)	0.76	(0.97)

The standard female lost 4% of its body water between Lummi Island and Lillooet and regained it between Lillooet and Forfar Creek (Table III). The flesh consistently lost water; 9.8% between Lummi Island and Lillooet and 9.6% between Lillooet and Forfar Creek. The remainder of the body gained 6.4% water between Lummi Island and Lillooet and 28% between Lillooet and Forfar Creek. The loss of water by the flesh is thus balanced by the water uptake of the remainder of the body resulting in no net loss of body water during the river migration.

The standard male lost 2% of its body water between Lummi Island and Lillooet and gained 24% between Lillooet and Forfar Creek for a net increase of 21%. The flesh lost 6.6% and gained 10.7% water between Lummi Island and Lillooet, and Lillooet and Forfar Creek, respectively, for a net increase of only 3.4%. The remainder of the body gained 6% water between Lummi Island and Lillooet and 45% between Lillooet and Forfar Creek for a net increase of 54%. It can thus be seen that the flesh is secondary to the remainder of the body in contributing to the increased water content of the body of the standard male during the river portion of the spawning migration. The flesh gains only 34 g of water as compared to 285 g by the remainder of the body.

Cholesterol

The standard male showed a steady increase in both the free and total cholesterol of the trimmings as the fish moved up the river (Table III). The standard

female showed a steady increase in total cholesterol of the trimmings as the fish moved up the river but the free cholesterol levels decreased slightly.

The standard female showed a constantly decreasing ratio of free to total cholesterol in the combined trimmings as the fish moved up the river (Table III). By contrast the ratio of free to total cholesterol in the flesh showed a steady increase at the same locations. The standard male also showed a constantly decreasing ratio of free to total cholesterol in the trimmings as the fish moved up the river; however, the ratio of free to total cholesterol in the flesh of the male reached a maximum at Lillooet.

The changes in flesh cholesterol (Table II) of the standard fish have been discussed in a previous report (Summary No. 2 of this Station's Annual Report for 1957-58).

Comparison of the Flesh to the Remainder of the Body as a Source of Fat

The flesh was the major source of fat for both sexes from Lummi Island to Lillooet (Table IV). The standard female used 41% more fat from the flesh than from the rest of the body while the male used 81% more from the flesh. The source of fat reversed from Lillooet to Forfar Creek. The female used 102% more fat from the remainder of the body as compared to the flesh while the male used 266% more from the remainder of the body. Over the entire migration route from Lummi Island to Forfar Creek the standard female drew 16% more fat from the remainder of the body as compared to the flesh while the male drew 35% more.

TABLE IV - Changes in fat and protein in the body of migrating sockeye salmon of standard length.

		Change in weight			
		Fat		Protein	
Site	Sex	Flesh	Trimmings	Flesh	Trimmings
		milligrams per kilometre of migration			
Lummi Island to Lillooet	M	-176	- 97	-137	-12
	F	-189	-134	-134	- 5
Lillooet to Forfar Creek	M	- 32	-117	- 65	+40
	F	- 53	-107	-101	+16
Lummi Island to Forfar Creek	M	- 82	-111	- 89	+22
	F	-100	-116	-112	+ 9

Comparison of the Flesh to the Remainder of the Body as a Source of Protein

The flesh was the major source of protein for both sexes in both segments of the river (Table IV). From Lummi Island to Lillooet the standard fish of both sexes used a small amount of protein from the remainder of the body. From Lillooet to Forfar Creek the portions of the body exclusive of the flesh of both sexes gained in total protein. The overall result from Lummi Island to Forfar Creek was a gain in protein of the remainder of the body for the standard fish of both sexes and the increase was three times as great for males as for females.

Comparison of Flesh Fat and Protein Consumption

The standard female withdrew 41% more fat than protein from the flesh between Lummi Island and Lillooet while the male withdrew 28% more fat (Table IV). The consumption of flesh fat as compared to flesh protein reversed between Lillooet and Forfar Creek; the standard female withdrew 91% more flesh protein than fat and the male withdrew 103% more flesh protein. The overall effect between Lummi Island and Forfar Creek was that on a weight basis, 8.5% and 12% more protein than fat was withdrawn from the flesh of the standard male and female, respectively.

Relative Fat, Protein and Total Energy Expenditures from the Entire Body of Both Sexes per Standard Fish

The standard female expended 132% and the standard male 83% more fat than protein from the entire body between Lummi Island and Lillooet. From Lillooet to Forfar Creek the female expended 88% more fat than protein while the male expended 495% more fat.

The standard female expended 18.1% more fat and 6.7% less protein from the entire body than did the male over the 403-km migration from Lummi Island to Lillooet. This resulted in an expenditure of 13.5% more energy from the body of the female as compared to the male.

The standard female expended 6.6% more fat and 247% more protein from the entire body than did the male over the 749-km migration from Lillooet to the spawning grounds. This resulted in an expenditure of 23% more energy from the body of the female as compared to male.

During the entire migration from Lummi Island to Forfar Creek the female expended 11.4% more fat and 53.7% more protein from the entire body than did the male. This resulted in an expenditure of 17.4% more energy from the body of the female as compared to the male.

Relative Fat, Protein and Total Energy Expenditures from the Entire Body of Both Sexes per Unit Weight of Live Fish

The preceding discussion does not take into account the differences in the live weight of the standard fish of the two sexes. The average live weight of the standard female was 2266 g and of the male 2468 g, from Lummi Island to Lillooet. The energy consumed from the body of the standard female was thus 1.57 Cal/kg/km as compared to 1.28 Cal/kg/km for the male or 23% more for the female than for the male.

From Lillooet to the spawning grounds the average live weight of the female was 2162 g and of the male 2477 g. The energy consumed from the body of the standard female was 0.85 Cal/kg/km as compared to 0.60 Cal/kg/km for the male or 42% more for the female than for the male.

In order to calculate the energy change from Lummi Island to Forfar Creek in terms of energy consumption per unit of live weight, it is necessary to consider the time the fish spent in the two phases of the migration. Lummi Island to Lillooet represents 10 days of travel at an average weight for males of 2468 g and 2266 g for females. Lillooet to Forfar Creek is 17 days and the average male weight 2477 g as compared to 2162 g for the female. Thus the best average weight of the standard male during the migration was 2475 g while that of the female was 2200 g. The energy consumed from the body of the standard female over the entire migration was 1.1 Cal/kg/km as compared to 0.84 Cal/kg/km for the male or 32% more for the female than for the male.

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. 34

MARINE STEROLS: ISOLATION OF 7,24(28)-ERGOSTADIEN-3β-ol FROM STARFISH

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

This investigation was started last year (Summary No. 14 of this Station's Annual Report for 1957-58). The sterols of starfish are generally conceded to be entirely of the C-7-unsaturated type, and 7-cholestenol, previously found in mammalian skin, recently has been isolated. Hitodesterol has been shown to be identical with α-spinasterol (7,22-stigmastadienol). 7-Stigmastenol a recognized plant sterol, has also been found in starfish. Indeed, the sterols of starfish have probably been more thoroughly investigated than have those of any other marine invertebrate.

Previous reports in the present series have described the isolation, characterization and partial synthesis of 24-methylenecholesterol and 24-dehydrocholesterol. The former sterol has been suggested to lie on a biochemical pathway common to sterols of the cholesterol (C-27) and ergosterol (C-28) type. The structure of the latter sterol suggests that the side-chain double bond of lanosterol has remained unchanged while the ring double bond has migrated from the 8(9) to the 5-position. The structural similarity of the new starfish sterol to 24-methylenecholesterol is apparent. If sterols with a C-7-double bond represent a transition between lanosterol and cholesterol, then the present sterol is of considerable interest because it not only has the C-7-double bond but also the 24-methylene group. The recently reported isolation of 4α-methyl-7,24(28)-stigmastadien-3β-ol from grapefruit suggests a link between the triterpenes and fucosterol in the C-29-series. By analogy the new starfish sterol would represent the next intermediate in the transition from triterpenes to 24-methylenecholesterol in the C-28-series. Further evidence that the C-7-double bond can be introduced prior to the removal of the 4-methyl group is afforded by the isolation of 4α-methyl-7-cholesten-3β-ol from rat feces and a cactus.

The sterols of marine invertebrates are of particular interest because they offer an isolation-characterization approach to the problem of sterol biogenesis and metabolism. The studies of these organisms are clearly showing that at least in most animals the evolutionary trend is toward cholesterol as the major or sole sterol component of most tissues and organs of the organism. These lower forms of animal life provide a means of studying sterols which thus far have been difficult or impossible to obtain in detectable amounts from more advanced forms of life such as the more common experimental animals.

The sterol reported in this summary was isolated by chromatography of the steryl azoates (p-phenylazobenzoyl esters), prepared from the sterols of the starfish Pisaster ochraceus, on silicic acid-Celite. The isolation of formaldehyde in good yield, following ozonolysis of the sterol, established the presence of a terminal methylene group and this was confirmed by the presence of a strong band at 890 cm^{-1} and a band of medium strength at 1640 cm^{-1} in the infrared spectra. The sterol and certain of its derivatives have molecular rotations (Table I) consistent with those of a C-7-double bond. The terminal methylene grouping was established to be in the 24(28)-position employing acid rearrangement followed by ozonolysis (see accompanying figure). A terminal methylene group at this position is known to not greatly influence the optical rotation of the sterol molecule. The modified Liebermann-Burchard reaction confirmed the presence of the double bond in the 7-position. It has previously been reported that the C-7-double bond takes up 2 moles of perbenzoic acid, under the conditions employed, and as expected the new sterol consumes a total of 3 moles of oxygen. Catalytic reduction of the sterol with Adams catalyst in glacial acetic acid resulted in the formation of a $\Delta^{8(14)}$-sterol and the uptake

of one mole of hydrogen. The 8(14)-double bond is readily characterized by means of the modified Liebermann-Burchard reaction and the "reduction" product of zone 2 sterol was thus established to have an 8(14)-double bond. The sterol was confirmed to be in the C-28-series by its molecular weight which was determined from the saponification equivalent of the acetate under carefully controlled conditions.

TABLE I - Properties of Zone 2.

	M.p.,°C	$[\alpha]_D$	M D
Sterol	131	+ 6.0°	+ 23.9°
Acetate	140	+ 6.4°	+ 28.2°
Benzoate	160	+ 11.9°	+ 58.0°

Preparation of crude sterols. The sterol content of a sample of macerated whole starfish weighing 755 g was obtained as follows: after moisture, 76.8%, had been determined on an aliquot, the material was extracted by shaking with 3 batches of acetone. The oil, 4.49 g (0.60%) was treated with acetone to remove phospholipids, 1.00 g (22.3%), and the residue yielded 1.01 g (22.5%) of non-saponifiable material. When this was chromatographed on an alumina column, 328 mg (32.5%) of crude sterol was obtained. In one experiment the gonads and digestive organs of mixed male and female animals were separated from the rest and found to contain 88.4% of the total sterols.

Chromatography. The azoyl esters of the crude sterol mixture were chromatographed on silicic acid-Celite columns with the use of 5.5:1 petroleum ether-benzene developer as previously described. Five zones developed. A column of double length (50 cm) was necessary to completely separate zones 3 and 4 from each other. Starting with the uppermost zone the composition was: zone 1, 9.0%; zone 2, 19.2%; zone 3, 29.5%; zone 4, 39.8%, and zone 5, 2.5%.

Zone 2 sterol and derivatives. The azoyl ester was crystallized from benzene-ethanol and hydrolyzed. The acetate was prepared and crystallized once from aq. ethanol. The acetate contained 0.3% of provitamin D as calculated from the ultraviolet spectrum. This was removed with maleic anhydride as previously described. After several crystallizations from aq. ethanol, the acetate melted at 140°C, $[\alpha]^{22}_D$ + 6.4°.

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 81.80; H, 10.66.

The acetate was hydrolyzed and the free sterol crystallized from aq. ethanol, m.p. 131°, $[\alpha]^{22}_D$ + 6.0°

Anal. Calcd. for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.28; H, 11.82.

The benzoate was crystallized from acetone, m.p. 160°, $[\alpha]^{22}_D$ + 11.9°; molecular rotational differences: 7,24-Ergostadien-3β-ol, Δ^{Ac} + 4, Δ^{Bz} + 34; Δ^7-sterols, Δ^{Ac}-15 ± 15, Δ^{Bz} + 20 ± 14.

Anal. Calcd. for $C_{34}H_{48}O_2$: C, 83.55; H, 9.90. Found: C, 83.64; H, 9.86.

Saponification equivalent. Zone 2 acetate, 257.766 mg, was saponified in 5.00 ml of 0.04736 N alcoholic NaOH for 1 hour and the residual alkali was back-titrated with 17.95 ml of 0.09968 N HCl. A correction factor, 0.9935, was employed

which was derived from several determinations of the molecular weight of highly purified cholesteryl acetate.

Anal. Calcd. for $C_{30}H_{48}O_2$: Mol. wt, 440.7. Found: Mol. wt, 442.6.

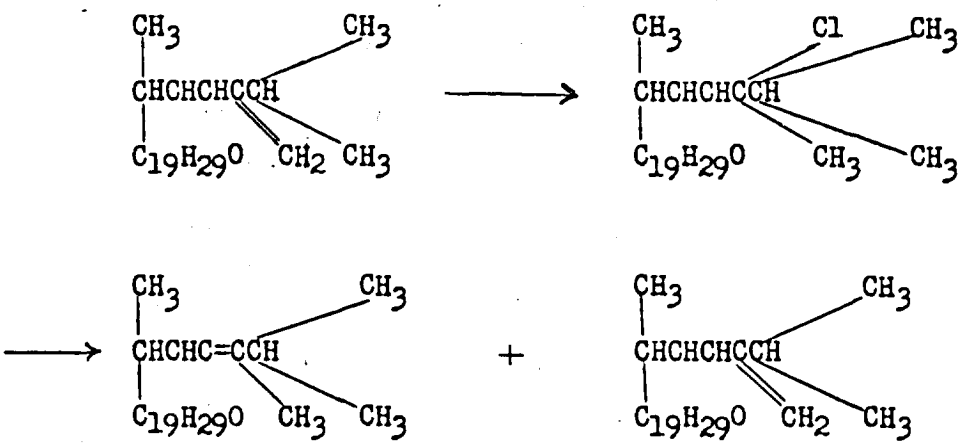
Perbenzoic acid titration. On standing for 5 days at -5° in an excess of perbenzoic acid in $CHCl_3$, 19.006 mg of zone 2 sterol consumed 2.11 mg of oxygen. The theoretical uptake for three atoms of oxygen is 2.07 mg.

Ozonolysis. Zone 1 sterol (100 mg) was suspended in 4 ml of acetaldehyde-free acetic anhydride - acetic acid (4:1) and cooled in an ice-bath. Ozonated oxygen was passed through the suspension at a rate of 10 mg ozone/l of oxygen until all of the solid was dissolved. Water and zinc dust were then added and the mixture was heated in a water-bath in order to decompose the ozonide. The mixture was steam distilled into a 0.5% dimedone solution. The pH of the solution was adjusted to 5.8 and the precipitate collected.

The dimedone derivative weighed 52.8 mg (71.8% of the theory for one methylene group), recrystallized m.p. 189°, mixed m.p. 190° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.91; H, 8.49.

The filtrate from the dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazine solution, which was then extracted with ethyl acetate. No hydrazone was obtained.



Rearrangement with HCl. Zone 2 acetate (430 mg) was dissolved in 5 ml of $CHCl_3$ and HCl gas was led through the solution for 6 hours. The solvent was evaporated and the residue was boiled for 10 hours with 6 ml of acetic anhydride. The solution was then diluted with water, the precipitate collected and chromatographed on an alumina column, from which 380 mg of material was recovered.

The product from the column was suspended in 10 ml of acetic anhydride - acetic acid (4:1) and ozonized as described above. The dimedone derivative weighed 71 mg (28.3% calculated as acetate), m.p. 189° after crystallization, mixed m.p. 189° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.85; H, 8.89.

The remaining dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazine solution. The solution was extracted with ethyl acetate and the evaporation

residue chromatographed on a silicic acid-Celite column (2:1). Skellysolve C-benzene (2:1) moved a band which was eluted (66 mg representing 28.7% calculated as acetate) and crystallized with aq. methanol, m.p. 123°, mixed m.p. 123° with authentic methyl isopropyl ketone.

Anal. Calcd. for $C_{11}H_{14}O_4N_4$: C, 49.62; H, 5.30. Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.75. Found: C, 49.68; H, 5.26.

The 2,4-dinitrophenylhydrazone of the ozonolysis product and of methyl isopropyl ketone had identical R_f values on a descending chromatogram run for 2.5 days in isopropyl alcohol-water (1:1) on Whatman No. 1 filter paper. Ethyl isopropyl ketone, isovaleraldehyde, methyl isopropyl acetaldehyde and ethyl isopropyl acetaldehyde had lower R_f values.

Mr. A. P. Ronald performed the saponification equivalent determination, the perbenzoic acid titration and the determination of rotations and C-H values.

SUMMARY NO. 35

MARINE STEROLS: COMPOSITION OF VARIOUS INVERTEBRATES

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

A number of marine invertebrates have been collected and the composition determined in regard to fat, phospholipids, nonsaponifiable portion and sterols. The oil was obtained by extracting three times with acetone. The nonsaponifiable portion in most instances was chromatographed on alumina (Brockmann Grade II) from which the sterol fraction was eluted with methylene chloride. Azoyl esters were chromatographed on silicic acid-Celite columns as described in previous reports.

	1	2	3	4	5	6	7	8	9
Name	Brine-shrimp	Crinoid	Sea-cucumber	Tunicate	Sea-anemone	Sea-urchin	Purple sea-urchin	Sun-flower star-fish	Chiton
A. Fat % of wet wt	0.65	0.72	0.17	0.83	0.58	0.65	3.70*		0.45*
B. Phospholipids % of A			56.6	34.4		33.8	15.5		25.4
C. Nonsap % of A	5.4	21.2	14.2	18.0	22.9	12.0	11.2	14.5	17.5
D. Sterols % " C	69.1	36.6	7.0	21.4	66.9	47.6	54.5	41.6	45.4
E. Moisture % of wet material				86.1				81.40	

	10	11	12	13	14	15	16	17	18	19
Name	Peri-winkle	Chiton	Star-fish	Nudi-branch	Octopus	Sea-cucumber	Sea-pen	Isopod	Bivalve	Sand-dollar
A.	2.24*	1.46*	0.85	0.48	0.16	2.13	0.55	2.80	1.29*	0.51
B.	16.0	18.5	21.7	9.6	5.6	18.3	25.5	49.9	8.9	
C.	16.8	19.6	26.6	33.6	47.6	22.5	29.5	7.3	26.6	
D.	65.1	49.0	28.6	47.5	14.4	14.4	46.1	29.2	46.0	9.35**
E.				60.0	89.3		86.8	75.2		

* Fat content as part of organic material (shell removed).
** Sterol content as part of fat.

Appendix to Table

1. Brine-shrimp (Artemia)
   Phylum: Arthropoda
   Class : Crustacea

The sterol azoates were chromatographed and separated into two zones. The upper zone was very small.

Lower zone:	Sterol	m.p. 145°,	$[\alpha]_D$ - 35.7°
	Acetate	" 110-11°,	" - 41.4°
	Benzoate	" 144 (170)°	

The lower zone is principally cholesterol.

2. A crinoid.
   Phylum: Echinodermata
   Class: Crinoidea

The crude sterols when crystallized out of aq. ethanol melted at 120-122°. The sterol azoates developed on a chromatogram into one wide zone, which was cut in half. The lower half melted when hydrolyzed at 125-133° after two crystallizations from aq. methanol. The infrared spectrum of the upper half shows absorption both at 890 cm^{-1} and 975 cm^{-1} (evidence for a terminal methylene group and a C-22(23) double bond respectively).

3. Sea-cucumber (Stichopus californicus)
   Phylum: Echinodermata
   Class: Holothuroidea

4. Tunicate (Halocynthia Haustor)
   Phylum: Chordata
   Class: Tunicata

The crude sterols were obtained by crystallization of the nonsaponifiable portion from aq. methanol, m.p. 120-125°, after two crystallizations m.p. 119-122.5°.

5. Sea-anemone (Metridium senile)
   Phylum: Coelenterata
   Class: Anthozoa

The m.p. of the crude sterols was 139.5-140.5°. The azoates separated into three zones.

	Sterol m.p.	Acetate m.p.	I.R. absorption
Zone 1	132-3°	126°	890 cm^{-1}
" 2	130-1°		975 "
" 3	144-5°		

6. Sea-urchin (Strongylocentrotus Drobackiensis)
   Phylum: Echinodermata
   Class: Echinoidea

The crude sterols were obtained by crystallization of the nonsaponifiable portion, m.p. 140-141°. The azoates were chromatographically separated into three zones.

7. Purple sea-urchin (Stronglyocentrotus franciscanus)
   Phylum: Echinodermata
   Class: Echinoidea

The azoates separated into three chromatographic zones which were hydrolyzed:

Zone 1	10.5%	m.p.	125-128° IR absorption 975 cm^{-1}
" 2	6.4%	"	115-117°
" 3	83.1%	"	145-146°

8. Sunflower starfish (Pycnopodia helianthoides)
   Phylum: Echinodermata
   Class: Asteroidea

The azoate chromatogram of this starfish was similar to that of the purple starfish (see Summary No. 34 of this Annual Report).

9. A chiton
   Phylum: Mollusca
   Class: Amphineura

10. Periwinkle (Thais lamellosa)
    Phylum: Mollusca
    Class: Gastropoda

11. Chiton (Katherina tunicata)
    Phylum: Mollusca
    Class: Amphineura

The azoylesters were separated into two zones.

12. A starfish
    Phylum: Echinodermata
    Class: Asteroidea

13. A nudibranch
    Phylum: Mollusca
    Class: Gastropoda

14. An octopus
    Phylum: Mollusca
    Class: Cephalopoda

The azoyl esters were separated into two zones.

15. A sea-cucumber
    Phylum: Echinodermata
    Class: Holothuroidea

16. Sea-pen (Leioptilum quadrangulare)
    Phylum: Coelenterata
    Class: Anthozoa

The azoyl esters separated into four chromatographic zones:

	% of total	Sterol m.p.	Acetate m.p.	% $\Delta^{5,7}$	IR Absorption
Zone 1	5.9	160-110°	119-129°	94.8	
" 2	22.2	116-117°		9.6	
" 3	22.2	125-126°	127-128°	1.2	975 cm^{-1}
" 4	49.7	140-142°	116-117°	-	

17. Isopod (Exosphaeroma oregonensis)
Phylum: Arthropoda
Class: Crustacea

The crude sterol was crystallized once from aq. methanol, m.p. 145-6°. The acetate when crystallized once melted at 115°.

18. Rockoyster (Hinnites giganteus)
Phylum: Mollusca
Class: Pelecypoda

19. Sand-dollar (Echinarachnius parma)
Phylum: Echinodermata
Class: Echinoidea

The azoyl esters separated into two chromatographic zones. The lower zone may consist of two compounds.

Upper zone: Sterol m.p. 125-129°
Lower " : " " 143-146°.

The upper zone sterol absorbs at 890 cm^{-1} in the IR spectrum, characteristic of a terminal methylene group. Calculated from the UV spectrum the sterol contains 22.5% of a $\Delta^{5,7}$-sterol.

SUMMARY NO. 36

ANDROGEN CONTENT OF SOCKEYE SALMON DURING SPAWNING MIGRATION

D.R. Idler
H. Tsuyuki
I. Bitners

The fish used in this study were male sockeye salmon captured at three locations during the 1957 Stuart Lake run (Summary No. 1 of this Station's Annual Report for 1957-58).

Milt samples (1000 g) of sockeye captured at Lummi, Lillooet and Forfar were extracted by the method of Gallagher and Koch with modifications. The tissue was thawed and homogenized with a sufficient quantity of 95% ethanol. The suspension was boiled for 5-10 minutes to coagulate proteins which may clog the continuous extractor. The clear supernatant was siphoned off and the suspension poured into a double thickness cheese-cloth sack in a 2-litre capacity continuous extractor. The turbid filtrate was re-cycled through the cheese-cloth several times till the filtrate became clear. Now the extractor was charged with 2 L. of absolute ethanol and the sample extracted continuously for four days.

The combined alcoholic extracts were concentrated to a volume of 3-400 ml (mostly water) and extracted three times with equal volumes of benzene. The combined benzene extracts were concentrated, re-dissolved in small portions of alcohol, and

again concentrated. This alternate solution in alcohol followed by concentration was repeated three times to remove as much of the benzene as possible. The residue was suspended in 1 litre of warm acetone, and the sticky suspension was stirred with a Hirschberg stirrer for 8 hours at -10°C. The solids were allowed to settle overnight, as much of the supernatant as possible was poured off and the remainder filtered. The residue was again suspended in a smaller volume of acetone, stirred for four hours, allowed to settle for 2 hours and the mixture filtered.

The combined acetone extracts were concentrated and the residue dissolved in 175 ml of hexane, 125 ml of 70% EtOH added and the mixture shaken in a separatory funnel. The troublesome emulsion which formed was finally broken by a combination of heat and centrifugation. The 70% EtOH layer was extracted five more times with hexane. The combined hexane phases were then extracted with 75 ml of 70% EtOH and in each case the alcohol layer was washed five times with fresh hexane. The 70% ethanol extracts were concentrated and the residue suspended in ether and extracted with 10 ml of 10% NaOH solution. The emulsion was again broken by centrifugation. The NaOH layer was washed five times with fresh portions of ether and the ether layers washed repeatedly with water. The ether phase was concentrated and the residue dissolved in warm chloroform. The remaining solids were transferred to the chloroform layer with a little water. The chloroform layer was washed once with water and the organic phase dried by shaking with anhydrous magnesium sulfate. After standing for 2 hours the drying agent was filtered off, washed well with anhydrous chloroform, and filtrate concentrated to a small volume under vacuum and finally quantitatively transferred to a 1-ml volumetric flask. This material was used for bio-assay.

Chick Bio-assay

Single-comb white male Leghorns hatched on June 27 were put on experiment July 1. Testosterone propionate was made up in corn oil at concentrations of 83.9, 143.1 and 289.7 µg/ml and 0.02 ml of each solution was applied to each comb for a period of 9 days. The control chicks were treated with 0.02 ml of corn oil per day. The entire alcohol soluble testes fraction from each location was made up to 1.0 ml in corn oil and 0.02 ml applied to each comb as above. The results are shown in the accompanying Table.

TABLE - Bio-assay of salmon testes extracts.

Sample	No. of chicks	Body weight (g)	Comb weight (mg)	Comb weight / Body weight (mg/100 g)
Testosterone				
0	9	87.0±4.17**	28.6±3	32.2
83.9*	9	71.0±3.42	37.6±3	52.6
143.1	9	79.0±2.96	68.0±7	85.0
289.7	8	87.0±2.92	150.1±20	171.3
Lummi Is	5	88.0±1.76	39.0±4	44.1
Lillooet	5	90.0±2.00	52.2±4	58.0
Forfar Creek	5	95.0±3.27	61.6±4	64.5

* Micrograms of testosterone propionate per ml of corn oil.
** Mean ± standard error.

The testes showed testosterone propionate equivalents of 41 µg, 106 µg and 133 µg/kg for Lummi Island, Lillooet and Forfar, respectively. Since all the salmon extracts gave a comb growth response most nearly approximating the lowest level of

the testosterone standard, the increment between this standard and the control group was used in the calculations. The hexane fractions showed no activity.

A previous bio-assay (Dr. W. Hoar, Dept. of Zoology, U.B.C.) of the androgen content of testes from spawning chum salmon (Oncorhynchus keta) gave a response equivalent to approximately 73 µg of testosterone propionate per kg of testes. The results obtained in the present study demonstrate a significant increase in the androgen content of the testes with increasing sexual maturity for the first 250 miles of the river migration ($p = .01-.05$ as obtained by the "t" test). The experiment should be repeated using 2-3 times the quantity of testes (ca 75-100 fish) in order to determine whether the apparent increased androgen content during the final 415 miles of the river migration is significant.

The authors wish to thank Dr. J. Biely and Mrs. B. March, of the Poultry Science Dept., U.B.C., for conducting the bio-assay.

SUMMARY NO. 37

BIOSYNTHESIS OF MARINE STEROLS

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

A preliminary report of this investigation was given in Summary No. 16 of this Station's Annual Report for 1957-58.

The biosynthesis of C-28 sterols from acetate and from other precursors has been demonstrated in yeast by several workers and it has been suggested that C-27 and C-28 sterols are biosynthesized from a common precursor. The present investigation concerns the biosynthesis of C-28 sterols isolated from starfish (Pisaster ochraceus) (Summary No. 1 of this Annual Report) and from a number of molluscs and the metabolic fate of exogenous sterols in these animals.

Starfish feed on molluscs, among them oyster, mussel and clam, which have been found to contain both Δ^5-monounsaturated C-27 sterols and diunsaturated C-28 sterols. As a tracer sterol the readily available 4-C^{14}-cholesterol was used in this investigation. Difficulty was encountered in introducing the compound to the starfish. In the first experiment the labelled sterol was dissolved in a few drops of salmon oil and the oil was injected with a needle into the stomach of the starfish.

The loss of oil was too great, therefore, in the following experiments (experiments 2-4) the oil was injected into the soft part of a live mussel, which was then fed to a starfish that had been kept in an aquarium for some time without food. The mussel was usually consumed in less than 10 minutes.

In experiment 2 a starfish was fed two small mussels the second and third day after the first radioactive feeding, followed by another radioactive feeding the fifth day. The starfish was sacrificed 8 days after the first radioactive feeding.

Activity of chromatographic zones

Zone 1	14 mg	870	cpm
" 2	55.5 mg	34,200	"
" 3	5 mg	6,300	"

The azoyl ester of zone 2 was crystallized four times from benzene with methanol without appreciable loss in activity. The high conversion to zone 2 could not be reproduced in subsequent experiments.

In experiment 3 the starfish was sacrificed three days after first radioactive feeding.

Activity of fractions and zones

Glycerol fraction	6,900 cpm
Fatty acid "	7,800 "
Digitonides	67,000 "
Zone 1	100 "
" 2	4,400 "
" 3	10,650 "

The glycerol fraction was obtained by extracting the combined water washings and original alkaline saponification residue with ether. The fatty acid fraction was obtained by extracting the acidified saponifaction liquid with ether.

In experiment 4 a starfish was sacrificed 9 days after the radioactive feeding.

Activity of fractions and zones

Oil	132,000 cpm
Nonsap	84,000 "
H_2O-wash of nonsap	46,800 "
Sterols from digitonides	84,000 "
Zone 1	700 "
Zone 2	17,800 "
Zone 3	51,600 "

Zone 2 was rechromatographed with carrier cholesteryl azoate. No appreciable activity moved with the cholesterol zone. Zone 2 was then crystallized once, hydrolyzed, acetylated and mixed with "cold" Δ^7-cholesteryl acetate and crystallized several times from ethanol. After an initial drop of 30% the specific activity (S.A.) remained constant. This was regarded as partial proof for a rearrangement of the 5-double-bond of cholesterol to the 7-position, a transformation which has been observed by other workers in a number of biological preparations. Of interest is the appearance of a large portion of the total activity (15-36%) in the water washes of the ether solution of the nonsaponifiable material.

In a subsequent experiment (experiment 5) gelatine capsules containing 4-C^{14}-cholesterol in salmon oil were force fed to three starfish, which were then held for 6 days in a tank. The gonads were separated from the rest of the organic material and extracted separately. It is possible that the activity in the gonads is due to contamination with the "rest" at the time the organs were separated from each other.

	Weight of nonsap	Cpm in nonsap
Gonads	0.3 g	15,000
Rest	1.3 g	240,000

A number of homogenates of starfish intestines were incubated with radioactive cholesterol. In the first experiment (experiment 6) the total intestines from one starfish were homogenized in a Waring Blendor with water drained from the intestinal cavity. Cholesterol-4-C^{14} was dissolved in 0.6 ml of ethanol and the solution was added to 50 ml of H_2O with stirring. The homogenate was then added to the suspension and the well-stirred mixture was incubated at 40°F for 20 hours. The total bacterial count of the homogenate was 400×10^6 per g. at the end of the incubation.

Activity of fractions and zones

Oil	540,000 cpm
Digitonides	510,000 "
H_2O washings of nonsap	27,000 "
Zone 1	3,500 "
Zone 2	22,900 "
Zone 3	336,000 "

It is possible that the activity in zones 1 and 2 is due to bacterial action on cholesterol.

Two homogenates were prepared in an ice-cooled Omni-Mixer in Bucher's medium (experiments 7-8) consisting of a potassium phosphate buffer (pH 7.4), 0.044 M; nicotinamide, 0.028 M; magnesium chloride, 0.007 M and sucrose 0.26 M. The intestines (50 g) were homogenized in 100 ml of medium for 15 sec and 50 mg of DPN and 2.5 µc of cholesterol-4-C^{14} in 1 ml of diluted Tween solution was added and incubation was carried out at room temperature in a shaker for four hours.

Activity of fractions and zones

	Expt. 7	Expt. 8
Nonsap	-	149,000 cpm
Zone 1	-	-
Zone 2	740 cpm	260 "
Zone 3	30,000 "	32,800 "

A total of 10 homogenates (experiments 9-18) was prepared of whole intestines of starfish and of gastro-intestinal tract and gonads separately in an ice-chilled, loose-fitting teflon homogenizer in a Bucher-type medium.

In some experiments the medium was made from a phosphate buffer (pH 7.4) and in some experiments from sea-water. The homogenates were centrifuged at 500 x g. In addition to glutathione, DPN and ATP, potassium hexosediphosphate and malonic acid were tried as additives. The homogenates were incubated with 2-C^{14}-acetate in a shaker at room temperature for 4-8 hours. Some of the incubations were carried out in an oxygen atmosphere. All these homogenates incorporated less than 0.1% of added activity into the neutral oil fractions. In a few instances, protein was precipitated with trichloracetic acid and washed a few times and was then void of activity.

Subsequent experiments were conducted with various molluscs. Two oysters (experiments 19-20) and two little neck clams (experiment 21) were kept from 18 to 20 hours in small amounts of sea-water to which had been added a Tween-suspension of 5 µc (about 500,000 cpm) of 4-C^{14}-cholesterol in each experiment. The two oysters were taken out after 3 and 4 days and extracted. The combined oil fractions contained 76,500 cpm.

Activity in chromatographic zones

Zone 1	1,340 cpm
Zone 2	7,000 "
Zone 3	39,800 "

Zone 1 was rechromatographed and then lost all its activity. The two clams in experiment 21 died after 2 days and contained then no activity.

In a subsequent experiment an oyster was anaesthetized with chloral hydrate. When the oyster had opened up the shell, the adductor muscle was severed and 0.5 ml of a Tween-suspension containing 2.5 µc (about 250,000 cpm) of 4-C^{14}-cholesterol was introduced with a blunt needle into the stomach through the mouth. The oyster was kept for 5 days in aerated cold sea-water, after which time it died. The ether extract then contained 87,500 cpm. A portion of the azoate was chromatographed together with 24-methylenecholesterol azoate.

Activity of zones

Zone 1 (24-methylenecholesterol)	230 cpm
Zone 2 (unknown	695 "
Zone 3 (Δ^5-monounsaturated sterols including cholesterol)	8,960 "

In experiments 23 and 24 about 500,000 cpm of 2-C^{14}-acetate were injected into a mussel and an oyster respectively. The acetate was dissolved in both cases in 0.05 ml of Bucher's medium in 0.05M buffer. After 21 hours the animals were extracted. The mussel sterols were precipitated with digitonin. The digitonide supernatant was evaporated, the residue was taken up in petroleum ether and chromatographed on a column with alumina deactivated with 5% water.

Activity of various fractions

	Expt. 23	Expt. 24
Total activity found in protein and acetone extract	459,400 cpm	964,800 cpm
Nonsap	8,600 "	7,960 "
Digitonides	200 "	
Column fractions:		
Petroleum ether insoluble	2,030 "	
Top of column after elution	2,440 "	
Petroleum ether fraction	730 "	
Benzene "	480 "	
Methylene chloride "	250 "	
Methylene chloride-Methanol (1/1) fraction	660 "	

	Nonsap activity % of total in flesh	Digitonide activity % of nonsap
Experiment 23	1.87	2.32
" 24	0.83	

One experiment was carried out with labelled formate, which is known to be incorporated into carbon 28 in ergosterol. Mevalonic acid (22.5 mg) and C^{14}-formate (about 5 million cpm) were dissolved in 0.5 ml of 0.05M phosphate buffer and injected into a clam. The clam was sacrificed and the fat extracted three days later.

Activity in various fractions

Total activity in flesh	2,500,000 cpm
Oil	910 "
Nonsap	440 "
Digitonides	150 "
Sterols obtained from digitonide	37 "

The subsequent experiments were carried out with butter clams (Saxidomus giganteus) that had been held up to two months prior to injection in an aerated aquarium supplied with running sea-water (filtered) and fed with mascerated frozen brine-shrimp. The animals used for each experiment were held in separate aquariums. Radioactive material was injected with a hypodermic needle, forced in between the closed shells, partly into the foot and partly aimed at the digestive gland adjacent to the stomach. The nonsaps. were obtained in the usual way and the sterols were precipitated with digitonin, reclaimed and chromatographed as azoylesters on SiO_2-Celite absorbent. In the experiments with C^{14}-cholesterol the bottom zone, containing the unreacted C^{14}-cholesterol, was removed and the remaining zone rechromatographed with carrier cholesteryl azoate. In this way the part of the strongly active bottom zone that usually contaminated the other zones was diluted. This process was repeated in a few instances.

Activity found after injection of 2-C^{14}-acetate in 0.1M phosphate buffer (pH 7.4)

Experiment number	Number of clams	Time between injection and extraction	Nonsap	Digitonides	Digitonides
			% of total activity in flesh		% of nonsap
26	1	10 days	0.78	0.024	3.1
27	2	11, 21 days	0.62	0.028	4.5
28	1	44 days	0.47	0.038	8.4

The results from experiments 26-28 indicate that the incorporation of acetate into nonsaponifiable matter reaches a maximum within 10 days after injection. After that time the activity decreases over a long period of time. Considering the results from experiments 23-24, it is probable that not much increase occurs in nonsaponifiable activity after the first 24 hours. Conversely the relative activity in the digitonides increases with time and the greatest value is obtained after 44 days. Schwenk et al. have similarly found a maximum in the nonsaponifiable C^{14}-activity after 24 hours in yeast homogenates. The conversion to sterols, however, reached a maximum between 96 and 118 hours. The sterol activity was then 36.5% of the nonsaponifiable while in the present experiments the highest value was 8.4% (after 44 days). The overall conversion of nonsaponifiable to sterols is rather small in comparison with the decrease in activity of the nonsaponifiable (in 34 days a loss of 31 cpm in the nonsaponifiable corresponds to an increase of 1.4 cpm in the sterols).

When the sterol fractions from these experiments were chromatographed no significant activity was found in zones other than the bottom zone (monounsaturated Δ^5-sterols).

Activity in the 24-methylenecholesterol zone after injection with 4-C^{14}-cholesterol

Experiment number	Number of clams	Time between injection and extraction	Injection medium	Activity in % of total activity on chromatogram
29	1	7 days	Tween suspension	1.34
30	2	18, 25 "	" "	2.28
31	2	2 "	Salmon oil	4.2
32	1	6 "	" "	3.6

The results from experiments 29-32 are inconsistent in regard to the influence of the time allowed between injection and extraction. Absorption from salmon oil seems to be more efficient than from Tween suspensions.

The clams used in experiment 31 were freshly caught. The upper zone sterol from experiments 29, 30 and 32 were acetylated and the acetate ozonized. The ozonide was decomposed with zinc dust in aqueous acetic acid, extracted with ether and washed with sodium bicarbonate solution. The evaporated residue was chromatographed on partially deactivated alumina (Brockmann Grade II). The ketone fragment from the column was in the form of the free steroid alcohol according to its IR spectrum. This fraction contained most of the activity present prior to ozonolysis. The specific activity dropped drastically in the first crystallization from aq. ethanol. Subsequent crystallizations, however, did not appreciably lower the specific activity. The material thus obtained had an mp of 140-5°. 24-Ketocholesterol melts at 136°. It is possible that due to the change in the composition of the food the clams have produced appreciable amounts of other sterols than 24-methylene cholsterol. However, the sterol from the upper zone of the clams used in these experiments exhibits a typical 24-methylene cholesterol spectrum.

The conversion of C^{14}-squalene was followed in one experiment. A clam was injected with C^{14}-squalene of biological origin in 0.2 ml of a Tween suspension and the oil was extracted 18 days later. The nonsaponifiable portion was obtained in the usual way and the sterols were precipitated with digitonin. The supernatant was evaporated and chromatographed on 100 g of alumina activity Grade II.

Activity of various fractions

Oil	133,000	cpm
Nonsaponifiable	133,000	"
Protein	24,500	"
Digitonides	2,090	"
Reclaimed sterols	1,500	"
Column fractions:		
Petroleum ether	64,800	"
Benzene-P.E. (1/1)	7,900	"
Benzene	11,100	"
Methylene chloride	3,400	"
Methylene chloride-ethanol (1/1)	29,900	"

A commercial sample of squalene on a similar column was eluted with petroleum ether. The corresponding radioactive fraction contained about half of the activity applied to the column (118,000 cpm). The rest, about half of the activity, has thus been converted to other compounds, but only 1.6% to digitonin precipitate material. The value for the protein fraction has yet to be confirmed.

SUMMARY NO. 38

ANALYSES OF CAPILANO RIVER COHO SALMON

I. Bitners
D.R. Idler

At the request of the Department of Fisheries, analyses were carried out on coho salmon prior to arrival at the Capilano River spawning area. This study was of an exploratory nature and the analyses were performed on fish captured by staff of the Department of Fisheries at the mouth of the Capilano River.

The lone female coho captured on October 6 was less mature sexually, as indicated by the ratio of the gonads to the live weight of the fish, than were the females captured on November 5. However, the body fat reserves were of the same order of magnitude as those of the later arrivals.

The plasma cholesterol levels of the October 6 males were much higher than those of the November 6 males. This is consistent with the effect observed for a pure stock of Fraser River sockeye at different stages in the migration (Summary No. 1 of this Station's Annual Report for 1957-58).

The data are recorded in the accompanying table.

Analyses of Capilano River coho salmon.

Date sample obtained	Sample No	Sex	Total wt in G	Wt of gonads in G	Gonads % of live wt of fish	Moisture % in body	Fat % in body	Protein % in body	Cholesterol µg/100 ml plasma Free	Cholesterol µg/100 ml plasma Total
Oct 6	1	M	3550	127	3.58	72.8	3.21	19.06	174.0	445.0
"	2	M	2110	100	3.12	73.4	2.97	19.38	199.0	430.0
"	3	M	2000	78	3.90	75.1	2.58	19.25	178.0	430.0
"	4	F	1710	200	11.70	72.6	4.43	18.88	156.0	315.0
"	5	M	1060	73	6.89	72.5	6.90	19.63	219.0	560.0
"	6	M	1300	69	5.31	72.6	5.81	19.75	194.0	490.0
Nov 5	1	F	1585	290	18.30	72.4	4.48	17.88	158.0	268.0
"	2	F	3790	845	22.33	72.3	2.09	17.88	193.0	308.0
"	3	F	3105	545	17.55	74.0	4.89	18.88	150.0	235.0
"	4	M	1965	85	4.33	74.0	2.49	18.00	60.0	120.0
"	5	M	1610	82	5.09	74.0	4.09	19.06	126.0	280.0
"	6	F	1810	335	18.51	77.2	2.29	17.94	115.0	243.0

SUMMARY NO. 39

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

Violet Chang
D.R. Idler

This is a continuation of the work reported in Summary No. 3 of this Station's Annual Report for 1957-58. Analyses have been completed in all the major tissues including those of the flesh and head, skin, bones and tail.

The methods of extraction and preparation for analysis of the various tissues with the exception of conditions of hydrolysis for total inositol are the same as those previously outlined in last year's report. It was found that the recoveries of inositol from tissue homogenates were higher when 22% HCl was used instead of 6 N HCl, and the duration of hydrolysis extended from 6 to 12 hours. It was also found that inositol was not decomposed when exposed to HCl concentrations of 22%, 30% or 37% at 110°C for 12 hours.

A. The distribution of phosphorus compounds in the tissues of migrating sockeye salmon

Female: Changes in percentage composition (See Table I.)

The total phosphorus content of the head, skin, bones and tail increases 10% in the journey from Lummi Island (560 µg/g) to Forfar Creek (630 µg/g), with the various components remaining in relatively the same ratios: 50% inorganic, 10% sugar-phosphate, 0% nucleotide phosphate and 40% phospholipid phosphate. In the flesh samples, the total

phosphorus content increases from Lummi Island to Forfar Creek by about 5%. This increase is reflected mainly as inorganic phosphate, since the other components, especially nucleotide and phospholipid phosphate are shown to decrease by 16% and 14% respectively per unit weight of tissue. There is very little change in the total phosphorus content per gram of tissue in the roe during migration. In roe, the major phosphorus component is the phospholipid phosphate which is 39% of the total at Lummi Island, and 53% at Forfar Creek. Inorganic and sugar phosphate occur in approximately the same ratio, the amounts being 20% and 16% of the total at Lummi Island and Forfar Creek, respectively.

TABLE I - Distribution of phosphorus compounds in the tissues of the migrating female salmon.

Tissue	Location*	Component (µg/g tissue)				
		Pt**	Pi**	Sugar PO_4	Nucleotide PO_4	Phospholipid P
Head, etc.***	L	561	262	54	-	245
	Lt	441	143	51	-	222
	FFC	629	313	62	-	245
Flesh	L	2158	1529	205	186	276
	Lt	2134	1580	58	201	291
	FFC	2259	1567	196	156	238
Gonads	L	2500	506	600	144	962
	Lt	2800	524	400	162	1500
	FFC	2546	404	400	112	1343
Gut	L	1000	597	85	118	273
	Lt	950	332	95	70	476
	FFC	666	162	92	32	405
Liver	L	1500	637	118	80	600
	Lt	1800	775	99	88	1080
	FFC	1609	603	124	58	1023
Kidney	L	1100	637	60	33	400
	Lt	1100	598	160	55	350
	FFC	1131	491	188	53	309

* L = Lummi Island; Lt = Lillooet; FFC = Forfar Creek.
** Total phosphorus, Pt; inorganic, Pi.
*** Head, skin, bones and tail.

The change in total phosphorus per gram of tissue during migration is much more marked in the gut and liver samples. The total phosphorus content of the gut decreases by one-third from Lummi Island to Forfar Creek. The changes involve a decrease in inorganic phosphate from 60% of the total at Lummi Island to 24% at the spawning grounds, a value which is only one-quarter of that at Lummi Island. There is a 7 µg/g increase in sugar phosphate from Lummi Island to Forfar Creek, but due to the general decrease in the total phosphate value, this gain accounts for the increase from 9% at Lummi Island to 14% of the total at Forfar Creek. Nucleotide phosphate in the alimentary tract (gut) shows a decrease of 75% during spawning migration, so that it accounts for only 5% of the phosphate at Forfar Creek as compared with 12% at Lummi Island. There is a doubling of the phospholipid phosphate content of gut at Forfar and it represents 61% of the total phosphate.

The total amount of phosphate per unit weight of tissue is shown to increase slightly in the liver samples from Lummi Island to Forfar Creek. An overall 7% increase is found, with the changes in the various components occurring as a decrease of inorganic and nucleotide phosphate, together with a 60% increase in phospholipid phosphate. The relative proportions of these constituents in the liver at Lummi Island are 43% inorganic phosphate, 8% sugar phosphate, 5% nucleotide phosphate, and 40% phospholipid phosphate. At Forfar, the distribution is found to be 38% inorganic phosphate, 8% sugar phosphate, 4% nucleotide phosphate, and 65% phospholipid phosphate.

In percentage composition, the total phosphate content of the female kidney samples remains essentially constant. However, there is a fluctuation in the ratio of the individual constituents from Lummi Island to Forfar Creek. Inorganic phosphate decreases from 58% to 43%, sugar phosphate increases from 6% to 17%, and phospholipid phosphate decreases from 37% to 27% of the total.

Changes in the standard female (See Table II)

The percentage composition data of Table I and the weights of the various tissues of the standard fish were used to calculate the overall change in phosphorus levels of the standard female (Summary No. 1 of this Station's Annual Report for 1957-58).

TABLE II - Distribution of phosphorus compounds in tissues of the standard female.

Tissue	Location	Sex	Component (mg/std fish)				
			Pt	Pi	Sugar PO_4	Nucleotide P	Phospholipid P
Head, etc	L	F	440	206	42	-	193
	Lt		337	109	39	-	170
	FFC		525	261	52	-	204
Flesh	L	F	2881	2041	264	248	369
	Lt		2422	1793	66	228	330
	FFC		2110	1464	183	146	222
Gonads	L	F	200	41	48	12	77
	Lt		386	72	55	22	207
	FFC		759	120	119	33	400
Gut	L	F	78	47	7	9	21
	Lt		25	9	3	2	13
	FFC		9	2	1	0.5	5
Liver	L	F	66	26	5	3	24
	Lt		80	35	4	4	48
	FFC		65	24	5	2	41
Kidney	L	F	24	14	1	0.7	9
	Lt		22	12	3	1	7
	FFC		22	10	4	1	6

There is a 20% increase of total phosphorus in the head, skin, bones and tail samples from Lummi Island to Forfar Creek. This is due to an increase in the percentage composition of phosphate accompanied by an increase in the total weight of the tissue. On the other hand, although the total phosphate of the flesh samples shows a

10% increase per gram of tissue at Forfar Creek, the total phosphate value of the standard female decreases by 25% throughout the entire river migration due to the great loss in weight of that tissue. Percent composition-wise, the total phosphate of the roe samples remains at a relatively constant level throughout migration; but because of the great increase in weight of roe, the net amount of total phosphate at Forfar Creek has more than tripled that present at Lummi Island.

Again, the changes encountered in the gut and liver are more pronounced when the total phosphorus of the standard fish is considered. The total phosphorus content of gut in the standard female fish at Forfar is only 10% of that at Lummi Island. Although there is a 45% rise in phospholipid phosphate in the liver at Forfar compared to that at Lummi Island, there is a slight decrease in total phosphate from Lummi Island to Forfar Creek. Total phosphate values of kidney for the standard female show neither increase nor decrease during the river migration. However, there is a shift in the relative proportion of the constituents which is reflected as a decrease of phospholipid phosphate and an increase in sugar phosphate.

It is evident that the major source of phosphorus in the standard female is the flesh. The decrease in total phosphate of the flesh more than accounts for the gain in total phosphate in the head, skin, bones and tail, and roe. However, the gain in phospholipid phosphate in the roe and head, skin, bones and tail is not balanced by the loss of phospholipid phosphate in the other tissues. Nevertheless, the tremendous drop in inorganic phosphate level of the flesh which is not balanced by any increase in any of the other tissues may be a significant indication that inorganic phosphate may have been mobilized in the formation of phospholipid phosphate in the roe.

Male: Changes in percentage composition (See Table III.)

TABLE III - Distribution of phosphorus compounds in the tissues of migrating male salmon.

Tissue	Location	Component µg/g tissue				
		Pt	Pi	Sugar PO_4	Nucleotide PO_4	Phospholipid P
Head, etc.	L	572	285	37	-	241
	Lt	446	164	69	-	211
	FFC	499	254	64	-	179
Flesh	L	2380	1626	228	189	291
	Lt	2128	1588	36	198	262
	FFC	2047	1444	221	152	286
Gonads	L	1300	714	152	46	348
	Lt	1250	777	133	40	240
	FFC	1111	551	187	40	353
Gut	L	900	450	85	95	323
	Lt	981	479	80	60	397
	FFC	685	175	110	34	393
Liver	L	1553	582	171	63	800
	Lt	1600	718	110	70	700
	FFC	1579	416	179	45	840
Kidney	L	1100	600	183	40	400
	Lt	1400	767	211	150	300
	FFC	1226	536	212	44	332

In contrast to the female head, etc., samples, the male head, skin, bones and tail show a decrease in the amount of total phosphorus on an equal weight basis for samples taken at Lummi Island and at Forfar Creek. As in the case of the female samples, the predominant type of phosphorus compound is inorganic (50%), the remaining components being phospholipid phosphate (40%), and sugar phosphate (10%). Again, the head, skin, bones and tail are conspicuous by their lack of detectable nucleotide phosphate. The male flesh samples differ from those of the female in that there is a decrease in the total phosphate on an equal weight of tissue, from Lummi Island to Forfar Creek, compared with a slight increase for the female flesh. The ratio of the various components in the flesh is the same for both sexes (inorganic phosphate 70%, sugar phosphate 10%, nucleotide phosphate 8%, and phospholipid phosphate, 12%). The milt contains only one-half the amount of phosphorus of roe. In milt, the dominant phosphorus component is inorganic (50%) followed by phospholipid phosphate (30%); sugar phosphate (17%), and nucleotide phosphate (3%). Total phosphate levels in male and female gut samples are almost the same, with the different components occurring in approximately the same proportions in both. The difference between the two sexes is that a gain in phospholipid is in the order of 30% for the female gut and 20% for the male gut.

Liver phosphorus values at Lillooet and Forfar Creek are higher in the female than in the male samples. The phospholipid content is 20% higher for the female than for male livers at these points. The amount and distribution of phosphorus compounds in the kidney are the same in both sexes (inorganic phosphate 44%, sugar phosphate 17%, nucleotide phosphate 4%, phospholipid phosphate 30%).

Changes in the standard male (See Table IV)

TABLE IV - Distribution of phosphorus compounds in tissues of the standard male.

Tissue	Location	Sex	Component (mg/std fish) Pt	Pi	Sugar PO_4	Nucleotide P	Phospholipid P
Head, etc	L	M	504	251	33	-	212
	Lt		389	143	60	-	184
	FFC		527	269	68	-	189
Flesh	L	M	3492	2385	335	277	427
	Lt		2771	2068	47	258	341
	FFC		2669	1883	289	198	373
Gonads	L	M	74	40	9	3	20
	Lt		103	64	11	3	20
	FFC		95	47	16	3	30
Gut	L	M	82	41	8	9	29
	Lt		30	14	2	2	12
	FFC		11	3	2	0.5	6
Liver	L	M	49	18	5	2	25
	Lt		41	18	3	2	18
	FFC		59	19	7	2	32
Kidney	L	M	29	16	5	1	10
	Lt		33	18	5	4	7
	FFC		35	15	6	1	9

As with the standard female, the head, skin, bones and tail of the standard male show a gain in total phosphorus at the spawning grounds, due to an increase in the total weight of the tissue. The depletion in the phosphorus of the flesh is of the same order as that for the standard female (approximately 800 mg/std fish). The flesh of the standard male is 25% higher in total phosphorus than the flesh of the standard female and contains 1.75 times more phospholipid phosphate. The flesh phospholipid phosphate expended by the standard male is not balanced by an increase in the phospholipid content of the gonads as is in the case of the standard female. The gut and kidney of the standard male have a higher total phosphorus content than does the standard female. On the other hand, the total phosphate level is consistently higher in the standard female liver than in that of the standard male. This is due primarily to the difference in weight of the organ in the two sexes. The total phosphorus of the standard female liver decreases only slightly in spawning migration, whereas the total phosphate content of the standard male liver increases by 20%, due again mainly to an increase in the weight of the liver at Forfar. In both sexes, the phospholipid phosphate content in liver is higher at Forfar Creek than at Lummi Island.

B. Changes in creatine levels in spawning migration of sockeye salmon

Changes in percentage composition (See Table V)

TABLE V - Creatine content of the tissues of migrating salmon.

(Creatine: µg/g tissue)

Tissue	Sex	Location		
		Lummi Is	Lillooet	Forfar Creek
Head, etc	M	1366	1296	1117
Flesh	M	5108	5174	5009
Gonads	M	1044	941	855
Gut	M	800	740	704
Liver	M	259	108	110
Kidney	M	476	288	183
Head, etc	F	1471	1465	1452
Flesh	F	5255	5304	5647
Gonads	F	140	116	125
Gut	F	712	739	721
Liver	F	203	163	269
Kidney	F	492	287	401

The creatine content of the flesh of both sexes is four times as high as that of the head, skin, bones and tail at Lummi Island, and throughout the entire river migration. The creatine content in the flesh of the male decreases slightly, and that of the female increases by 7.5% from Lummi Island to Forfar Creek.

The creatine level is considerably higher in milt than in roe, the levels being 1044 µg/g tissue and 140 µg/g tissue, respectively at Lummi Island. At the end of the migration journey, the percentage of creatine in milt had decreased by 20%, whereas that of roe had decreased by only 10%.

The creatine content of the head, skin, bones and tail is about twice that of the gut on an equal weight basis. The male samples of gut are about 10% higher than those of the female at Lummi Island. During the migration up the river, the creatine level of the male gut decreases by 10% to approximate the female gut level which has

remained essentially unchanged.

The creatine content of male liver samples shows a decrease from 259 µg/g tissue at Lummi Island to 110 µg/g tissue at Forfar Creek. In contrast, the female liver samples show an increase from 203 µg/g tissue at Lummi Island to 270 µg/g tissue at Forfar Creek. As in the case of the liver samples, male kidney samples exhibit a decrease in creatine at Forfar Creek at 60%, but female kidney samples show a 20% drop only.

Changes in the standard fish (See Table VI)

TABLE VI - Creatine content of the tissues of the standard fish (mg).

		Location			
Tissue	Sex	Lummi Is	Lillooet	Forfar Cr	Change: Lummi→Forfar Cr
Head, etc	M	1203	1129	1181	- 22
Flesh	M	7493	6737	6532	- 962
Gonads	M	59	77	73	+ 14
Gut	M	72	22	12	- 61
Liver	M	8	3	4	- 4
Kidney	M	12	7	5	- 7
				Total:	-1043
Head, etc	F	1155	1121	1211	+ 56
Flesh	F	7015	6020	5274	-1741
Gonads	F	11	16	37	+ 26
Gut	F	56	19	9	- 46
Liver	F	8	7	11	+ 3
Kidney	F	11	6	8	- 3
				Total:	-1705

As with the data on the distribution of phosphorus compounds, the total creatine content of the various tissues of the standard fish was calculated from the data given in Table V and the weights of the various tissues (Summary No. 1 of this Station's Annual Report for 1957-58).

The major source of creatine is the flesh. Although the percentage composition of creatine in female flesh samples is shown to increase with migration to the spawning grounds, there was an actual loss of 1741 mg from the flesh of the standard female. The loss from the flesh of the standard male was 961 mg during migration from Lummi Island to Forfar Creek.

In contrast, although the creatine content per unit weight of tissue was shown to decrease in the gonads of both sexes, there is an increase of 14 mg and 26 mg in milt and roe respectively of the male and female standard fish due to the great increase in total weight of these tissues at the spawning grounds.

The standard male was found to lose 22 mg of creatine from the head, skin, bones and tail, whereas the overall creatine content of the same tissues of the standard female were found to increase by 56 mg.

In both sexes, the creatine content of the gut decreased drastically from 72 mg to 12 mg for the standard male and 56 mg to 9 mg for the standard female from

Lummi Island to Forfar Creek. The greater part (80%) of this loss occurred in the portion of the journey from Lummi Island to Lillooet. The total creatine level in liver is the same for both standard male and female at Lummi Island. However, at the end of migration, the standard male liver had lost 50% of its original creatine but the standard female had increased by 25% its original creatine level.

As with most of the other tissues, the kidney creatine content is higher in the standard male than in the standard female at Lummi Island, but is shown to be depleted to a greater extent than the standard female, with the result that the creatine content in kidney is higher in the standard female than it is for the standard male at Forfar Creek. In none of the samples analysed was evidence of creatine-phosphate found. The fish were caught in reef nets to minimize struggle but this precaution would probably not protect against the depletion of the supply of creatine-phosphate.

C. Qualitative and quantitative changes in inositol in the tissues of migrating sockeye salmon

Changes in percentage composition (See Tables VII, VIII)

TABLE VII - Distribution of the inositol in the tissues of the migrating female salmon.

		Components µg/g tissue			
			Acid-soluble		
Tissue	Location	Total	Free	Total	Phospholipid
Head, etc	L	155	52	53	42
	Lt	133	58	56	34
	FFC	233	54	46	37
Flesh	L	202	13	22	103
	Lt	209	22	27	121
	FFC	195	19	27	108
Gonads	L	461	47	48	80
	Lt	468	32	33	209
	FFC	474	32	60	186
Gut	L	335	244	154	22
	Lt	308	167	116	83
	FFC	246	97	99	79
Liver	L	666	124	122	102
	Lt	662	82	72	535
	FFC	683	87	91	509
Kidney	L	287	140	116	40
	Lt	604	302	269	51
	FFC	463	302	294	38

The total inositol content of the head, skin, bones and tail at Lummi Island is slightly higher in the female (155 µg/g tissue) than in the male (143 µg/g tissue). In both sexes, the acid-soluble is the predominant form. The total inositol of the male head, skin, bones and tail sample remains essentially the same at Forfar, with a change only in the relative proportions of the constituent types, with an increase

in the acid soluble and a decrease in the phospholipid fractions. The female head, skin, bones and tail samples show a 50% increase in total inositol at Forfar Creek, and this is accompanied by a slight increase in phospholipid inositol.

TABLE VIII - Distribution of inositol in the tissues of the migrating male salmon.

Tissue	Location	Component µg/g tissue			
			Acid-soluble		
		Total	Free	Total	Phospholipid
Head, etc	L	143	59	51	25
	Lt	156	60	58	28
	FFC	143	67	40	15
Flesh	L	203	16	20	105
	Lt	203	25	36	107
	FFC	144	19	26	122
Gonads	L	245	119	123	60
	Lt	268	252	176	15
	FFC	200	140	118	5
Gut	L	352	183	216	30
	Lt	299	234	167	38
	FFC	208	83	87	90
Liver	L	529	100	112	300
	Lt	630	130	133	237
	FFC	641	113	83	421
Kidney	L	325	157	222	31
	Lt	665	726	539	27
	FFC	581	304	258	63

Both male and female flesh samples contain approximately the same amounts of total, acid-soluble and phospholipid inositol at Lummi Island. The decrease of total inositol in flesh at Forfar is more drastic in the male (30%) than in the female (3%). Phospholipid inositol is the major type found in flesh.

On an equal weight basis, roe samples are twice as high in total inositol as in milt samples. On the other hand, milt samples contain more than twice the amount of acid-soluble inositol than do roe samples at Lummi Island and at Forfar Creek. The phospholipid inositol of milt decreases from 60 µg/g tissue at Lummi Island to negligible amounts at Forfar Creek. In contrast, the phospholipid inositol of roe is shown to increase from 80 µg/g tissue at Lummi Island to 186 µg/g tissue at Forfar Creek.

At the start of migration, acid-soluble is the principal form of inositol in the gut of both sexes, the amounts being more than seven times those of the phospholipid type. At the end of migration, the total inositol of the gut is depleted by one-third, the acid-soluble inositol by more than one-half, but the phospholipid inositol is increased by 3-fold.

On a percentage composition basis, the liver samples of both sexes are richest in inositol, with a ratio of more than 4:1 of phospholipid to acid-soluble at Forfar

Creek. In contrast, the kidney samples of both sexes are substantially higher in acid-soluble than in phospholipid inositol.

Changes in the standard fish (See Tables IX, X)

Although it would appear from the foregoing that the major sources of inositol are the liver and kidney samples, and that the minor sources are the head, skin, bones and tail, and flesh samples, the reverse is true when the weights of these tissues in the standard fish are considered. Thus, it is seen that because the flesh accounts for 57% of the total weight in the standard fish, this tissue appears as the major source of inositol.

TABLE IX - Distribution of inositol in the tissues of the standard female.

		Components mg/std fish				
			Acid-soluble		Phospho-	Change:
Tissue	Location	Total	Free	Total	lipid	L→FFC
	L	122	41	42	33	
Head, etc	Lt	102	44	43	26	
	FFC	194	45	38	31	+72
	L	270	17	29	138	
Flesh	Lt	237	25	31	137	
	FFC	182	18	25	101	-88
	L	37	4	4	6	
Gonads	Lt	65	4	5	29	
	FFC	141	9.5	18	55	+104
	L	26	19	12	2	
Gut	Lt	8	4	3	2	
	FFC	3	1	1	1	-23
	L	27	5	5	4	
Liver	Lt	30	4	3	22	
	FFC	28	4	4	21	+1
	L	6	3	3	1	
Kidney	Lt	12	6	5	1	
	FFC	9	6	6	1	+3

There is a decrease of 30% in the total inositol of both sexes in the flesh during the entire river migration. The ratio of total inositol of the flesh to that of the head, skin, bones and tail is 2.2:1 at Lummi Island and 0.95 and 1.2:1 at Forfar Creek for the standard female and male, respectively. The total inositol of the head, skin, bones and tail rises 20% in the standard male and 50% in the standard female.

The increase in the weight of the gonads is again reflected in an overall increase in the total inositol content of these tissues in the standard fish. Phospholipid inositol is the most important form of inositol in roe, and increases from 6 mg at Lummi Island to 55 mg per standard female at Forfar Creek. The change in milt is not as dramatic, the increase being from 14 mg at Lummi Island to 17 mg at Forfar Creek per standard male.

TABLE X - Distribution of inositol in the tissues of the standard male.

Tissue	Location	Components mg/std fish				Change: L→FFC
			Acid-soluble		Phospho-	
		Total	Free	Total	lipid	
Head, etc	L	126	52	45	22	
	Lt	136	52	51	24	
	FFC	151	71	42	16	+25
Flesh	L	298	23	29	154	
	Lt	264	33	47	139	
	FFC	188	25	34	159	-110
Gonads	L	14	7	7	3	
	Lt	22	21	14	1	
	FFC	17	12	10	0.4	+3
Gut	L	32	17	20	3	
	Lt	9	7	5	1	
	FFC	3.4	1.4	1.4	2	-29
Liver	L	17	3	4	9	
	Lt	16	3	3	6	
	FFC	24	4	3	16	+7
Kidney	L	8	4	6	0.8	
	Lt	15	14	13	0.6	
	FFC	16	9	7	2	+8

The gut of the standard fish shows an expenditure of almost 90% of its total inositol reserve from Lummi Island to Forfar Creek, the greater portion (70%) of this being spent between Lummi Island and Lillooet. Liver inositol values of both sexes increased as the fish approached the spawning grounds. The increase is reflected as an increase in phospholipid inositol, two times for the standard male, and five times for the standard female. The kidneys are also found to increase in total inositol, with the exception that in this case the increase is mainly in the acid-soluble portion.

It is of interest to note that the concentrations of total phosphorus, inorganic phosphorus, inositol and creatine are identical in both sockeye salmon and human muscle. The distribution of these compounds are as follows: total phosphorus 0.2%, inorganic phosphorus 0.15-0.16%, inositol 0.02%, and creatine 0.5%.

D. Changes in the lipid content in the major tissues of the migrating salmon

The total lipid content of all the major tissues has been determined and the results have been summarized in Table XI and XII. Values for the standard fish have been calculated from the percentage composition data and the absolute weights of the various tissues of the standard fish (Summary No. 1 of this Station's Annual Report for 1957-58).

TABLE XI - Changes in total lipid in the tissues of migrating male sockeye salmon.

Tissue	Location	Fat in tissue (%)	Fat in standard fish (g)
Head, etc	L	20.48	180.43
	Lt	14.47	126.03
	FFC	4.06	42.91
Flesh	L	8.87	130.12
	Lt	6.14	79.94
	FFC	3.15	41.08
Gonads	L	1.58	0.89
	Lt	1.76	1.44
	FFC	1.95	1.67
Gut	L	6.06	5.48
	Lt	5.59	1.68
	FFC	2.84	0.47
Liver	L	4.55	1.44
	Lt	4.82	1.22
	FFC	3.30	1.24
Kidney	L	2.52	0.66
	Lt	2.54	0.59
	FFC	2.43	0.68

TABLE XII - Changes in total lipid in the tissues of migrating female sockeye salmon.

Tissue	Location	Fat in tissue (%)	Fat in standard fish (g)
Head, etc	L	21.33	167.44
	Lt	14.14	108.17
	FFC	4.40	36.70
Flesh	L	10.53	140.58
	Lt	6.36	72.19
	FFC	2.83	26.43
Gonads	L	10.33	8.27
	Lt	13.37	18.45
	FFC	10.85	32.33
Gut	L	5.49	4.30
	Lt	5.05	1.32
	FFC	2.93	0.38
Liver	L	4.70	1.89
	Lt	3.75	1.67
	FFC	3.76	1.52
Kidney	L	2.25	0.49
	Lt	2.25	0.45
	FFC	3.52	0.69

Changes in percentage composition

The total lipid content of the head, skin, bones and tail in both sexes at Lummi Island is more than twice that of the flesh. In both tissues, the female samples are richer in total fat than are the male samples. During the initial phase of the migration journey (from Lummi Island to Lillooet) the demand on the flesh fat is of a somewhat greater order than that for the head, skin, bones and tail - e.g., there is utilization of 40% of the flesh fat as compared with 34% of that of the head, skin, bones and tail for the female, and a 31% expenditure of the flesh fat as compared with 29% for the head, skin, bones and tail of the male. The reverse is true during the remaining half of the migration journey wherein the greater portion of the lipid expenditure per unit weight tissue is from the head, skin, bones and tail (cf. 80% depletion in total lipid of the head, skin, bones and tail for both sexes with 73% and 65% depletion of flesh lipid in the female and male, respectively). The greater concentration of expendable fat in the head, skin, bones and tail of the fish compares with the subcutaneous tissues in human beings as fat storage depots.

The lipid content of gut on a unit weight basis is approximately one-half of that found in flesh for the female samples, and approximately two-thirds of that found in the flesh for the male samples. The greater decrease in the lipid content of gut occurs, as with the head, skin, bones and tail, during the latter half of the river migration. On a unit weight basis, the amount of total lipid decreases by 8% in both sexes from Lummi Island to Lillooet; but on arrival at Forfar Creek, the amount of total lipid contained in these tissues has been reduced to 53% and 47% of that at Lummi Island for the female and male, respectively.

Percentage composition-wise, the liver and kidney samples do not reflect the dramatic changes found in the flesh and head, skin, bones and tail samples. In the male livers, the total lipid content increases slightly at Lillooet, but a final decrease of 28% from the level at Lummi Island is shown at Forfar Creek. The liver of the female has a lipid content of 4.7% at Lummi Island. This level decreases by 20% by the time the fish have arrived at Lillooet, and this new level is then maintained for the remainder of the journey to Forfar Creek. Only a very slight increase in lipid content is demonstrable with respect to the kidney samples of the male during spawning migration. On the other hand, there is an increase of 50% per unit weight of tissue with those of the female samples.

Milt samples at the spawning grounds increase almost one-third over the level at Lummi Island in total lipid per unit weight. In the roe, there is an initial rise in the total lipid level during that portion of the migration from Lummi Island to Lillooet, from which point there is a decrease, so that the level of total lipid at the spawning grounds returns to approximately that at Lummi Island. The increased total lipid content of roe at Lillooet is accompanied by an increase in the phospholipid content.

Changes in the standard fish

Although the flesh of the fish accounts for 56-57% of the total weight of both males and females at Lummi Island (Summary No. 1 of this Station's Annual Report for 1957-58), the greater source of expendable lipid is contained in the tissues of the head, skin, bones and tail. Throughout the entire river migration, the head, skin, bones and tail of the standard female contribute 130 g of total fat as compared with 114 g from the flesh; the head, skin, bones and tail of the standard male contribute 137 g of total fat as compared with 89 g from the flesh. Although the standard male draws somewhat more fat from the head, skin, bones and tail than does the female, the combined fat consumption of the flesh and head, skin, bones and tail is 18 g more for the standard female fish than it is for the standard male.

The samples from the gut of both sexes show a decrease of approximately 50% in total lipid per unit weight of tissue on arrival of the fish at the spawning grounds. However, when the total lipid content of the gut of the standard fish is considered, the change from Lummi Island to Forfar Creek is much more dramatic and 90% of the total available lipid content of the alimentary tract has been utilized for the migration journey.

In contrast, there is an increase of total fat in the gonads of both sexes which is due mainly to the increase in weight of these tissues. In the standard male, the absolute increase of lipid in the milt is in the order of 88%, while that of the roe in the standard female is in the order of 291% at Forfar Creek.

Total lipid undergoes relatively little change in the liver and kidney samples of the standard male. The liver lipid decreases by 14% during migration from Lummi Island to Forfar and a very small increase of 3% is manifested in the lipid content of the kidney of the standard male. The liver of the standard female decreases 20% in total lipid during the entire river migration. Like the standard male, the kidney of the standard female exhibits an absolute increase in total lipid, but of a greater magnitude, the increase at Forfar Creek being 41% above the level at Lummi Island.

The relative stability to change in total lipid levels of the liver and kidney suggests that the composition of lipid in these tissues is, in the main, essential lipid which is considered to represent the fundamental components of protoplasmic structure, whereas the lipid of the gut, flesh, head, skin, bones and tail are primarily expendable storage fats, which serve as sources of reserve energy as well as insulation. Therefore, the total lipid content of these latter tissues is seen to decrease from a high level during good nutrition (the state at Lummi Island) to practically nothing in the tissue of starvation (the state during migration, and especially at Forfar Creek). This last observation is further substantiated by the fact that there is comparatively little change in the phospholipid content per unit weight of tissue throughout the entire migration (Tables I and II).

SUMMARY NO. 40

PHOSPHORUS COMPOUNDS IN SALMON: THE ROLE OF NUCLEOTIDES IN INOSITOL METABOLISM

H. Tsuyuki
D.R. Idler

The investigation of acid-soluble phosphorus compounds has been continued in relation to the metabolism of inositol in salmon intraperitoneally injected with 2-C^{14}-myo-inositol. Methods used in the isolation and identification of the nucleotides and the abbreviations have been adequately outlined in Summary No. 17 of this Station's Annual Report for 1957-58, except where deviation was necessary.

Injection of 2-C^{14}-myo-inositol* into Coho

A large coho held in captivity at the Vancouver aquarium was transferred to a 40-liter tank containing 30 litres of fresh water to which 30 ml of quinaldine (100 mg/ml of acetone) had been added. After the fish had been quiescent under anesthesia for 30 minutes, 2 ml of a solution of radioactive inositol containing

* 2-C^{14}-myo-inositol was kindly supplied by Professor Laurens Anderson of the University of Wisconsin.

3.98 mg of 2-C^{14}-myo-inositol of 22 µc total activity was injected intraperitoneally. Seven hours later another 6.0 ml of quinaldine solution was added and after waiting a few minutes, the tail was removed and the fish bled. The liver was excised and the remainder of the fish frozen immediately in dry ice together with the liver and blood. After thawing overnight in the refrigerator, the carcass was separated into kidney, milt, guts, heart, spleen, flesh, head, skin and bone. An untreated coho was also anesthetized and divided into the various components exactly as before. Due to the small amount of sample the liver, kidney, guts, and heart tissues from the two fishes were combined. The weights of the various tissues from the two cohoes are summarized in Table I.

TABLE I - Weights of organs (gm).

Tissue	Coho(1)^{C14}	Coho(2)
Liver	23.5	17.9
Kidney	25.4	17.6
Milt	119	109.8
Guts	33.5	18
Heart	5.5	8.8
Spleen	3.0	
Flesh	1314	971
Head	370	355
Skin and bone	405	328
Blood	39.3	

The acid soluble extracts of the tissues were prepared as previously described (Summary No. 17 of this Station's Annual Report for 1957-58) and the amounts of tissue extracted are shown in Table II.

TABLE II - Perchloric extracts of organs.

Tissue	Amount (gms) Used for Perchloric Acid Extraction		Final Volume (mls) of Perchloric Acid Extract
	Coho(1)^{C14}	Coho(1)^{C14}+ Coho(2)	
Liver		41.4	55
Kidney		43	40
Milt	50		46
Guts		51.5	40
Heart		14.3	10.5
Flesh	55		30
Head	50.9		40.5
Skin and bone	53.9		33
Blood	39.3		21.5

In view of the experiments of Charalampous who formulated the following scheme as one of the pathways of inositol metabolism in rat kidney - Inositol ⟷ glucose ⟷ UDP glucose ⟷ UDP glucuronic acid ⟷ glucuronic acid, our experiments were partly designed to isolate and investigate these intermediates. Anderson et al. found some conversion of 2-C^{14} inositol to liver glycogen and urinary uronic acids of rat. Consequently, these were also investigated.

In our procedures, glycogen, inositol and hexoses are not retained on the Dowex-1-formate column and appear in the water wash of the column. The reference standard of galacturonic acid has been shown to appear in the same position as fraction 'A' on Dowex-1-formate columns and in the water wash of charcoal columns. UDP glucose and UDP glucuronic acid are eluted from Dowex-1-formate in the usual manner. In this way all of these components may be isolated in the acid extract of liver or other tissues of the fish, in addition to other unknown intermediates.

The acid soluble extracts from the various tissues of the coho were therefore separated into groups of compounds by the charcoal and Dowex columns as described above, and their radioactivity was measured roughly (Table III). To determine which of the tissues contained the highest activity in the nucleotides the ethanol/NH_3 fractions from the charcoal columns were examined for activity. The liver extract was the most active in this respect and consequently was selected for more detailed studies.

TABLE III - Distribution of radioactivity in the tissues.

		Charcoal column		Dowex-column on charcoal water wash		
Tissue	Total cpm	Water wash	Alcohol/NH_3	Water	0.4 N (Formic	4.0 N Acid)
Head	5,875	7,860	0	1,920	860	0
Heart	357					
Blood	1,535					
Skin, bone	28,020	35,600	0			0
Flesh	5,250	1,950	240			
Liver	9,000	6,325	2,882	2,340	3,610	
Kidney	106,000	77,500	700	78,800	1,500	1,100
Milt	75,900	57,500	0	56,800	0	747
Gut	262,000	191,000	0	208,000	575	

Behaviour of Galacturonic Acid on Dowex-1-formate

10 mg of galacturonic acid was put on a 1x20 cm column of Dowex-1-formate and eluted first with 250 ml of water followed by 0.4 N formic acid. The uronic acid was not eluted until after 50 cc of 0.4 N formic acid was passed through. This formic acid concentration is not attained in the gradient elution columns until early in fraction 'A'.

Treatment of Perchloric Acid Extract on Charcoal

The radioactive acid soluble extracts were separated into two broad groups by using charcoal:celite (1:1) columns. By this technique the water wash will include inositol, free sugars, sugar phosphates and uronic acids and the ethanol/NH_3/water (40/1/59) eluate included the nucleotides and nucleotide-X compounds where X may be pentoses, hexoses, hexosamines, N-acetyl hexosamines, uronic acids, to mention only a few examples of this type of compound.

A 2 ml aliquot of each extract was put on a previously water washed 1 g charcoal/celite column and the column was washed first with 100 ml of water, followed by 100 ml of ethanol/NH_3/H_2O. Each of these two fractions were concentrated by means of a dry-ice-acetone trapped flash-evaporator at 40°C in successively smaller round-bottom flasks and finally transferred to 5.0 ml volumetric flasks. The radioactivity was determined on an aliquot of this volume and calculated on the

basis of total activity in the tissue (Table III).

Treatment of Charcoal Water Wash on Dowex-1-formate

The water wash from charcoal/celite column was separated into free sugars and uronic acid and/or sugar phosphates by passage through a 1x20 cm column of Dowex-1-formate. The material was eluted from the column by washing successively with 500 ml water, 350 ml of 0.4 N formic acid, and finally with 350 ml of 4 N formic acid. The eluates were concentrated in a flash evaporator, cooled by acetone-dry ice trap and the final volume of concentrate brought to 1.0 ml after a series of transfers into progressively smaller flasks. Radioactivity was measured on aliquots of these 1.0 ml fractions and calculated on the basis of the entire tissue (Table III).

Separation of Nucleotides of Liver

The perchloric extract of liver was passed through a 1x20 cm column of Dowex-1-formate at 0°C as previously reported for the separation of nucleotides. The column was initially washed well with 300 ml of water before eluting with the formic acid system.

The water-wash from the Dowex-column was deionized by passing it through a mixed bed of 1x12 cm of IR-120(H) (top half) and 1x12 cm of IRA-400(OH) (bottom half) and the eluate concentrated under vacuum to a glass-like residue similar in appearance to glycogen. The residue was treated with 1x2 volumes of 95% ETOH containing a crystal of LiCl and the glycogen centrifuged off. This procedure was repeated. Most of the activity (1640 cpm in 41.4 g liver) was located in the supernatant which was again deionized as above. The activity in the glycogen fraction could not be measured. Dry weight of glycogen = 297 mg from 41.4 g of liver. The supernatant which was concentrated to a small volume was streaked on Whatman #3 paper and the chromatogram developed (descending) with n-butanol/acetic acid/water (40/10/50). The only components detectable were inositol and glucose, which were both radioactive. No other hexoses or pentoses were present in detectable quantities. Glucose was distinguished from other hexoses by a second paper chromatogram which was developed with ethyl acetate/water/pyridine (2/2/1).

Of the nucleotide fractions designated A to K inclusive obtained by formic acid elution of Dowex column only fractions A (1070 cpm), B (320 cpm), I (330 cpm), and K (90 cpm) displayed any activity. Fraction I was ion-exchanged with IR-120, lyophilized, and re-chromatographed on Dowex-1-formate using the ammonium formate system as previously reported. Two fractions, I_1 (0 cpm) and I_2 (330 cpm) were obtained, I_2 containing all of the activity. I_2 moved as a single component in Pabst #1 system (isobutyric acid/c. ammonium hydroxide/water 66/1/33) with an Rf corresponding to that of UDP-glucuronic acid. When I_2 was hydrolysed with .01 NHCl for 20 minutes at 100°C and chromatographed on ethanol/1M ammonium acetate pH 4.5 (65/35) UMP and gluconolactone were obtained. Glucuronic acid under these conditions of hydrolysis is readily lactonized. I_2 was hydrolysed in .02 N HCl for 20 minutes at 100°C, chromatographed on the same system and when the band corresponding to gluconolactone was eluted, ion-exchanged and counted, all the activity was recovered in the gluconolactone. Thus all the radioactivity of fraction I has been located in the glucuronic acid moiety of UDP-glucuronic acid.

Dowex Column Fraction 'A'

One half of fraction 'A' from the Dowex-1-formate column was put through a 5 g charcoal/celite column. The water-wash showed no activity while the ethanol/NH_3 fraction contained all the activity (644 cpm). The uronic acids which should appear in the water-wash of charcoal did not show any measurable activity.

The ethanol/NH_3 fraction was then separated by a 1x40 cm column of Dowex-1-formate as described in Table IV. Most of the activity was concentrated in fraction A_9. When A_9 was chromatographed on Pabst #1 and the inositol region eluted and re-chromatographed on n-butanol/acetic acid/water (4:1:5) two bands were obtained. The major band which moved like galactose showed no activity but the minor band which corresponded to inositol contained 180 cpm of the original total of 216 in A_9. The nucleotide in A_9 behaved like AMP-2'3' in two solvent systems (Pabst #I and III) and formic acid hydrolysis produced adenine as the sole base.

These data bring out the fact that the inositol obtained in fraction 'A' must have been combined in some fashion to impart absorptive properties toward Dowex-1-formate as well as to charcoal. Free inositol under these conditions is eluted from both Dowex-1 and charcoal with water alone. However, inositol in its combined form no longer exists after gradient elution with formic acid. The compound then is highly unstable in dilute acid. This acid instability of the compound would also be applicable during the perchloric acid extraction, though enough of it survives this treatment to enable isolation of the inositol derivative from the Dowex-1-formate column. The presence of inositol in the same fraction as AMP-2'3' (A_9) does not necessarily indicate that this is the parent nucleotide or for that matter if it is a nucleotide at all. Rather, this indicates that some fraction beyond AMP-2'3' in fraction 'A' may have been the parent compound.

TABLE IV - Re-chromatography of one-half of fraction 'A' on Dowex-1-formate.

Fraction	Tube No.	U.V. absorption	cpm
		Water-wash (10 ml/tube)	
A_1	1-4	+	20
A_2	5-7	+	22
A_3	8-16	+	15
A_4	17-25	+	9
A_5	26-45	-	0
	Gradient elution with 4 N formic acid (5 ml/tube)		
A_6	1-21	-	32
A_7	22-47	+	60
A_8	48-56	+	12
A_9	57-86	+	216
A_{10}	87-128	-	26
	Direct elution with 4 N formic acid (5 ml/tube)		
A_{11}	129-142	-	16
Total recovery off Dowex column = 428 cpm			

1x40 cm Dowex-1-formate column was used at 0°C. A concave upward type of gradient elution technique was carried out with 180 ml of 4 N formic acid in the reservoir running into a mixing chamber containing 1000 mls of water in a precise manner as defined by the equation:

$$C = C_2-(C_2-C_1)(1-\text{[illegible]})^{A_2A_1}$$ where:

C = conc. of acid in the eluate

$A_2/A_1 = \frac{\text{cross-sectional area of the reservoir}}{\text{cross-sectional area of the mixing chamber}} = \frac{27\ cm^2}{127\ cm^2}$

C_2 = conc. of acid in reservoir = 4 N

C_1 = conc. in mixing chamber = 0

$\alpha = \frac{\text{volume (ml) of eluate which passed through the column}}{\text{total initial volume (ml) present in the system}}$

The tracer experiments have enabled us to show that a highly unstable inositol compound does exist in fraction 'A'. Consequently, further studies were carried out on non-radioactive liver extracts in an attempt to obtain the inositol compound intact. Acid extracts of sockeye livers were used in these experiments. Extracts from 70 g of powdered livers were used for each column. The length of the columns were doubled to 1.4x40 cm to enable better separation of fraction 'A', and in each case the sample on the column was washed thoroughly with 1000 ml of water to insure removal of any free inositol before elution with the 4 N formic acid system according to the method of Hurlbert and co-workers. Elution was continued until fraction 'B' just appeared and the remainder discarded. In this way, 5-6 discrete fractions, tentatively designated, A_1, A_2, A_3, A_4, A_5 and A_6 were obtained in fraction 'A' without resorting to a second column as previously (Summary No. 17 of this Station's Annual Report for 1957-58). Microbiological assay using Saccharomyces carlsbergensis showed 18 µg of inositol in A_4 and 65 µg in A_5 from 70 g of liver. The major component of A_3 has been identified as AMP-5'; A_4, AMP-2'3; and A_5, succinoadenine. Together with the DPN, AMP-5' and succinoadenine found previously (Summary No. 17 of this Station's Annual Report for 1957-58), these bring to four the total number of fractions identified definitely in fraction 'A'. That inositol in sockeye liver is associated with AMP-2'3' and succinoadenine fractions has also been shown by paper chromatography though it cannot be stated that these were their respective parent compounds.

The conversion of myo-inositol to glucose in the rat has been demonstrated by many investigators. Recently, Charalampous reported that kidney extracts are capable of converting inositol to DL glucuronic acid and postulated three schemes by which this conversion occurs, one of which is as follows: inositol $\longleftrightarrow$ glucose $\longleftrightarrow$ UDP-glucose $\longleftrightarrow$ UDP glucuronic acid $\longleftrightarrow$ glucuronic acid. Anderson isolated labelled glucuronic acid from the urine of rats fed 2-C^{14}-myo-inositol and also demonstrated that it was converted to liver glycogen to a certain extent. In neither case has it been shown that the glucuronic acid moiety of UDP-glucuronic acid carries label as we have shown in the case of coho livers. When our results are compared to those from mammalian tissues, several important differences become evident. Liver glycogen did not show any significant activity. However, the relatively short (7 hr) period experiment may not have allowed sufficient conversion of inositol to glycogen. A more likely possibility is that the fasting condition of the coho in captivity would not favour deposition of glycogen. This condition of the fish is in contrast to Anderson's experiments in which well-fed rats were used. The absence of any measurable activity in the nucleotide fraction 'G', which in the sockeye liver has been identified to be composed mainly of UDP galactose and in which UDP-glucose should appear (if present), came as a surprise, for if Charalampous' postulation is correct and if the same scheme is applicable to coho, this fraction should have been labelled. The lack of activity in the uronic acid fraction, though a deviation from the results of other workers, seems rather strange since UDP-glucuronic is present in the liver. This is particularly so in view of the demonstration by Ginsberg et al that particulate fractions from rat kidney catalyses the hydrolysis of UDP-glucuronic acid to glucuronic acid. However, whether such a hydrolysis occurs in situ is problematical. The possibility that labelled glucuronic acid is present in other tissues of the coho has not yet been investigated.

The presence of label in inositol in fraction 'A' indicated that another derivative of inositol exists in this fraction. Free inositol is completely washed off the Dowex column with water and indeed it has been isolated from the water wash. Fraction 'A' was also treated with charcoal to remove any remaining traces of free inositol. The possibility that it is coming from inositol-P is not likely since drastic acid hydrolysis is necessary to free the inositol and furthermore inositol-P has been shown to elute in fraction 'B'. The specificity of the inositol in fraction 'A' has been verified by its response to microbiological assay. This inositol derivative is acid labile since it is already decomposed during gradient elution when the formic acid concentration reaches 0.2 N. The fact that the nucleotide and base fractions in which the labelled inositol is found have been identified as AMP-2'3' and succinoadenine, respectively, does not necessarily indicate that these are the cleavage products.

The problem of the identification of this acid labile inositol derivative is being continued in an effort to assess its possible role in the metabolic pathway of inositol utilization.

SUMMARY NO. 41

STEROID HORMONES IN THE PLASMA OF MIGRATING SOCKEYE SALMON

D.R. Idler
A.P. Ronald
P.J. Schmidt

This report is a continuation of the study described in Summary No. 4 of this Station's Annual Report for 1957-58.

Cortisol has been conclusively established to be the principal adrenal cortical hormone in the peripheral plasma of the normal human. In general total 17-hydroxysteroid determinations have given higher values than have paper chromatographic methods involving the isolation of cortisol. A recent report describes a mean cortisol concentration in normal male plasma of 7.7 ± 3.5 µg/100 ml when cortisol was determined by paper chromatography as compared to 20.0 ± 8.7 µg/100 ml when total 17-hydroxysteroids were determined. Cortisol is also reported to be the principal steroid in the blood of several animals and has been tentatively identified in carp plasma. Extracts of the plasma of the Atlantic salmon (Salmo salar) have been demonstrated to possess considerable glycogenic activity. The same investigators determined the total 17-hydroxycorticosteroid levels by the Nelson-Samuels method and reported that there was considerably more glycogenic activity than could be accounted for on the basis of the chemical analytical procedure. Attempts to find cortisone in adrenal vein blood where the level of cortisol is extremely high and in human peripheral blood have been unsuccessful. Occasional blood samples have been reported to contain small amounts of a "cortisone-like" substance but the evidence is inadequate to establish identity. The isolation of 17 α-hydroxyprogesterone from the human ovary has very recently been described. This is the only reported occurrence of this substance in a natural product.

The present report is concerned with the isolation and characterization of cortisol, cortisone and 17 α-hydroxyprogesterone from sockeye salmon plasma and with the quantitative determination of plasma steroids in sockeye at various stages of sexual maturity.

Collection of Samples

All sockeye were captured with a dip net. The locations at which the various samples were taken are shown in Tables I and II. The isolation and characterization studies were carried out on the plasma obtained from several hundreds of female

salmon captured October 28, 1958 near Lytton, British Columbia, where the fish were about one week from the spawning grounds.

The 1957 Stuart Lake fish were captured at Lillooet, 415 miles from the spawning area and on arrival on the spawning grounds at Forfar Creek, British Columbia. All fish were bled by severing the caudal artery and care was taken to exclude slime. Heparin was used as an anti-coagulant for the samples taken on the Stuart Lake run while a mixture of ammonium and potassium oxalates was used on the Adams River samples. All samples were centrifuged immediately and the plasma stored at the temperature of dry ice until ready for analysis. The temperature of the plasma samples did not exceed 4°C during the thawing process.

Purification of Materials

Solvents were reagent grade. All low-boiling solvents, chloroform, methanol, benzene, etc, were distilled through a suitable fractionating column to assure no residue on evaporation. Chloroform was always distilled immediately before use. Formamide and ethylene and propylene glycols were distilled under high vacuum. The distilled formamide was stored in a desiccator over concentrated sulphuric acid. Specially purified Whatman No. 1 filter paper was used for all paper chromatograms. The paper was extracted in a Soxhlet apparatus for one day with 5% ammonium hydroxide; this was followed by a 2-day extraction with 5% aqueous acetic acid and a 3-day extraction with methanol. The paper was then dried *in vacuo* at a relatively low temperature and stored in Mylar plastic bags to exclude atmospheric contaminants.

Analytical Methods

The dihydroxyacetone sidechain determinations were carried out with the phenylhydrazine reagent. The α-ketol determinations were carried out with blue tetrazolium. Steroids with a Δ^4-3-ketone structure were detected on paper chromatograms with ultraviolet light and the location confirmed with the Δ^4-3-ketone specific alkali spray. A blue tetrazolium spray was used to detect steroids with the dihydroxyacetone or α-ketol sidechain on paper chromatograms. The Zimmermann reagent was used to locate 17-keto steroids on paper chromatograms and examination under ultraviolet light before and after alkali spray was used to locate those 17-keto steroids which also contained a Δ^4-3-ketone grouping. All spectra were recorded with a Beckman DK spectrophotometer. Micro (1 ml) cells were employed for most determinations.

Quantitative Analysis of Steroids

A highly specific acid fluorescence method was used for the determination of cortisol and corticosterone. Quantitative determinations of cortisol and cortisone were also carried out with the Porter-Silber reagent. Total 17-hydroxysteroids were determined by the method of Silber and Porter. It was observed that a slight yellow colour was formed immediately on the addition of the phenyhydrazine reagent to the plasma extracts. This colour was not formed by the addition of acid alone nor was it produced by pure reference steroids, therefore a small additional correction, not described in the published procedure was applied.

The authentic steroids referred to are USP chromatographic reference standards.

Isolation and Characterization of Cortisone, Cortisol and 17 α-Hydroxyprogesterone

In a typical isolation 1760 ml of plasma obtained from female sockeye captured at Lytton, B.C. was extracted four times with 2000 ml portions of ethyl acetate. The ethyl acetate was concentrated to 2000 ml on a flash evaporator at approximately 40°C with the receiver immersed in dry ice-acetone. The ethyl acetate (2000 ml) was washed

3 times with 100 ml portions of 5% aqueous sodium carbonate in order to remove estrogenic substances and other impurities. The ethyl acetate was then washed with 3 100 ml portions of water and the ethyl acetate phase clarified by the addition of small quantities of benzene. The residue was then taken to near dryness in vacuo. The residue was taken up in 300 ml of 70% aqueous methanol and extracted 4 times with equal volumes of n-hexane. The hexane phase was then washed with 200 ml of 70% methanol which in turn was washed 4 times with hexane. The methanol phases were combined with 2 volumes of water and the mixture continuously extracted with peroxide-free ether for 24 hours.

Whatman No. 1 filter paper was impregnated with a 30% solution of formamide. The residue obtained after removal of the ether was taken up in a small volume of chloroform:methanol 1:1, divided in two portions, and each was applied at the origin on a 12 cm strip of paper. Cortisone, cortisol and corticosterone were run as reference standards. The chromatograms were developed with chloroform:benzene 1:1 until the solvent had reached the bottom of the paper. A strip containing the standards and 1/40 of the plasma strips was examined under ultraviolet light, then sprayed with the alkali fluorescence reagent, and finally with the blue tetrazolium reagent. The plasma strips showed 2 strongly positive areas for all three tests. One corresponded with cortisol and the other with cortisone (or aldosterone). Visually cortisone appeared to be the major component. The remainder of the plasma paper strips were divided into 6 sections: 1 contained the origin and residual fatty material; 2 contained cortisol; 3 contained cortisone; 4 contained the region between cortisone and corticosterone; 5 contained corticosterone and the lower limit of corticosterone to the bottom of the paper was number 6.

The materials in sections 1 to 6 were eluted from the paper with three portions of hot methanol. Approximately 20 ml portions were used for the smaller strips and 40 ml for the larger. The methanolic extracts were transferred to a titerlenmeyer (a 50-ml erlenmeyer flask with a well 0.8 mm inside diameter and 3 cm deep attached to the bottom), and evaporated to dryness at 59°C under an atmosphere of nitrogen. The formamide were then removed under high vacuum at a temperature of about 70°. The contents of the flask were rinsed into the well with chloroform: methanol 1:1 and evaporated again under nitrogen. This procedure was repeated two or three times. The residue in the well was then taken up in several small portions of hot chloroform and the residual paper removed by filtration. The filtrate was evaporated to dryness.

Fraction 2. Fraction 2 showed an absorption maximum at 239 mμ, and calculated on $\epsilon 15.8 \times 10^3$ and using the molecular weight of cortisol there were 491 μg of steroid. However, by the highly specific acid fluorescence method for cortisol there was only 311 μg or 63.4%. Fraction 2 was then run on paper in the toluene-propylene glycol system for 78 hours. Three ultraviolet absorbing bands were then observed, 2A, 2B and 2C. The major component was 2C and it had an Rf value identical with authentic cortisol. The solvents were removed as described above and substance 2C was characterized as cortisol as described below.

Fraction 3. Fraction 3 showed an absorption maximum at 237 mμ and based on $\epsilon 15.8 \times 10^3$ and using the molecular weight of cortisone there were 650 μg of steroid. Fraction 3 was re-chromatographed in ethylene glycol-n-butylacetate along with authentic cortisone and aldosterone. A test strip showed that there was only 1 ultraviolet absorbing compound and this corresponded in position to cortisone. This substance was also the only material which exhibited alkali-fluorescence or reduced blue tetrazolium. The area corresponding to authentic aldosterone will be referred to as Fraction 3A and the area corresponding to authentic cortisone as Fraction 3B. Fraction 3A was re-run in toluene-propylene glycol and the aldosterone-cortisone area was bio-assayed for aldosterone activity as described below.

Fraction 3B was homogeneous in the toluene-propylene glycol system and in 48 hours travelled exactly the same distance as cortisone.

Fraction 5. Corticosterone if present would be in Fraction 5. The plasma strip exhibited no ultraviolet absorption in this area, so to aid in the detection of corticosterone, a further 200 ml of plasma was worked up as described above giving a total Fraction 5 from 2160 ml of plasma. Fraction 5 was re-chromatographed in toluene propylene glycol, and the area corresponding to authentic corticosterone (Rf 0.2) was cut out and worked up as described above. The sample exhibited no maximum in the region of 240 mμ and a calculation applying Allen's correction indicated a total of only 1.2 μg of ultraviolet absorbing material as compared to an appropriate paper blank. If this entire absorption was due to corticosterone it represented a level of only 0.05 μg/100 ml of plasma. The remainder of the paper contained no alkali fluorescent compounds.

Fraction 6. Even after hexane extraction the 70% methanol fractions always contained a certain amount of coloured fatty material which ran near the solvent front in the chloroform-benzene system. Therefore Fraction 6, which contained all steroids less polar than corticosterone was re-run in benzene-heptane 1:1 -formamide. Three ultraviolet absorbing compounds were observed only one of which will be considered in the present report. This material designated Fraction 6B ran in a similar position to authentic 11-desoxycorticosterone (Rf 0.45). This material exhibited an absorption maximum at 239 mμ, and corresponded to 204 μg (19 μg/100 ml for 60% recovery) based on the constants for 11-desoxycorticosterone. However, the material was observed to exhibit a sulphuric acid chromogen different from 11-desoxycorticosterone. Fraction 6B was re-chromatographed in normal hexane-propyleneglycol and the ultraviolet absorbing compound was found to have an Rf value relative to 11-desoxycorticosterone of 1.73, a value identical to that obtained for authentic 17-α-hydroxyprogesterone. There was no ultraviolet absorbing substance corresponding to 11-desoxycorticosterone.

Identity of Fraction 2B with cortisol and 3B with cortisone. Once the chromatographic homogeneity of 2C and 3B had been established, a sample of each was treated for 2 hours at 22°C with concentrated H_2SO_4. Substance 2C exhibited absorption maximum at 237, 282, 395 and 478 mμ and O.D. ratios of 0.98:1:0.47:0.55 and substance 3B exhibited maximum at 284, 343 and 420 and O.D. ratios of 1:0.38:0.31. These values were identical to those obtained from authentic cortisol and cortisone, respectively. Aliquots of 2C and 3B gave quantitative Porter-Silver reactions for the dihydroxyacetone sidechain of the magnitude expected from the concentration based on ultraviolet absorption measurements and the chromogens gave the expected λ maximum at 410 mμ. Authentic cortisol and 2C were treated with fuming sulphuric acid for 1.5 hours. The spectra of the resulting chromogens were identical with absorption maximum at 240 mμ and 490 mμ and a shoulder at 270 mμ.

Oxidative Removal of the Dihydroxyacetone Group of 2C and 3B

Sodium bismuthate (1 g) was added to ca 30 μg of 2C, 3B and authentic cortisol and cortisone, each of which was previously dissolved in 5 ml of 50% aqueous acetic acid. The mixtures were shaken for one half-hour and then filtered through purified Whatman No. 1 paper. The residues and the papers were washed thoroughly with water. The aqueous acetic acid filtrates were extracted with chloroform. The chloroform was removed under a stream of nitrogen and the residues were run in heptane:benzene-formamide. The reaction products of authentic cortisone and 3B produced a single ultraviolet absorbing spot with the same Rf value as Δ^4-androstene-3,11,17-trione. Authentic cortisol and 2C both contained a major UV absorbing substance corresponding in Rf value to 11-β-hydroxy-Δ^4-androstene-3,17-dione, and both gave also a very minor component corresponding to Δ^4-androstene-3,11,17-trione. The major ultraviolet

absorbing components were eluted from the paper as previously described and re-run in benzene:hexane:methanol:water 33:66:80:20. The oxidation products of authentic cortisol and cortisone had Rf values of 0.16 and 0.23 respectively and both compounds absorbed in the ultraviolet and gave positive Zimmerman reactions. The oxidation products of substance 2C and 3B corresponded identically with those from the respective reference standards.

The infrared spectra of substances 2C and 3B confirmed their identity with cortisol and cortisone respectively.

Identity of Substance 6B with 17 α-Hydroxyprogesterone

A solution of substance 6B in methanol contained 18.3 μg/ml 17 α-hydroxyprogesterone equivalents as indicated by the ultraviolet absorption at 240 mμ. The solution of substance 6B and an equivalent solution of authentic 17 α-hydroxyprogesterone gave the same quantitative Porter-Silber reactions. The maximum in each case was at 340 mμ. The sulphuric acid chromogens produced by aliquots of the two solutions were also identical and exhibited absorption maxima at 285 and 430 mμ as compared to 285, 370 and 446 for authentic 11-desoxycorticosterone. Substance 6B and authentic 17 α-hydroxyprogesterone were each treated with 20 mg of sodium borohydride in 0.5 ml of 80% aqueous tert-butanol for 18 hours. The mixtures were then shaken with 1 g of sodium bismuthate in 4 ml of 50% aqueous acetic acid for 1 hour. The steroids were extracted as described above and chromatographed in the benzene: hexane:methanol:water system. Substance 6B and 17 α-hydroxyprogesterone both gave Δ^4-androstene-3,17-dione as the major product as well as three minor Zimmermann positive substances with Rf's 0.03, 0.17 and 0.35.

Substance 3A

The strip corresponding to authentic aldosterone from 2160 ml of plasma was applied as a spot and run in the toluene-propylene glycol system for 48 hours. There was no evidence for an ultraviolet absorbing substance on the paper. The area corresponding to authentic aldosterone was cut out and extracted as described above. There was a negligible residue when the solvent was removed and when the fraction was run against an appropriate blank there was no absorption maximum near 240. However, there was a shoulder in this region but if the total absorption was due to an aldosterone it would represent less than 2 μg. The sample was taken up in ethanol and divided among six 5-day adrenalectomized assay rats, along with three dose levels of desoxycorticosterone acetate (1,4 and 16 μg) at 8 rats per dose. The index of activity was the Na/K ratio in the urine sample collected 0-5 hours after injection. The sample proved to be virtually inactive giving a response less than that obtained with 1 μg of 11-desoxycorticosterone (DOCA). A sample of free aldosterone was also included in the assay at a dose of 0.25 μg and gave the expected response, with a potency of approximately 50 x DOCA. (Dr. G. Farrell, Western Reserve, did the bioassay.)

Quantitative Determination of Steroids in Adams River Sockeye Plasma

Ethyl acetate extracts were prepared from 100 ml samples of the plasmas listed in Table I. The only modification from the large scale isolation was in the extraction of the 70% methanol fraction after the hexane partition. Three volumes of water was added to the 70% methanol and the steroids were extracted three times with 200 ml portions of chloroform. Each residue was then placed on a 14 cm length of pre-washed Whatman No. 1 paper impregnated with formamide and benzene: chloroform 1:1 was used as the developer. Cortisol, cortisone and 17-α-hydroxyprogesterone were run on an adjacent length of paper. The material from the plasma zone corresponding in position to authentic cortisol and cortisone was eluted from the paper as described above and run for 48 hours in the toluene-propylene glycol system. The areas of the plasma

chromatogram corresponding to cortisone and corticosterone were extracted from the paper, and made up to volume for analysis. The cortisol was analyzed both by acid fluorescence and the Porter-Silber reaction, while cortisone was analyzed by the latter method. The results are shown in Table I. Recovery experiments were carried out in duplicate with authentic standards which were added to 100 ml samples of Lytton male plasma at levels of 15 and 30 µg per 100 ml. The recovery of added cortisone through the entire procedure varied from 64 to 68% and averaged 65%. The recovery of added cortisol averaged 50%.

TABLE I - Cortisone and cortisol in Adams River sockeye plasma.

Date 1958	No. of fish	Location	Sex	Cortisone* µg/100 ml	Cortisol* µg/100 ml	Cortisol**	Total 17-‡ Hydroxy-corticosteroids µg/100 ml
Oct 16	34	Lower	M	-	-	8	-
" 18	32	Fraser	F	15	19	28	50
" 27	21	"	M	27	12	11	33
	57	"	F	19	21	26	112
" 29	80	Lytton	M	22	11	11	30
	80	"	F	41	26	24	126
Nov 2	55	Adams Lake	M	27	64	66	154
	15	"spawned"	F	44	129	141	231

* Ethyl acetate plasma extracts purified by chromatography in chloroform:benzene 1:1-formamide and the toluene-propylene glycol and the steroids measured by the Porter-Silber reaction.

** Chloroform plasma extracts purified by chromatography in toluene-propylene glycol and the steroid measured by acid-fluorescence.

‡ Steroids determined directly on chloroform extracts of plasma with the Porter-Silber reagent.

Quantitative Determination of Steroids in Pre-spawning Stuart Lake Sockeye Plasma

The plasma was pooled to give 4 male and 4 female samples (Table II). To 10 ml of each plasma 0.5 ml of 1 N sodium hydroxide was added and the mixture shaken and then extracted three times with 20 ml portions of chloroform. The chloroform extracts were chilled at 4°C overnight and filtered through glass wool. The chloroform was removed under nitrogen and the residue chromatographed in toluene propylene glycol for 48 hours. The area of the paper containing the cortisol was extracted with hot methanol and suitable aliquots were taken for acid fluorescence measurements.

The recovery of added cortisol to two of the samples treated as above was 65%. The results corrected for recovery are shown in Table II.

Others have shown that the salmon pituitary has been shown to elaborate a substance with ACTH activity and this gave rise to the hypothesis that the degenerative changes following spawning might be due to intolerable concentrations of an adrenal cortical hormone produced under the influence of large amounts of ACTH. These studies have now demonstrated that Pacific salmon show extensive degeneration of the pituitary gland at full maturity in spawning, and this is accompanied by marked hyperplasia (and presumed increased secretory activity) of the adrenal gland. In contrast, mature rainbow trout, which spawn repeatedly, generally show little, if any, pituitary degeneration.

TABLE II - Cortisol in Stuart Lake sockeye plasma.

Location	Sex	No. of fish	Cortisol µg/100 ml	Sex	No. of fish	Cortisol µg/100 ml
Lillooet	M	10	18	F	10	42
"	M	10	27	F	10	37
"	M	10	18	F	10	37
"	M	10	21	F	-	-
Average			23			39
Forfar	M	10	9	F	14	22
Creek	M	5	16	F	14	31
"	M	6	13	F	13	40
"	M	4	11	F	14	28
Average			13			30

The greatly increased cortisol concentration in the plasma of both sexes following spawning is apparent by both chromatographic procedures, whether the final analysis was carried out by the Porter-Silber or the acid-fluorescence method (Table I). The total 17-hydroxycorticosteroid levels as determined directly on chloroform extracts of plasma confirmed the greatly increased steroid content of the plasma following spawning. These data support the suggested high secretory activity of the adrenal gland at this stage in the life cycle. It certainly appears plausible that these extremely high hormone plasma levels may play a role in the degenerative changes and death of the fish after spawning. Now that the qualitative and quantitative data are available it may be possible to design an experiment to artificially produce and maintain these hormone blood levels and attempt to gain an insight into this problem.

The presence of detectable quantities of cortisone was unexpected but even more surprising was the finding that it was a major plasma steroid in pre-spawning fish and the predominant one in several samples. Cortisol is the principal plasma steroid in both sexes after completion of the spawning act. The data in Table I do not answer one important question - "Is the increased cortisol level coincident with the completion of the spawning act or was there a very significant increase between Lytton and spawning, a period of ca 13-15 days?" Plasma samples were available from fish captured on arrival at the Stuart Lake spawning grounds. These fish were due to spawn in ca 4-5 days. Male plasma (100 ml) averaged 13 µg (Table II) as compared to 11 µg and female plasma averaged 30 µg as compared to 26 µg at Forfar Creek and Lytton, respectively. These data show that there was a very sharp increase in the cortisol concentration in sockeye plasma during the brief period immediately preceding spawning and at the completion of the spawning act. There appeared to be some decrease in plasma cortisol between Lillooet and Forfar Creek for the Stuart Lake sockeye. However, it should be emphasized that these samples were obtained in 1957 and while they were stored at low temperature, some protein denaturation had occurred. It is probably desirable at this time to interpret data on the Stuart Lake samples only to the point of indicating the order of magnitude of the cortisol concentration in plasma particularly since techniques were improved for the analyses of the Adams River samples.

The total 17-hydroxysteroid levels are in most instances considerably higher than the combined cortisol and cortisone concentrations. There are many factors possibly contributing to this result, among which may be mentioned the small

contribution of 17 α-hydroxyprogesterone and the possible presence of non-steroid substances in the chloroform extracts of salmon plasma which react with phenylhydrazine. It has not yet been established whether the two steroids which accompany cortisol in the chloroform:benzene 1:1 - formamide system, but separate from it in the toluene-propylene glycol, react with Porter-Silber reagent. In any event the total 17-hydroxycorticoid levels in human plasma have generally been found to be higher than the cortisol level even following chromatographic purification of the chloroform extracts. The levels of cortisone and cortisol or the total 17-hydroxycorticoid levels found in the spawned fish or the total 17-hydroxycorticoid levels found in nearly all the female samples in the present study are sufficiently high to account for the glycogenic activity found in Salmo salar plasma. The report of this study was too abbreviated to permit an assessment of the possible reasons that the biological assay did not agree with the chemical (Porter-Silber) method. One conceivable explanation is that the less polar cortisone was not retained on the adsorbent along with the cortisol.

Another very important factor which would be expected to be under hormone control during the spawning migration is the maintenance of electrolyte balance during and after the transition from salt to fresh water. Aldosterone is the most potent electrolyte regulating hormone secreted by the human adrenal cortex. Aldosterone has been identified in the adrenal vein blood of hypophysectomized dogs. The level in normal human systemic blood is reported to be 0.08 µg/100 ml and others report that sheep blood contains 0.35 µg/100 ml. Since aldosterone has a biological potency of approximately 50 times that of 11-desoxycorticosterone in the assay method used in the present study, it would be expected that as little as 0.02 µg in the 2160 ml would have been detected. If aldosterone were present at a concentration comparable to that in human plasma, approximately 1.7 µg should have been present, or 0.8-0.9 µg allowing for ca 50% recovery.

Corticosterone has been reported to be the principal steroid in the circulating plasma of the rat, and is probably present at a level of approximately 1/5 that of cortisol in normal human plasma. However, little if any corticosterone appears to be present in the female sockeye salmon used in the present study. Both male and female sockeye salmon captured 415 miles from the spawning ground at Lillooet, B.C. on the 1956 Stuart Lake run and at Forfar, B.C. (Table II), were worked up as described in the experimental section and run in the toluene propylene glycol system. The areas corresponding to authentic corticosterone were extracted from the paper and assayed by the fairly specific acid fluorescence method. The level of corticosterone was less than 1 µg/100 ml. Although it will be necessary to carry out studies on more samples before it can be concluded that the concentration of corticosterone is always extremely low in salmon plasma, it is highly unlikely that corticosterone plays a significant role in regulating ion balance. Another important mineral regulating hormone, 11-desoxycorticosterone, does not appear to be present in detectable quantities in the adrenal vein blood of the dog or in normal human plasma. In the present study there was no evidence for the occurrence of 11-desoxycorticosterone in a large sample (1760 ml) of plasma. It appears, therefore, that none of the anticipated electrolyte regulating hormones are present in significant quantity in salmon plasma.

Because of the relatively high levels of cortisol in normal human plasma as compared to aldosterone, it has been suggested that cortisol may play a significant role in maintaining normal mineral metabolism. If this conclusion is valid for human plasma, then it is indeed possible that the extremely high levels of cortisone and cortisol regulate mineral metabolism in salmon at least during the river migration.

It is believed that this is the first isolation of 17 α-hydroxyprogesterone from blood. The physiological effects of this steroid have only recently been investigated but it is already apparent that it represents a rather unique case where the

free alcohol generally has very slight biological activity while a simple derivative (the caproate) has 60 times the biological potency of progesterone in one biological assay. The analytical methods which are so sensitive for compounds such as cortisol and cortisone are not nearly so effective for this steroid because of the absence of the hydroxyl group on C21. For this reason and because of the difficulty in highly purifying the more non-polar steroids, it has not yet been possible to obtain reliable quantitative estimations on relatively small volumes of plasma.

There is a considerable difference in the timing of the various Fraser River sockeye runs which brings up the question, "What triggers the move to the spawning grounds. Is it under hormone control?" The fish going to Stuart Lake arrive just a few days prior to spawning, while those going to Chilco Lake arrive a month early and spend this time waiting for the gonads to ripen. The huge Adams River run delays for several weeks in brackish water before moving up river to arrive just prior to spawning. Fortuitously, this delay coincides with unfavourable temperatures in the spawning area. The Adams River run provides an excellent opportunity for studying the plasma hormone level immediately before, during, and after the commencement of the river migration. In 1959 100,000-200,000 sockeye are expected to make the migration to Adams River and a fairly extensive sampling program is contemplated.

STAFF OF THE VANCOUVER TECHNOLOGICAL STATION

ADMINISTRATIONAL

Director	H.L.A. Tarr, Ph.D. (McGill; Cantab.), F.R.S.C.
Executive Assistant (Admin. Officer, Grade 2) (Employment extended after age 65, from February 7, 1959 to April 15, 1959.)	J.W. Kilpatrick, M.C.
Executive Assistant (Admin. Officer, Grade 1) (from December 2, 1958)	P.N. MacLeod
Secretary (Clerk, Grade 4)	Nellie E. McBride
Stenographer and Executive Clerk (Stenographer, Grade 3)	Cecilia L. Robertson

INVESTIGATIONAL

Biochemistry:

Senior Scientist	R.A. MacLeod, Ph.D. (Wisconsin)
Associate Scientist	C.A. Claridge, Ph.D. (Iowa State)
Assistant Scientist	J.R. McBride, M.A. (Brit. Col.)
Junior Scientist	Eva Onofrey, B.Sc. (Manitoba)
Junior Scientist	Aiko Hori, B.S.A. (Brit. Col.)

Chemistry:

Senior Scientist	D.R. Idler, Ph.D. (Wisconsin)
Associate Scientist	H. Tsuyuki, Ph.D. (Wisconsin)
Associate Scientist	P.J. Schmidt, B.E. (Sask.)
Assistant Scientist	U.H.M. Fagerlund, M.Sc. (Åbo, Finland)
Junior Scientist	Violet M. Chang, B.A. (Brit. Col.)
Technician, Grade 1	A.P. Ronald

Engineering:

Associate Scientist (Transferred to Nanaimo Station, November 21, 1958.)	P.J.G. Carrothers, S.M. (Massachusetts Inst. Tech.)
Associate Scientist	J.S.M. Harrison, B.A.Sc. (Brit. Col.)
Associate Scientist	S.W. Roach, B.A.Sc. (Brit. Col.)
Assistant Scientist (Employment terminated by death, October 18, 1958.)	N.A.W. LePage, B.A.Sc. (Brit. Col.)
Assistant Scientist (from Nov. 24, 1958)	F.G. Claggett, B.A.Sc. (Brit. Col.)
Technician, Grade 1	E.G. Baker

Microbiology and Food Technology:

Director	H.L.A. Tarr, Ph.D. (McGill; Cantab.), F.R.S.C.
Associate Scientist	N. Tomlinson, Ph.D. (California)
Associate Scientist	J.D. Wood, Ph.D. (Aberdeen)
Assistant Scientist	Burnett A. Southcott, B.S.A.(Brit. Col.
Assistant Scientist	Rudolph H. Moyer, B.S.A. (Brit. Col.)
Junior Scientist	Vera M. Creelman, B.A. (Brit. Col.)

STAFF OF THE VANCOUVER TECHNOLOGICAL STATION
(Continued)

LIBRARY

Librarian (Asst. Technician, Grade 3) part time (resigned Feb. 28, 1958)	Alix G.A. Richards, B.A. (McGill)
Librarian (Asst. Technician, Grade 3) part time (from Feb. 17, 1958)	Lois M. Warren, B.A. (Brit. Col.)

MAINTENANCE

Maintenance Supervisor, Grade 4	F.C. Freeman
Caretaker, Grade 2	P.E. Enright
Cleaning Service Woman (part time)	Rita Reichert
Cleaning Service Woman (part time)	Agnes M. Hunter

TEMPORARY STAFF

Bitners, I.I. Student Assistant. Term May 1 to Sept. 30, 1958. Continuing part time as Technician 1. (Chemistry)

Buttkus, Hans. Student Assistant. Term May 5 to Aug. 29, 1958. (Biochemistry)

Duncan, D.W., B.S.A. (Brit. Col.). Student Assistant. Term June 9 to Sept. 5, 1958. (Microbiology)

Ferguson, B.C. Stenographer. Part time from Aug. 26 to Sept. 19, 1958. (Administration)

Grajcer, Don, B.A. (Calif. at L.A.). Technician 1. Part time from Nov. 7, 1958, continuing. (Biochemistry)

Hodgson, Jean D. Cleaning Service Woman. Part time from July 15 to Aug. 1, 1958. (Buildings)

Hrushowy, E.J., B.A. (Brit. Col.). Technician 1. Part time from April 1 to Oct. 30, 1958. (Chemistry)

Jackson, Michael, B.Sc., M.Sc. (Manchester). Technician 1. Part time from Nov. 17, 1958, continuing. (Microbiology).

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

Katin, Adele. Cleaning Service Woman. Part time from Aug. 22 to Sept. 16, 1958. (Buildings)

Leung, Jock. Student Assistant. Term May 16 to Aug. 18, 1958. Continuing part time as Technician 1. (Chemistry)

Lukey, L.J. Student Assistant. Term May 5 to Sept. 30, 1958. Part time as Technician 1 from Oct. 8, 1958 to March 26, 1959. (Biochemistry)

Mullick, Dharam B., B.Sc. (Delhi). Technician 1. Part time from Nov. 6, 1958, continuing. (Biochemistry)

Reid, K.G., B.A. (Brit. Col.). Student Assistant. Term May 5 to August 29, 1958. (Biochemistry). Part time as Technician 1 from Oct. 9, 1958 to Feb. 12, 1959. (Microbiology)

PUBLICATIONS

VANCOUVER TECHNOLOGICAL STATION

1958

1. Baker, E. G., B. A. Southcott and H. L. A. Tarr. Effect of chlortetracycline (CTC) antibiotic on the keeping quality of lingcod stored in refrigerated sea water. Pacific Prog. Rept., No. 112, pp. 15-17.

2. Carrothers, P. J. G. The approach to fishing gear problems. Canadian Fisheries Annual for 1958, p. 53.

3. Duncan, D. W., and H. L. A. Tarr. Biochemical studies on sockeye salmon during spawning migration. III. Changes in the protein and non-protein nitrogen fractions in the muscles of migrating sockeye salmon. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 8, pp. 799-803, 1958.

4. Fagerlund, U. H. M., and D. R. Idler. Marine sterols. V. Isolation of 7,24(28)-ergostadien-3β-ol from starfish. Journal of the American Chemical Society, Vol. 81, No. 2, pp. 401-403, 1958.

5. Idler, D. R., and I. Bitners. Biochemical studies on sockeye salmon during spawning migration. II. Cholesterol, fat, protein and water in the flesh of the standard fish. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 8, pp. 793-798, 1958.

6. Idler, D. R., and H. Tsuyuki. Biochemical studies on sockeye salmon during spawning migration. I. Physical measurements, plasma cholesterol, and electrolyte levels. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 8, pp. 783-791, 1958.

7. MacLeod, R. A., H. Hogenkamp and E. Onofrey. Nutrition and metabolism of marine bacteria. VII. Growth response of a marine flavobacterium to surface-active agents and nucleotides. Journal of Bacteriology, Vol. 75, No. 4, pp. 460-466, 1958.

8. MacLeod, R. A., C. A. Claridge, A. Hori and J. F. Murray. Observations on the function of Na^+ in the metabolism of a marine bacterium. Journal of Biological Chemistry, Vol. 232, No. 2, pp. 829-834, 1958.

9. Possible role of Na^+ in metabolism of a marine bacterium. Federation Proceedings, Vol. 17, p. 267, 1958.

10. MacLeod, R. A., R. E. E. Jonas and J. R. McBride. Variations in the Na^+ and K^+ content of the flesh of Pacific salmon with particular reference to migration. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 11, pp. 1257-1268, 1958.

11. March, B. E., J. Biely, J. R. McBride, R. A. MacLeod and D. R. Idler. A comparison of the nutritive value of condensed herring solubles prepared by acid and enzyme treatments. Pacific Prog. Rept., No. 111, pp. 23-28.

12. Moyer, R. H., B. A. Southcott and H. L. A. Tarr. Distribution of chlortetracycline (CTC) antibiotic in ice made from hard water. Pacific Prog. Rept., No. 112, pp. 21-22.

13. Roach, S. W., and J. S. M. Harrison. Application of refrigerated sea water fish holding to a small combination fishing vessel. Pacific Prog. Rept., No. 112, pp. 3-6.

14. Southcott, B. A., R. H. Moyer, E. G. Baker and H. L. A. Tarr. Some recent experiments in preservation of fish with tetracycline antibiotics. Pacific Prog. Rept., No. 110, pp. 16-18.

15. Tarr, H. L. A. Dogfish. Pacific Prog. Rept., No. 110, pp. 13-16.

16. Lingcod muscle purine nucleoside phosphorylase. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 6, pp. 517-530, 1958.

17. Biochemistry of fishes. Annual Review of Biochemistry, Vol. 27, pp. 223-244, 1958.

18. Tarr, H. L. A., and D. C. Gillespie. Method of producing block ice. Canadian Patent No. 552,878, February 4, 1958.

19. Thomson, W. A. B., R. E. E. Jonas, R. A. MacLeod and D. R. Idler. Measurement of the viscosity of herring solubles. Fisheries Research Board of Canada, Technological Station, Circular No. 21, 5 pp., April, 1958.

20. Tomlinson, N. Ribonuclease activity of fish muscle extracts. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 6, pp. 633-643, 1958.

21. A nuclease from lingcod muscle. Proceedings of the British Columbia Academy of Science, Vol. 2, p. 11, 1958.

22. Tsuyuki, H., V. M. Chang, and D. R. Idler. The acid-soluble nucleotides of salmon liver. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 5, pp. 465-473, 1958.

23. Wood, J. D. The non-protein nitrogenous constituents of the muscle of sockeye salmon during spawning migration. Proceedings of the Canadian Federation of Biological Societies 1st Annual Meeting, p. 55, 1958.

24. Biochemical studies on sockeye salmon during spawning migration. IV. The non-protein nitrogenous constituents of the muscle. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 8, pp. 833-838, 1958.

25. Nitrogen excretion in some marine teleosts. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 12, pp. 1237-1242, 1958.

26. Wood, J. D., and B. B. Migicovsky. The effect of dietary oils and fatty acids on cholesterol metabolism in the rat. Canadian Journal of Biochemistry and Physiology, Vol. 36, No. 4, pp. 433-438, 1958.

SUBMITTED FOR PUBLICATION

1. Carrothers, P. J. G. The impact of synthetic fibres in the fishing industry. (B.C. Professional Engineer. Submitted for publication.)

SUBMITTED FOR PUBLICATION (Cont'd.)

2. Creelman, Vera M., and N. Tomlinson. Biochemical studies on sockeye salmon during spawning migration. VI. Ribonucleic acid and deoxyribonucleic acid. (Journal of the Fisheries Research Board of Canada. Submitted for publication.)

3. Idler, D. R., and I. Bitners. Biochemical studies on sockeye salmon during spawning migration. V. Cholesterol, fat, protein and water in the body of the standard fish. (Journal of the Fisheries Research Board of Canada. In press.)

4. Idler, D. R., and W. A. Clemens. The energy expenditure of sockeye salmon during their spawning migration to Stuart and Chilco Lakes. (Progress Report of the International Pacific Salmon Fisheries Commission. In press.)

5. Idler, D. R., A. P. Ronald and P. J. Schmidt. Isolation of cortisone and cortisol from plasma of Pacific salmon. (Journal of the American Chemical Society. In press.)

6. Biochemical studies on sockeye salmon during spawning migration. VII. Steroid hormones in plasma. (Canadian Journal of Biochemistry and Physiology. Submitted for publication.)

7. McBride, J. R., R. A. MacLeod and D. R. Idler. Studies on the conversion of fish stickwater to solubles. III. Identity of the gel factor in herring solubles and means of overcoming its effect. (Journal of Agriculture and Food Chemistry. Submitted for publication.)

8. Ronald, A. P. An automatic semi-micro zone melting apparatus. (Analytical Chemistry. In press.)

9. Tarr, H. L. A. Lingcod muscle phosphoriboisomerase and ribulose 5-phosphate 3-epimerase. (Canadian Journal of Biochemistry and Physiology. Submitted for publication.)

10. Chemical control of microbiological deterioration. (Chapter for a book entitled "Fish as Food", to be published by Academic Press, Ed. G. Borgstrom.)

11. Changes in nutritive value through handling and processing procedures. (Chapter for a book entitled "Fish as Food", to be published by Academic Press, Ed. G. Borgstrom.)

12. New horizons for food preservation. (Food in Canada. Submitted for publication.)

13. Tomlinson, N. Lingcod muscle nuclease. (Canadian Journal of Biochemistry and Physiology. Submitted for publication.)

14. Wood, J. D. Lipase activity in lingcod muscle preparations. (Canadian Journal of Biochemistry and Physiology. Submitted for publication.)