FISHERIES RESEARCH BOARD OF CANADA

ANNUAL REPORT

for

1961 - 62

of the

TECHNOLOGICAL STATION
Vancouver, B. C.

H. L. A. TARR, Director

FISHERIES RESEARCH BOARD OF CANADA

TECHNOLOGICAL STATION

VANCOUVER, B.C.

INTRODUCTION

Technological research has been an important segment of this Board's operations for over thirty-five years and during this time many original contributions have been made which are being used actively by the Canadian fishing industry. Thus, refrigerated sea-water holding of fish, first used experimentally at the Halifax Station before 1929, is now being employed on a very large scale for salmon in the Pacific Northwest, while studies on fish preservation by antibiotics, initiated about 1944 at the Vancouver Station, have resulted in quite extensive fish fillet treatments in the Maritimes. These examples have been taken partly because they show the typical time lag which may elapse between an idea and its final application and partly because they represent advances which can be made by intelligent application of existing knowledge. Though similar useful applications may be made in the future, it is probable that they will occur less frequently. Services heretofore often performed by Board scientists have involved work which is becoming increasingly the responsibility of other organizations within the Department of Fisheries. This, it is hoped, will enable the Board scientist to carry out his primary function, namely research. Therefore, the future trend will undoubtedly be toward development of new knowledge with the final objective of its application to fisheries. Indeed, the present report emphasizes this trend. It is unlikely that more than a very small percentage of this work is going to prove financially profitable, and that which does may well have to go through a long stage of preliminary development. This situation is not unique for it applies to most of the research conducted by large industrial organizations. Continued close co-operation with other fishery organizations, particularly with the Department and its Industrial Development Service, must be maintained.

H.L.A. Tarr,
Director.

GENERAL SUMMARY OF INVESTIGATIONS

TABLE OF CONTENTS

Refrigerated Sea Water

Refrigerated sea-water transportation and storage of salmon has continued to experience a very favourable reception in the Pacific northwest. Indeed, it can be stated that this method has almost revolutionized the handling of these fish in certain areas. The past year has seen the completion and thoroughly satisfactory performance of a vessel "Western Express" as a refrigerated sea-water packer. This vessel, which has a capacity for 450,000 lb of fish, has been an outstanding success in all respects; fish are loaded and chilled very rapidly and thus excellent quality is maintained. Employed in her design were all the engineering principles developed here over the past several years, including the means of driving equipment, the design of heat exchange units, and construction of tanks and piping with a view to ease of sanitation. In this field, though activities were limited to observation and guidance, the refrigerated sea-water applications on the halibut vessel "Silver Viking II" and the salmon troller "Chiller" have performed well. Three further conversions of salmon packers with capacities of from 150,000 to 300,000 lb of fish are slated for the present year, and because of the wide practical and theoretical experience in this field, this Station continues to be consulted closely.

Freezing Fish at Sea

As a result of a number of meetings with prominent fishermen and representatives of the Department of Fisheries and of its Industrial Development Service, this Station has been involved in a proposal to equip four seiners with freezing equipment in order to investigate the possibilities of developing an active tuna fishery. Following a visit to California by Station engineers, freezing systems suitable for local vessels were designed and costs estimated. The Freon system proposed differs in certain important respects from that currently employed on United States tuna vessels, and it is felt has many advantages. Three seiners are presently being equipped with this system and one seiner with the brine spray system using ammonia compressors.

Herring Canning. Salmon Unloading

One company has undertaken a commercial venture into canning herring employing the vacuum method which was developed several years ago at this Station. Another engineering project to which some time has been devoted is that of a "pneumatic" unloading system for larger fish such as salmon. The principle was found to work well with herring, and one vessel, the "Nootka Chief", has demonstrated the effectiveness of this system in handling salmon under practical conditions.

Biochemistry of Fresh, Chilled and Frozen Fish

As a consequence of the fact that tuna, and possibly halibut, may be frozen at sea on British Columbia vessels within the next few years, the need for a better understanding of the post-mortem biochemical changes which occur in fish under different storage conditions has become apparent. Therefore, much of the basic research in biochemistry has been diverted to studies of changes which proceed in fish after death, and how these may relate to the condition of the subsequently frozen and stored fish. Since it was realized that one of the most undesirable changes in frozen stored fish was the development of toughness, a program has been initiated in order to study this problem. A method recently developed at the Torry Research Station was not found entirely satisfactory, and so far toughness has been determined with the aid of a modified Mangold sclerometer. However, the need for more sensitive mechanical methods for objective texture evaluation has been recognized and is being explored. Certain treatments of fish prior to slaughter appear to partly inhibit the onset of toughness of the muscle when the fish are frozen and stored. However, it is as yet too early to judge the potential value of this investigation. It has been found that the brown lateral muscle of fishes under certain conditions contracts much more strongly post mortem than does the white muscle, and this phenomenon is being investigated actively.

With the aid of certain radioactive compounds such as glycogen and adenosine triphosphate, certain of which have been prepared in the laboratory, the

origin of the sugars, glucose and ribose, and of related sugar phosphates which occur in fish flesh post mortem has been determined with considerable accuracy.

Careful experiments have proven beyond doubt that the fructose monophosphate which occurs in fish muscles is entirely fructose 6-phosphate and not, as British investigators have maintained, a mixture of fructose 1- and 6-phosphates. Also, in contradiction to British results, no ribose 1-phosphate has been found in fish muscles post mortem.

An entirely original method of evaluating the comparative contribution of the hexosemonophosphate shunt system (pentose cycle) in carbohydrate utilization in fish has been studied. This has involved an investigation of the metabolism in living fish (Salmo gairdnerii) of radioactive phosphogluconic and gluconic acids which have been prepared specifically C^{14} labelled in the C_1, C_6 or all carbon atoms. The results indicate a very small participation of the shunt system and it appears that in fish the Embden-Meyerhof glycolytic pathway is of greatest importance.

One study with lingcod has shown that phospholipid phosphorus is hydrolyzed more rapidly at 14°F than at -4°F and is very slowly hydrolyzed at -22°F. Another investigation has shown that with Pacific gray cod, a species which is related to Atlantic cod, the phospholipids hydrolyze more rapidly during the first few weeks of frozen storage than do those of lingcod. Thus it has been established that free fatty acid formation from muscle phospholipids occurs in local species of fish, but it cannot be stated with certainty that this phenomenon has an adverse effect on quality. It has been impossible to detect formation of lactic acid in lingcod muscle held at -22°F (-30°C) during six months storage.

An exploratory examination has been made of the bound nucleotides of fish muscle in relation to denaturation of muscle proteins during frozen storage. Over 90% of the total protein-bound nucleotide of washed lingcod muscle residue has been found to be accounted for as adenosine diphosphate in both freshly frozen and severely denatured frozen muscle. It appears that there is a small decrease in the

amount of this nucleotide during frozen storage. With actin extracted from either freshly frozen or severely denatured frozen muscle adenosine triphosphate was found to be the principal bound nucleotide. A small loss of this nucleotide during frozen storage was also observed.

An investigation of changes in muscle protein solubility during the course of rigor mortis is in progress. Early results with rainbow trout held at 72°F (22°C) suggest that there is no significant denaturation of protein until the fish has begun to soften, or has become quite soft again following rigor.

Investigations in this laboratory which have extended over the past few years have shown that the lipid fraction of the muscle of active fish such as migrating salmon is rapidly depleted and is presumably used for energy purposes. The enzymic mechanisms responsible for this utilization are being studied. It has been found, employing muscle slice techniques, that the muscle contains enzymes capable of oxidizing lipids and that these enzymes are about 50 times more active in the dark lateral muscle than in the ordinary muscle as judged by in vitro oxidation of octanoic acid.

Fish Attractants and Fish-holding Facilities

Further progress has been made in the difficult problem of the homestream odour attractants for adult migrating sockeye salmon. Using a bioassay system developed last year, fish from Great Central Lake responded positively in 14 of 15 tests to water from that lake, and in 5 of 16 tests to other water supplies. When water from three creeks that flow into Great Central Lake was tested, in only 3 of 5 tests were the results in accordance with expectations based on known runs of sockeye. In testing fish from Cultus Lake, results were in accordance with expectations in 10 of 11 tests. Attempts to concentrate the active attractant compounds from the water have been partially successful and indicate that they are volatile. Bi-weekly salt baths and weekly intramuscular injections of terramycin (oxytetracycline) have been found to control both fungus infections and furunculosis

in these fish. The results indicate that after a few weeks, treatment may be discontinued without reappearance of either infection.

Tests with juvenile sockeye salmon indicate that olfactory perception towards natural foods is probably conditioned by previous exposure to the food in question. Fish can detect concentrations of the active substances in these foods in concentrations as low as one part of the wet weight of the whole food in 800,000,000 parts of water. Plugging of the nasal sacs of these fish prevents this food response, the fish becoming sensitive again after the removal of the plugs.

An annex constructed last year to house live fish-holding facilities has been equipped with an air compressor, water dechlorinators, seven 400-gallon circular fiberglas tanks, ten 10-gallon oblong fiberglas tanks, and one large and four small glass-faced experimental observation tanks. The equipment has given excellent service since completion of the installation.

Nutritive Value of Fish Products

The investigation of the hypocholesterolemic activity of the unsaponifiable fraction of certain fish oils as related to its vitamin A content has been concluded with the finding that the vitamin A structure is necessary for this activity and that all types of vitamin A are effective. A number of compounds related to vitamin A and not having the same structure were inactive. There are indications that the hypocholesterolemic activity occurs before or during absorption of the vitamin into the intestinal wall of the chicks. An abnormally high intake of vitamin A by mature hens, especially if this is taken as fish oil concentrate, causes a serious decrease in egg production.

It has been shown that the sterol fractions from two brown algae (Fucus and Sargassum species) lower the serum cholesterol level in chicks fed a high cholesterol diet. The sterols fucosterol and sargasterol are believed to be responsible for this effect.

Completion of the first year's study of the nutritional value of the lipid fraction of stored herring meals has shown that this fraction is not appreciably toxic to young chicks, that there is some loss in its digestibility during a year's storage of the meals, that much of it can still be metabolized even after such extensive storage periods and that antioxidant (BHT) treatment appears to protect this fraction against deterioration. Further work is necessary to ascertain whether the addition of antioxidant can be considered a safe and useful procedure economically. Results of the comparative nutritional evaluation of British Columbia herring meals, Peruvian anchovy, and menhaden meals have indicated that the herring meals tend to have higher nutritive value. However, there was some variation in the nutritive value of different herring meals and the reason for this is being investigated.

A chemical study has been initiated in order to determine the chemical changes which occur in the lipid fraction in stored herring meals.

Steroids and Pituitary Hormones

All the major blood steroids of sockeye salmon have now been determined quantitatively in fish caught at different stages of sexual maturity. Adrenosterone and 20β-dihydrocortisone have been isolated from sockeye salmon plasma, and testosterone, which is normally thought to be a male sex hormone, has been identified as the free compound and acid glucuronide in female as well as in male salmon plasma. The suspected aldosterone fraction of spawned sockeye salmon plasma has been shown to be mainly saturated steroids. If aldosterone is present it is below 0.5 µg/100 ml of plasma.

In contrast to the wealth of knowledge available on the physico-chemical and physiological functions of the six or more hormones present in mammalian pituitary glands little is known about those from piscine sources. Investigations have been undertaken during the past year with a view toward the isolation and characterization of fish pituitary hormones as well as towards determining their physiological

functions. Thousands of salmon pituitary glands were obtained by methods developed at this Station from Canadian and United States west coast commercial fisheries and United States fish hatcheries.

One of the important purposes of this investigation is a study of gonadotropic hormones involved in gonadal maturation and reproduction of Pacific salmon. Because of evidence pointing to species specificity, the development of a suitable bioassay was mandatory. One such assay was based on the increase of the weight of juvenile gonads of immature rainbow and steelhead trout following thrice weekly injections of extracts of salmon pituitary glands. The increase in weight of the testes was found to be considerably greater than that of the ovaries and consequently offered greater potential as a bioassay. The temperature at which the assay fish were held influenced greatly the degree of response of the gonads to the pituitary extracts. It varied from a good response at a water temperature of 15°C to a low response at 5°C.

The above assay method is very slow and, in an attempt to find a more rapid bioassay, tropical fish with a short spawning cycle (e.g. Betta splendens with a three-week cycle at 25°C) were used. In preliminary experiments the pituitary extracts appeared to stimulate ovarian development. In addition to the weight gain over the controls, the gonads of the injected fishes were also examined histologically for evidence of cell differentiation during the onset of maturation. Both the testes and the ovaries showed the presence of cells in their early stages of maturation. Histological examinations were extended to other internal organs to determine the effect of pituitary hormones on them. Preliminary studies showed that the adrenal cortical cells of fishes with immature or precociously mature testes were not distinguishable.

The Galli-Mainini frog pregnancy test was investigated as a possible rapid assay for gonadotropic hormones. This method involves the injection of male frogs (Rana pipiens) with pituitary extracts followed by a microscopic examination of the

cloacal fluid for the first appearance of sperms. Although a gametokinetic dose-time response was obtained in frogs injected with human chorionic gonadotropins, results with salmon pituitary extracts were inconsistent. Consistent results could not be achieved even by varying the temperature of the experiments or by increasing the extract dose. This method of bioassay is being reinvestigated with salmon pituitary extracts purified by starch gel electrophoresis. The various separated protein fractions thus obtained will be tested for gonadotropic activity by both frog and fish assays. Large scale isolation and characterization studies of fish pituitary hormones will be commenced as soon as one of these assay methods is considered sufficiently reliable and rapid.

Base Composition of Salmon Sperm Deoxyribonucleic Acids (DNA)

A new project is concerned with the isolation and characterization of the nucleic acids of marine species. Because of the genetic nature of nucleic acids, it is very probable that their chemical composition will reflect phylogenetic relationships. A study of the deoxyribonucleic acids from the sperm of three species of Pacific salmon revealed that O. gorbuscha sperm DNA contains approximately 1.5% more guanine than sperm DNA of O. kisutch or O. nerka, as determined by measurement of Tm values. The latter two species are closely related in this respect and this is in agreement with the relationship between them as demonstrated by a study of their water soluble muscle proteins (see below).

Physical Separation of Salmon Flesh Proteins and its Probable Significance

Most investigations of fish muscle proteins have been carried out with Atlantic cod and a few white-fleshed species of fish. Since on this coast salmon (Oncorhynchus) are singly the most important fish economically, the present investigations are largely concerned with the five species of Oncorhynchus as well as with Salmo species. After preliminary experiments two methods, column chromatography for large-scale operations, and starch gel electrophoresis for finer resolutions, were selected for

the separation of the flesh proteins soluble in dilute neutral salt solutions (myogens). By gel electrophoresis the myogens of the Pacific salmon, trout, and Atlantic salmon resolved into many different protein components thus permitting exacting comparisons between fishes. In contrast the Atlantic cod and the Pacific lingcod myogens were characterized by fewer components as well as major differences in their isoelectric points and other physical characteristics. With the exception of the rainbow trout and the anadromous steelhead trout, which were identical, all the other fishes studied were clearly distinguishable by their characteristic protein patterns. Of the Pacific salmon species, the sockeye, pink and chum differed strikingly from the coho and spring salmon, and thus could be separated into two general groups. Direct chemical evidence based on the myogen proteins can now be provided to complement a similar grouping arrived at through behaviour studies by other workers. The rainbow and steelhead trouts showed patterns very similar to the Atlantic salmon which in turn were sufficiently similar to the coho and spring salmon as to indicate a possible evolutionary development of the genus Oncorhynchus from the genus Salmo. So far, sex and sexual maturity do not appear to influence these protein patterns.

Microbiology

In lingcod treated with either CTC or OTC antibiotic there is a poor correlation between the trimethylamine (TMA) content and viable bacterial counts during spoilage. Viable bacterial counts may indicate poor quality when organoleptic rating and TMA content of these antibiotic-treated fish indicate acceptable quality. However, a reasonable correlation was obtained between these three tests in untreated lingcod.

In collaboration with the International Pacific Salmon Fisheries Commission some success has attended isolation and infection experiments in connection with an apparent bacteriological disease which has been causing high mortality in the Horsefly River spawning area. At present this disease appears to be somewhat unique but further work is necessary in order to establish definitely the nature of the disease

and whether the bacteria isolated are really responsible for it. Further progress has been made in the study of the deleterious effect of certain cations on activity of tetracycline antibiotics in fish preservation. Current investigations on shrimp preservation indicate that a comparatively high content of divalent ions such as magnesium in shrimp may be responsible for the high levels of antibiotic required to preserve them.

Miscellaneous Investigations

An entirely new investigation concerned with evaluation of the desirable flavour and odour components of fish has been initiated, since it is felt that too much emphasis has heretofore been given to studies on undesirable flavour and odours. Studies indicate that volatile, odoriferous compounds are responsible for the flavours of cod and salmon and that non-volatile compounds play little if any part in the flavour. Salmon oil was found to contain a very high proportion of the compounds involved in the flavour of the fish.

The work on dogfish gelatins has been completed. A comparison has been made which has indicated that cartons insulated with polystyrene foam are better and actually more economical to use for air shipment of fresh fish than uninsulated cartons. Work on the biological formation of trimethylamineoxide which was commenced at the Gaspé Station has been extended here.

A study of the relationship between blood pH and mortality in rainbow trout has shown that these fish die when the pH falls a little below 7. There was no apparent correlation between the concentration of the lactate ion in the blood and mortality.

A particulate bacterial enzyme has been prepared by means of which both glucose and ribose may be removed simultaneously from fish muscles, thereby facilitating research on Maillard browning reactions in heated or dried fish muscle. The enzyme oxidizes the sugars directly to the corresponding hexonic and pentonoic acids or their lactones.

An improved method of preparation from lingcod muscle of the enzyme phosphoserine phosphatase has been developed and further studies of the properties of this enzyme have been made.

A herring flour has been produced from air-flow dried herring meal by extracting the lipid with hot anhydrous ethanol. The final product is free flowing, has very little moisture, less than 0.5% lipid (by chloroform-methanol extraction), little odour and a bland taste. Bread has been made using up to 10% of this product. At 5% level the bread was quite palatable, but at 10% level was somewhat objectionable to most tasters.

Several methods to overcome scaling of evaporator tubes during condensed fish solubles production were proposed. These included direct addition of sulfuric acid to the stickwater to lower the pH to about 5.5, or a periodic flooding of the evaporators with a 10% inhibited hydrochloric acid solution.

A study of the energy consumption of migrating pink salmon is now under way. This investigation, as with the recently completed sockeye salmon migration studies, is being sponsored by the International Pacific Salmon Fisheries Commission. Salmon from Seton Creek (early) and Harrison River (late) runs have been collected and physical measurements on these fish are being carried out prior to chemical analyses.

A higher rate of conversion of radioactive cholesterol to 24-methylene-cholesterol in butter clams (Saxidomus giganteus) has been achieved through changes in the experimental technique.

INDEX

INVESTIGATORS' SUMMARIES

NEW FISH HOLDING FACILITIES

SUMMARY NO. 1

PLANS AND SPECIFICATIONS FOR LIVE FISH HOLDING FACILITIES — J.S.M. Harrison

As a result of the necessity of live fish holding facilities in connection with many Station programmes, the engineering Department was asked to make preliminary designs and estimates for an addition to the building to provide the required space at lowest possible cost. The proposal was to build a single storey wing on the south side of the main building. The building was to be 56' x 40', one section 28' x 40' enclosed, another section 28' x 40' an open but fenced car port and sheltered storage area. The enclosed portion was to be on a concrete slab and have 8" concrete block walls, glulam beams with 4" T & G decking with built-up tar and gravel roof. All services were to be exposed for easy access for alteration and addition. The open portion was to have the same roof structure supported at the outside by three steel columns. Fencing and gates were to be chain link.

Because of the urgency of this work the proposals were used as final drawings and specifications by the construction firm. The resulting addition has proved most useful.

SUMMARY NO. 2

FISH HOLDING FACILITIES — N. Tomlinson, J.R. McBride

The following equipment, together with the necessary plumbing, has been installed: one glass-faced, fibreglass-lined observation tank, 12' x 3' x 3'; four glass, iron-framed observation tanks, 4' x 2' x 2'; ten fiberglass, self-cleaning holding tanks, 4' x 2' x 2'; seven fiberglass, circular holding tanks, 6' (diameter) x 3', of which four are self-cleaning; two filters (charcoal) capable of delivering dechlorinated water at the rate of 30 gpm each; one air compressor.

The water and air supplies to each tank are controlled by individual valves and all water supply pipes and valves are plastic. The equipment has given continuous, trouble-free service since completion of the installation.

MECHANICAL ENGINEERING

SUMMARY NO. 3

BRINE SPRAY SYSTEM OF FREEZING TUNA ON TUNA SEINERS — J.S.M. Harrison, S.W. Roach

During the week of July 31 to August 4, 1961, a study of brine spray freezing of tuna on seiners at Terminal Island, California was made. Several vessels were examined, unloading operations were seen, and discussions were held with boat skippers and engineers and with canning company personnel.

Design of equipment

The operating principle of the system is that the hold is made watertight and lined with cooling coils on all sides and bulkheads. Brine is made in the hold, then pumped, spraying from overhead throughout the hold, running over the cooling coils and fish, in turn being cooled and cooling the fish. The operation is continued until all fish are frozen, then the brine is pumped out. Refrigeration is continuously applied to the coils to maintain temperatures below freezing.

The vessels equipped with this system have a fairly uniform design. The hold is plywood lined, then fibre-glassed, making it completely watertight below deck. Removable fibreglas panels cover the shaft log. These are bolted on about 4" centres, the sealing faces being rubber gasketed. Brine intakes are on either side of the shaft log. They are perforated plate of expanded metal sheet about 1' wide laid obliquely from the hold floor to the top of the shaft log extending from the horn timbers to the engine room bulkhead.

The pump is usually a 4" centrifugal type delivering about 600 gpm. Three main distributor pipes of about 3" nominal size Kralasitic pipe run the length of the hold on port and starboard sides and to the hatch on the centre line. The pipes are drilled at intervals or have spray heads of different types.

The hold refrigerant piping is $1\frac{1}{4}$" nominal iron pipe galvanized on the outside. Pipes are on 8" centres covering all sides, bulkheads and deckhead. Two single row pipe coils run from the hatch to engine room, their function being to cool the centre of the load and act as pens preventing load shift. The bulkhead is the height of the hold. The coils are divided in about 5 refrigerant circuits such as port and starboard side bulkhead coils, bottom coils, centre coils and top coils. Each circuit is continuous, served by liquid and suction shutoffs, thermal expansion valve and hand expansion valve. Compressors are one or two in number, about 30 hp in size. Where two are used, one may be smaller, serving to give a better balance on holding after freezing with the small compressor only and employing the large compressor for freezing only. The two compressors, where installed, are in parallel permitting use of either or both, increasing the reliability of the system.

Compressors are driven electrically or mechanically. Mechanical drives are from an auxiliary engine; in some vessels provision is made to drive from the main engine as well. Speed variation available with the auxiliary engine is employed to balance the refrigeration load in holding.

Operation requires considerable knowledge and judgment by the ship's engineer in its different steps. The first operation is filling the vessel with sea water. About 3,000 gallons are taken aboard, the actual amount being dictated by that required to cover adequately the suction intakes. The sea water is then precooled to its freezing point. In some vessels ice is allowed to build up on the coils. However, some operators feel that the loss of coil efficiency by ice build-up and the loss of load carrying space by some of the ice remaining unmelted offsets any gains in stored refrigeration.

As fish are caught and put in the hold, the sea-water circulation and cooling continues. The fish are merely dumped in the hold and spread out but with no attention particularly being paid to any penning or stacking. This operation will continue until the vessel is full, at which time the sea-water salt concentration is increased and the cooling is carried on into the freezing range and finally to about 22°F. If, however, fishing is suspended for bad weather or fish not showing, it may be necessary to move the catch to date, stowing it in the forward part of the hold

to avoid to some extent thawing when the rest of the catch is made.

After freezing with the brine the hold is dried up and the temperature is held by the ammonia coils alone, maintaining about 22°F; no attempt is made to obtain lower temperatures, one reason being that it is impossible to cool the centre of the load quickly and secondly, that any further cooling increases the problem of thawing.

The final operation is a partial thawing of the load to permit unloading. This is done by circulating sea water, which is salted to a freezing point about 6°F lower than the temperature of the fish. No heat is applied, the only sources being the heat of sea water initially added and the leakage heat to the hold and circulation piping and the heat of pumping.

In discussing the system with those working with it we found fishermen very satisfied with the system. It permits them to handle fish with a minimum of attention and bring in a load in marketable condition. People concerned with canning of tuna do not share the fishermen's enthusiasm. They maintain that the quality of tuna is poorer by this method than by ice boats or tuna clippers. They believe that much poor quality fish results from the handling of large sets of fish where the refrigeration system is inadequate and the circulation of brine is impeded by the large quantities of warm fish packing together. The effect resulting from the first caught fish being thawed to some extent, then refrozen, is a further cause for concern among canners but the extent of this damage cannot easily be seen.

It appears to us that this general system of handling tuna would be adequate for our present interest as the major problem encountered with it of very large sets is not one we are too likely to come across in albacore seining. We believe a modification could profitably be made to suit our own particular requirements. We propose that the pipe coils in the hold should be dispensed with, employing an external heat exchanger for the cooling and freezing operation and employing the fibreglas lining which must be installed as a jacket for the holding of the load at freezing temperatures.

The use of a heat exchanger would help solve many of our problems. Firstly, we estimate it would be cheaper. It would permit the use of Freon refrigerant circumventing our Steamship Inspection regulations regarding ammonia. It would eliminate the accident hazards of ammonia. It would permit the use of brine of lower salinity, reducing salt penetration in the fish.

The use of a jacket would also have many advantages besides lower cost. It would provide a simpler system to operate. It would help in the thawing operation by avoiding the present freezing to the coils and permit the use of warm air to accelerate thawing. Furthermore, the jacket would provide something of great value to our vessels for other fisheries. It would give an excellent ice meltage retarding system for halibut and salmon fishing and prove a less obstructed hold for herring fishing. For all fishing it would give a hold which could be easily cleaned, solving a long-standing problem in the industry.

The problem of using ammonia with regard to our Steamship Inspection Regulations is a serious one. They forbid the placing of ammonia equipment in the engine room; thus the only conceivable place on our vessels where the compressor might be accommodated is the forepeak. The costs of locating equipment there and the required sealing of the space from the engine room and provision for adequate ventilation cannot be estimated except for a specific vessel.

Our estimate of the costs for the equipment is as follows:

Ammonia System

Refrigeration	$ 18,000.00
Auxiliary engine and drives	3,000.00
Pumps and piping	2,500.00
Hold lining and other shipyard work	3,000.00
Fibreglassing of hold	2,000.00
	$ 28,500.00

Freon System

Refrigeration	$ 11,000.00
Auxiliary engine and drives	3,500.00
Pumps and piping	2,000.00
Insulation	1,000.00
Hold lining jacket and other shipyard work	4,000.00
Fibreglassing of hold	2,000.00
	$ 23,500.00

SUMMARY NO. 4

TUNA FREEZING EQUIPMENT FOR BRITISH COLUMBIA SEINE BOATS

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

During the past year the Department of Fisheries has considered ways in which the development of a Canadian tuna fishery may be effected. One of the principal factors in development of such a fishery is the necessity for suitable refrigeration for the preservation of the catch. It is considered that demonstration of freezing equipment used in California might facilitate the development of a Canadian fishing fleet. Accordingly, the Department, through its Industrial Development Service, is prepared to arrange for the installation of freezing equipment in three or four selected B.C. purse seiners. This Station's enginerring department has designed the refrigeration system which will be used on these vessels and will supervise its installation. These are the specifications which have been prepared.

Tuna Freezing Equipment for Seiners "Pacific Harvester", "Dominator" and "Blue Pacific No. I"

Brine Cooling Equipment

Compressor

A compressor shall be installed in the engine room on mounting provided. It shall use Refrigerant 22 having an output of 25 tons at 10°F suction and 100°F condensing temperatures. It shall have a minimum of 4 cylinders. It shall not exceed 26" in width and 24" in one other dimension, exclusive of valves, flywheel and removable accessories. The compressor shall be equipped with the following accessories: pressure control switch for high and low pressure; oil pressure safety switch; capacity reduction equipment for steps of 75, 50 and 25%.

Condenser

A condenser shall be installed on the port side of the deck house. It shall have a capacity of 50 tons at 110°F condensing temperature with 100 usgpm of sea water at 85°F for cooling. The condenser shall be a marine cleanable type with cupronickel tubes and tube sheets, if any.

Brine cooler

A brine cooler shall be installed on the port side of the deck house. It shall be a dry expansion shell and tube type with copper or cupronickel tubes and shell of same material or plastic. Spacing between tubes shall be not less than 3/8"; total tube outside surface shall be not less than 250 square feet. Multiple units may be used.

Liquid refrigerant receiver

A liquid receiver shall be mounted on the port side of the deck house. It shall be a vertical type of sufficient capacity to hold the entire refrigerant change.

Accessories and Piping

All the above-mentioned equipment shall be connected with copper tubing sized and arranged as required with regard to pressure loss and oil return. Included in the piping arrangement shall be: thermal expansion valve or valves; refrigerant drier with shut-off and by-pass valves; main suction and liquid valves located near the compressor; isolating liquid and suction valves for each unit of the brine cooler; liquid sight glass with moisture indicator; high and low pressure gauges. The unit shall be charged with an adequate supply of refrigerant for all operating circumstances.

Jacket Air Cooling Equipment

A condensing unit shall be installed in the engine room and an air cooling coil in the hold. The condensing unit shall have an open type 4 cylinder compressor belt driven from the existing auxiliary engine. The compressor shall have a capacity of 2 tons at 10°F suction with 100°F condensing temperature. The condenser shall be a marine cleanable type with cupronickel tubes and shall condense 2 tons at 100°F with 85°F sea water.

The cooling coil shall be arranged for a vertical downward air flow of 3000 cfm with not more than $\frac{1}{2}$" H_2O static pressure drop. It shall have a capacity of 2 tons at 10°F$\triangle$t.

The unit shall have: automatic hot gas defrost equipment; high and low pressure cutout connected to an alarm bell; high and low pressure gauges; air temperature thermostat and liquid line solenoid valve; liquid sight glass with moisture indicator; liquid line drier.

All the above equipment shall be connected with copper tubing sized and arranged as required with regard to pressure drop and oil return.

Tuna Freezing Equipment for Seiner "Nanceda"

The vessel "Nanceda" is to be equipped for the cooling and freezing of tuna by means of a brine spray system in which sea water or brine is drawn from intakes in the bottom of a watertight lining of the hold, then pumped and distributed by spray piping over the fish and over refrigeration coils in the hold. The refrigeration equipment and brine pump are powered by a direct current generator now in the vessel.

Hold lining

The hold shall be lined with waterproof plywood 3/4" thick or two courses of 3/8" where required to accommodate curves. This shall include sides, bulkheads, floor and shaft tunnel sides. This lining shall be fibreglassed. The shaft tunnel shall be made watertight by plywood and polystyrene sandwich covers, fibreglassed on top edges and surfaces where sealed to the tunnel. These covers shall be secured to the tunnel by hanger bolts.

Door to forepeak

The door from engine room to forepeak shall be replaced with a steel gasketed gas-tight door, its purpose being to keep ammonia gas from entering the engine room.

Ventilation of forepeak

The forepeak shall be ventilated by a blower fan driven by a $\frac{1}{2}$-hp 110-v dc motor. Supply ducts to the forepeak, and exhaust from the fan, shall be 9" diameter or equivalent rectangular.

Brine circulation

A pump shall be installed in the engine room below the level of the hold. It shall be a 3" all-iron, non-clogging centrifugal pump delivering 500 usgpm at 40' head. Paramount model 3SHD with 8-3/4" 2-port impeller or equivalent. The pump shall be driven by a 10-hp 110-v dc motor. Piping shall be as in drawing, "Piping and mechanical equipment for 'Dominator' and'Pacific Harvester'", excluding heat exchanger connections, and shall include overboard discharge line with shut-off valve.

Condenser cooling pump

A pump supplying condenser cooling water shall be installed below the water line and piped to a suitable sea cock and the condenser. Piping shall be 2" galvanized iron below water line and polyethylene or galvanized iron above.

The pump shall be a $1\frac{1}{2}$" all-iron centrifugal, delivering 70 usgpm at 40' head, Paramount $1\frac{1}{2}$ B or equivalent. A $1\frac{1}{2}$-hp 110-v dc motor shall be supplied and installed to drive this pump.

Compressor motor

A 20-hp 1800 rpm, 110-v dc motor shall be supplied.

Wiring and motor control

All necessary wiring, starters, and overload controls for the three motors shall

be supplied and installed.

Refrigeration equipment

A competent refrigeration contractor shall be employed for the supply and installation of the following equipment:

An ammonia compressor shall be installed in the forepeak of the vessel. It shall have a capacity of 15 tons at 20°F evaporating temperature and 100°F condensing temperature. The compressor shall be complete with base and pulleys for driving from an 1800 rpm motor.

A condenser and liquid receiver shall be installed on the port side of the deck house. It shall be a special galvanized marine type with a minimum of 500 lineal ft of $1\frac{1}{4}$" tubing as condensing surface. The receiver shall accommodate the entire refrigerant charge.

The evaporator shall consist of $1\frac{1}{4}$" pipe coils on $6\frac{1}{2}$" centres, lining the entire hold, on deckhead, sides, floor, bulkheads, shaft box sides and hatch combing. Two double rows of pipe coils shall be located on either side of the shaft box, extending from the forward edge of the hatch to the engine room bulkhead and from floor to deckhead.

The evaporator shall be divided into at least six circuits each with separate thermal expansion valves, each with hand-operated by-pass and isolation valves, and suction shut-off valves. All the above valves shall be located in the forepeak compartment. All piping between the hold and forepeak shall be run on the port side of the deckhouse.

An oil trap shall be installed on the discharge line. A back pressure regulator shall be installed on the suction main. High and low pressure gauges shall be installed.

SUMMARY NO. 5

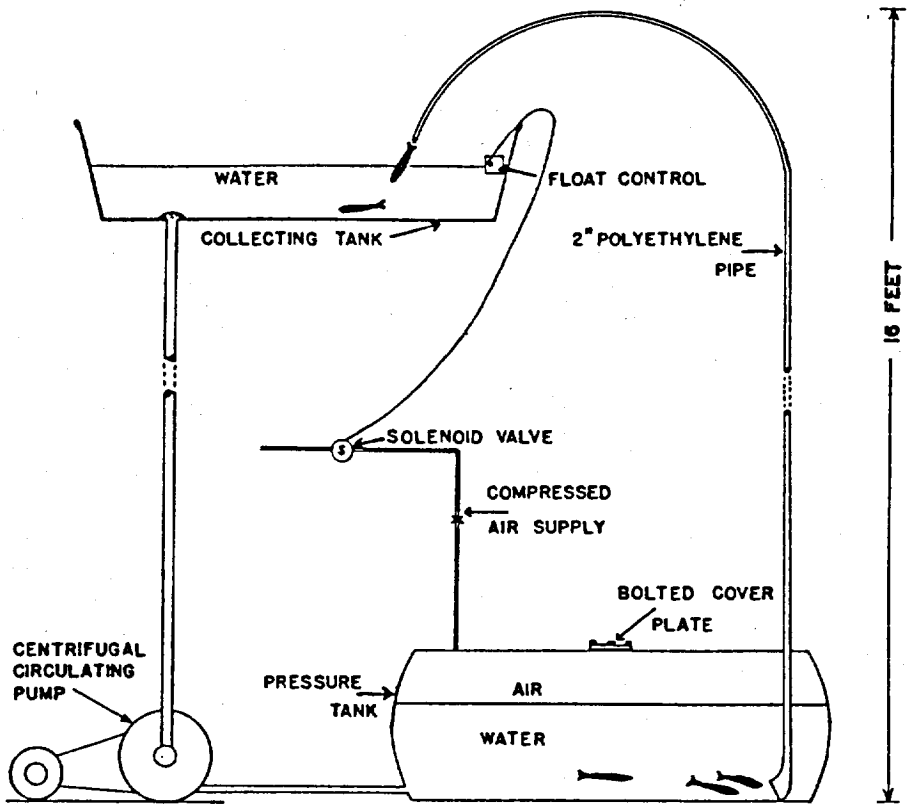
A NEW METHOD FOR UNLOADING FISH

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

In recent years the fishing industry has been seeking improved methods of unloading fish. The development of large pumps for unloading herring at the reduction plants has been a success but no new method has yet been developed for efficiently unloading larger fish such as salmon. A method such as pumping which would facilitate lifting the fish from the hold of a vessel onto the cannery wharf regardless of tidal conditions would be most desirable.

In the spring of this year we were asked by Mr. R. Payne of J.H. Todd & Company to assist in the development of such an unloading method. His idea as presented was to unload salmon through a pipe by imposing air pressure on a closed tank of fish being held in refrigerated sea water. The company were preparing to install such an unloading system on a fish packer for the coming salmon season but had no experimental data on which to base their design.

Because little time was available, a small-scale installation to move herring was set up. The equipment arrangement is shown in the attached figure.



Our primary aim was to ascertain if fish could be moved by this method and secondly to determine the minimum or optimum size of unloading pipe as it seemed likely that with sufficient air pressure some fish could certainly be lifted. Design of the equipment was based on the assumption that the fish could be lifted by the frictional force exerted by the circulating water or, in other words, by vertically fluming. A compressed air line was connected to the tank so that a volume of air could be injected to replace the fish as they left the tank and to maintain the static pressure required. The line through which the fish were to move was a 2" polyethylene pipe. This size was chosen as being the minimum size through which it could reasonably be expected that the herring could pass. A funnel-shaped fitting was attached to the entrance of the pipe inside the tank to expedite the entry of fish into the pipe but this was of dubious value. The centrifugal pump used to circulate the water delivered approximately 30 imperial gallons per minute. Air pressure was supplied at 20 psi and was regulated by the float valve to maintain a sufficient level of water in the collecting tank. The difference in level between the collecting tank and the closed pressure tank was 16 feet which corresponds to a hydraulic pressure difference of approximately 7 psi.

The equipment operated very successfully in all the trials. The largest batch of fish used was 200 lb of herring which were very rapidly transported to the upper collecting tank. There was no apparent damage to the fish and virtually all of them were transferred. The latter was a very significant finding for the removal of a residue of fish from such a tank could be a serious problem in a commercial installation.

Report on the First Trip of the "Nootka Chief" After Equipping for Refrigerated Sea Water and Pneumatic Unloading

The fish packer "Nootka Chief" has been equipped with closed tanks and other equipment for holding salmon in refrigerated sea water and self-unloading by means of compressed air. Her first trip was made in the week July 8-14, 1960. Fish were collected in the Milbank Sound, Rivers Inlet area, about 60,000 lb being taken aboard in her forward hold only.

The vessel has installed four cylindrical tanks, two port and starboard forward, and two port and starboard aft. The tanks each have a capacity of about 35,000 lb of fish. They are built to withstand about 30 psi air pressure, having eliptical ends and pressure tight loading manholes. Each has a 12" steel fish discharge pipe extending from an intake boot near the bottom of the tank to a point above and outside the tank where it connects with a 12" discharge hose for delivery of fish to the dock. Each tank is also filled with compressed air supply lines and water supply lines.

Two refrigerated sea-water circulating pumps, electrically driven, are installed on board for circulation, filling and emptying sea water, one for forward and one for the aft tanks. There is no mechanical refrigeration at the present time; ice and sea water is being used for cooling.

In unloading, compressed air is applied to the top of a tank forcing fish and water into the intake boot and up the discharge pipe and hose. As the water has a greater velocity than the fish, some additional water must be added through the water supply lines.

In this trial run the temperatures were maintained at 30-32° by ice and sea water. The quality of the fish delivered showed a marked improvement over that delivered by conventional ice packers.

Unloading was reasonably successful in that virtually all fish were discharged to the dock by this method, the height of the dock above the fish being about 12 ft. Facilities for supplying additional water to the tanks were inadequate, however, and the unloading was not a single continuous operation. It was necessary to stop the air supply and release compressed air in the tank at intervals to permit recharging with water. Modifications are now being made to correct this.

This trial has proved conclusively that fish can be unloaded by this method. The economic feasibility remains to be seen. It would appear that the scheme would be feasible in new construction where tanks could be built to withstand this pressure without sacrificing space to use cylindrical tanks. It is a very promising development, not only as a labour- and time-saving device but as a means of delivering fish without exposure to higher temperatures, this being accomplished by recirculating the water from the tanks without additional makeup. It would also permit storage of the fish ashore in the same refrigerated water.

SUMMARY NO. 6

REFRIGERATED SEA WATER

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

Details of the installation on the "Western Express" were given in Summary No. 44 of this Station's Annual Report for 1960-61.

The conversion of this large salmon packer to RSW was completed just in time to permit its use during the peak week of this year's Rivers Inlet sockeye run. A near capacity load of about 400,000 lb was taken aboard on July 17, 18, 19 and 20 and unloaded at Steveston on July 22. The appearance of the fish upon delivery was excellent and it was judged by cannery personnel to be far superior in quality to that delivered by conventional ice packers.

Since this operation was carried out during a period of heavy fishing it afforded an excellent opportunity to observe performance of the equipment at full load. The following observations were made:

(1) Transfer of fish from the collector boats to the "Western Express" was efficient and rapid, four collectors being unloaded simultaneously. The increased speed of this operation over conventional methods is due to the fact that brailer loads of fish are dropped into the tanks of RSW and no further handling is required.

(2) Heaviest refrigeration load occurred during precooling of sea water and with these very large tanks it was necessary to run the compressors almost continuously for the first two days of the trip. However, with the compressors running during fish loading, very little temperature rise occurred. During the trip to the cannery the compressors were run only for about an hour at 12-hour intervals. RSW temperatures were constantly in the range of 30° to 32°F.

(3) Temperature rise in the off-cycle due to heat leak into the tanks was very slight. Although these tanks are not insulated their cubic capacity relative to their external surface area is very large so that the temperature rise was only 1 or 2 degrees after 12 hours without additional cooling.

(4) Chilling of the fish was very rapid and complete. Even after periods of rapid loading of fish there was little or no rise in temperature after the initial cooling period. This is due to the very large capacity of the tanks and the high rate of circulation of RSW through the tanks.

(5) Fish were unloaded by brailing. The trunk openings through the deck are large, which makes unloading by brailing simple and effective. Unloading with a vertical bucket elevator was also tried but modifications to the elevator must be made if it is to be successful.

During the remainder of the salmon season which continued until late in the fall the "Western Express" made weekly trips, picking up fish in the central area, in Johnstone Straits and on the west coast of Vancouver Island. Over 2 million pounds of salmon were transported by the vessel during this period. Company officials are

completely satisfied with the performance of the vessel and its equipment and are proceeding to equip two more large salmon packers "Hesquiat" and "Western Star" with refrigerated sea-water equipment.

The success of the "Western Express" has also prompted another major fishing company to equip two of its largest salmon packers, the "Kimsquit" and the "Quatsino" with refrigerated sea water. These vessels will use tanks and refrigeration equipment of the type and design developed for the "Western Express".

SUMMARY NO. 7

PACKAGING AIR SHIPMENTS OF FRESH FISH — F.G. Claggett

This study was undertaken at the request of local interests who at present are shipping fresh fish by air to eastern Canada and Europe.

The normal shipping method is to chill fresh fish to 32°F, pack them in kraft cartons of 100-lb capacity and ship them shortly before they are required. This has proved satisfactory for short-distance shipments but for longer shipments and even the shorter ones during the summer months there is some danger of spoilage. As a precaution, small polyethylene bags of a frozen gel are included in these shipments to help maintain a low temperature.

The suggestion was put forward that cartons of laminated kraft board and polystyrene foam might be of use to eliminate the need of these frozen gel packs. The purpose of this study was to compare the insulating properties of these laminated cartons with the standard kraft carton to obtain a basis for economic evaluation.

Four cartons were tested for their insulating properties by following the temperature rise of cold water in containers sealed in the cartons. This method was chosen over the actual packing of fresh fish because the comparative insulation efficiencies are of interest for products other than fresh fish. A description of the cartons is given in Table I.

TABLE I - Description of cartons.

Carton No	Material	Box description	Size, inches
1	Corrugated kraft board	Single walled, single overlapping top and bottom	10 x 14 x 30 thickness 178 mils
2	Kraft polystyrene laminate	Single walled, single overlapping top and bottom	10 x 14 x 30 thickness 242 mils
3	Kraft polystyrene laminate	Double walled, full overlapping, top and bottom	10 x 14 x 30 each thickness 242 mils
4	Kraft polystyrene laminate	Full telescoping, liner on top and bottom	10 x 14 x 30 each thickness 242 mils

These cartons were found to hold three 4-gallon tins exactly. Copper constantan probes were soldered to the middle of one side of each tin so that when the tins were packed a temperature near two interior walls and near the centre of each carton could be recorded. The tins were chilled to 32°F, ice water was added and they were then packed into the cartons. The cartons were sealed with tape and the thermocouples attached to a recorder. The ambient temperature was maintained at 80°F and the temperatures were recorded for 18 hours.

In equation form the expression for the transmission of heat through any barrier is, -

$$Q = UA\ (t_o - t)$$

where Q = heat transmitted, BTU/hr
U = overall coefficient of heat transfer, BTU/hr/sq ft/F°
A = area of transmitting surface, sq ft
t_o = outside air temperature, °F
t = inside temperature, °F

The data obtained were plotted on the accompanying graph and used to obtain the overall coefficient of heat transfer of each carton. These values are found in Table II. The segment of data used was that obtained until the contents of each carton reached 40°F.

TABLE II - Overall coefficients of heat transfer.

Carton No	Overall coefficients of heat transmittance BTU/hr/sq ft/F°
1	0.27
2	0.16
3	0.16
4	0.13

As may be seen from the values of U obtained, and a study of the accompanying graph, the laminated material in the cartons provides a significantly better thermal barrier than does the corrugated kraft material.

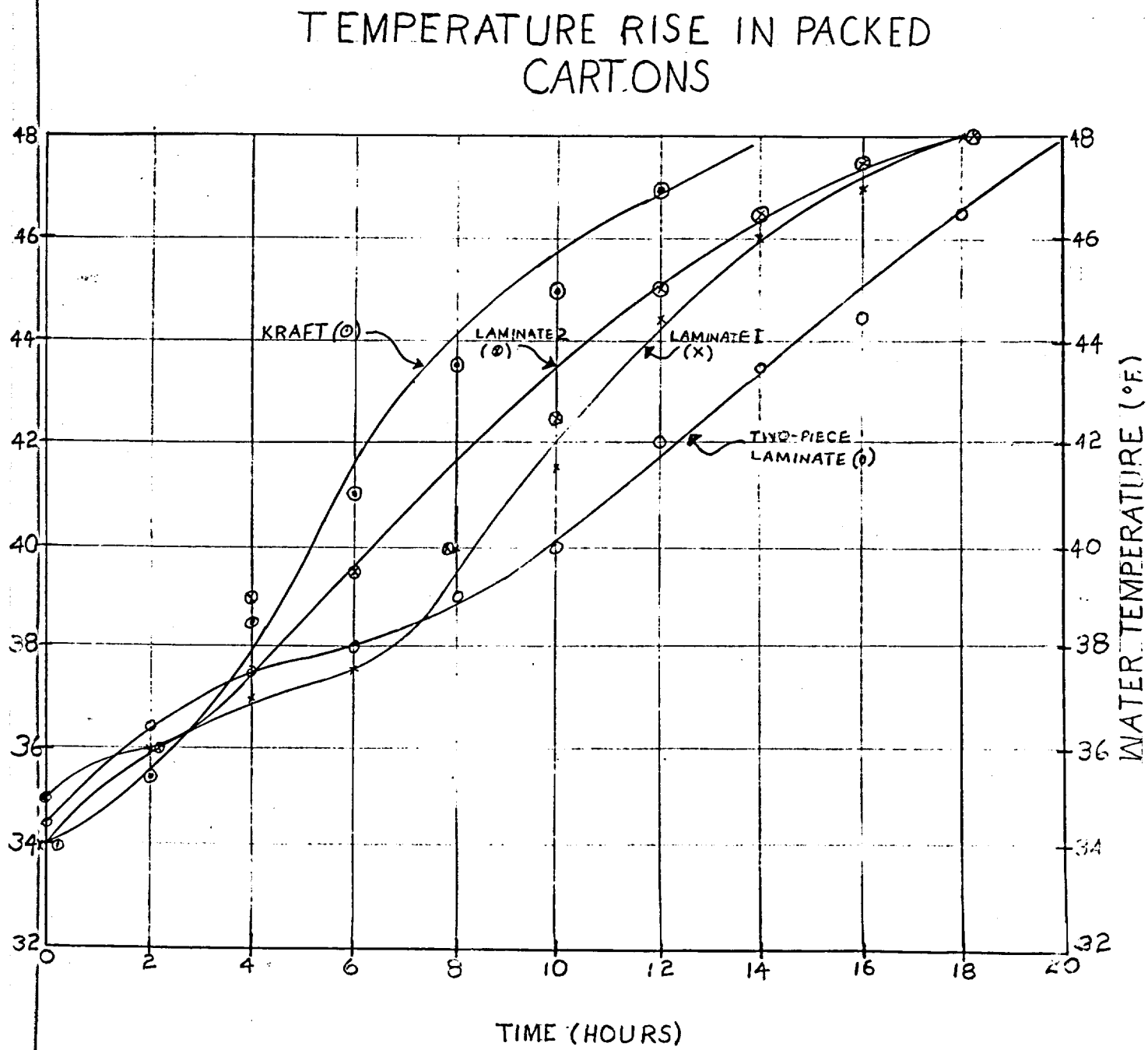
Using the calculated values of U, it may be seen that the heat absorbed by the contents of the kraft box in one hour would melt almost a pound of ice, where that absorbed by the contents of the laminated cartons would melt only about one-third of a pound. For distance shipping and during hot weather the laminated cartons would more than pay for themselves in saving in the number of icing packs required.

There are considerations to be taken into account other than insulating properties when choosing a shipping carton. The weight, moisture absorption, abrasion resistance, tensile and compressive strengths, and cost are all important factors. The Product Technical Information Bulletin No. 2 provided by the Fome-Cor Corporation claims that for laminated cartons these properties are all equal to, or better than, the corresponding ones for corrugated kraft board, with the exception of cost. As may be expected, the cost of the laminated cartons is almost twice that of the kraft board cartons.

Cartons of the kraft-polystyrene laminates tested are superior to the standard kraft cartons when used as insulators. The former are also stronger, lighter and more durable, which make them more acceptable cartons for air shipment of fresh fish.

The extra cost of these cartons is offset by the safety margin in shipping time for short hauls, and by the savings in the cost and transport of icing packs in longer hauls or during hot weather.

The writer wishes to thank Mr. James Collard of British Columbia Packers Ltd. and Mr. Arthur Stringer of Trans-Canada Air Lines for the aid and suggestions given.



FISH MEAL AND FISH FLOUR

SUMMARY NO. 8

NUTRITIONAL AND CHEMICAL CHANGES IN THE LIPID FRACTION OF HERRING MEALS WITH AND WITHOUT ANTIOXIDANT TREATMENT

B.E. March, J. Biely, (Dept. Poultry Science, U.B.C.)
F.G. Claggett, H.L.A. Tarr

Considerable information is available on the nutritive value of fish meals with respect to protein quality and vitamin content. There is, however, little or no data regarding the nutritive properties of the fat present in fish meals. Herring meals contain 3.7 to 12.6% ether extract on a dry weight basis. The amount of fat contributed by herring meal to a poultry ration may therefore be sufficient to be of nutritional significance. Because of the oxidative conditions to which the fat is subjected during meal processing and storage, it has been felt that there is a possibility of toxicity from oxidized and polymerized fatty compounds in the meal. Finding have been summarized in the literature in regard to the production of toxic compounds in autoxidised and heated fats. Furthermore, there is doubt as to the digestibility of fats which have been oxidised or polymerized. It has been found that heat polymerization of a number of oils, including herring oil, resulted in some factor(s) which depressed rat growth and efficiency of feed utilization. Others found that the rate of absorption of hydrogenated fat by the rat varied inversely with the degree of oxidization. The effects of thermally polymerized and autoxidized fish oil when fed to rats at a level of 10% of the diet were compared and it was concluded that thermally polymerized fish oil was more harmful than was oxidatively polymerized oil.

March and Biely fed normal and defatted fish meals to chicks and found that the fat content of the meals did not affect the growth response to the meals. Others working with meat meals obtained equally good results when meals of high or low free fatty acid content were fed and concluded that rancidity of the fat in animal protein concentrates does not adversely affect the nutritive properties of the meals when they are fed in well-balanced rations. In neither of these two studies was any data obtained on the utilization of the fat contained in the animal protein concentrates fed. Neither was it possible to assess the effect of the meal fat as distinct from possible variations in the nutritional properties of the different meals apart from their fat content.

The following investigation was conducted to determine (a) whether the inclusion in chick diets of fat extracted from herring meal has any toxic properties for the chick and (b) the extent to which herring meal fat is utilized by the chick.

Experimental

Experiment 1. Three extracts of herring meal were tested in experiment 1. A batch of freshly manufactured herring meal was drawn from commercial production. A sample of the meal was extracted within a few days of manufacture with a 2:1 mixture of chloroform and methanol and the solvent removed.

A second sample of the meal was stored at warehouse temperature for 7 months and extracted at the end of this time with chloroform-methanol. Butylated hydroxytoluene (BHT) at a level of 0.11% was added to a third sample of the meal. The antioxidant-treated meal was likewise stored for 7 months and then extracted with chloroform-methanol.

The three extracts and a sample of commercial herring oil were fed to chicks at a level of 10% in place of the ground cellulose in the control diet shown in Table I. Each of the fats was also fed at the same level in the control diet supplemented with wht following vitamins: 2.6 mg riboflavin, 0.5 mg folacin, 8.4 mg calcium pantothenate, 24 mg niacin, 2.6 mg pyridoxine HCl, 0.08 mg biotin and 2000 USP units Vitamin A per lb. Each diet was fed to a lot of 30 chicks to 5 weeks of age. In this and subsequent tests the chicks were kept in battery brooders and were given the experimental diets ad libitum.

TABLE I - Composition of basal diets.

Ingredient	Experiment 1 /100 lbs	Experiment 2 /100 lbs
Ground wheat	31.5 lb	65.1 lb
Ground yellow corn	20.0	-
Soybean oil meal 44% protein	30.0	10.0
Herring meal 70% protein	5.0	6.0
Distillers dried solubles	-	2.5
Dehydrated cereal grass	-	2.5
Boanmeal	2.0	2.0
Limestone	1.0	1.0
Iodized salt	0.5	0.5
Choline chloride 25%	-	0.4
Ground cellulose	10.0	10.0
Vitamin A	200,000 USP units	240,000 USP units
Vitamin D_3	12,000 ICU	18,000 ICU
Menadione	0.027 gm	-
Manganese sulphate	10.0 gm	10.0 gm
Chlortetracycline HCl	-	8.0 gm
Vitamins	-	*

* 24 mg niacin, 2.6 mg riboflavin, 8.4 mg calcium pantothenate, 0.5 mg folacin, 2.6 mg pyridoxine HCL, 0.08 mg biotin per lb.

Experiment 2. Chloroform-methanol extracts of meals stored for different lengths of time and under different conditions were tested. A freshly manufactured batch of herring meal was stored at 25.5°C. BHT was added to a second lot at a level of 0.11% and the meal stored at 25.5°C. The third lot of meal was chilled quickly at -20°C and stored at this temperature. Extracts of the meals were made a few days after preparation and after storage for 2 and 11 months.

The percentages of chloroform-methanol extract obtained from the meals were determined at the time of each extraction and each extract was analyzed for nitrogen content. In addition, the meals after storage for 11 months were analyzed for ether-extractable fat. The ether-extracted meals were then acidified and further extracted with acetone as in the method of Almquist and adopted by the AOAC for the determination of total fat in fish meal.

Chick biological tests on the extracts were made separately on each set of extracts. The formula of the basal diet is shown in Table I. The extracts were fed at a level of 10% to replace the ground cellulose in the basal diet. For comparative purposes diets containing a herring oil and hydrogenated vegetable oil, respectively, were included in each test. An additional oil was included in the third test in the experiment. This was a sample of the herring oil fed in Test 2 which had been kept at room temperature during the intervening 9 months.

The extracts were so viscous that, in order to distribute the material uniformly in the diets, it was necessary to pass the diets through a meat grinder.

In the three tests each of the diets was fed to duplicate lots of chicks. The chicks were fed a chick starting diet for 11, 19 and 17 days respectively before being put on experiment in tests 1, 2 and 3. Duplicate lots of 15 to 17 chicks were standardized as to weight. The chicks were then fed the experimental diets for 19, 17 and 21 days in the respective tests.

Growth rate and efficiency of feed utilization were measured. In addition to determining the effect of the fats on the efficiency with which the diets as a whole were utilized, the amounts of fat excreted by the chicks on each diet were also determined.

TABLE II - Average weights (grams) at 5-weeks of age of chicks in Experiment 1.

Fat supplement to diet	Without vitamin supplement	With vitamin supplement
None	356	408
Herring oil	168	370
Extract of freshly prepared herring meal	147	421
Extract of normal herring meal after 7 months storage	161	396
Extract of BHT-treated herring meal after 7 months storage	117	396

Fat excretion was determined on feces collected on two days during the last week of each test. The values given Table III for fat excretion in test 1 and 3 are the average of 4 values obtained from feces collected on two days from each of the duplicate lots. The values for test 2 are the average of the analyses on two days of feces from one lot of chicks on each diet. At the time of each feces collection feed was withheld from the birds overnight, i.e. approximately 16 hours. The dropping trays were then cleaned and the birds fed. The amount of food consumed during the subsequent 8 hours was measured and the feed again withheld overnight. Feces were collected and weighed on the following morning after 16 hours starvation.

Because of the nature of the fish meal extracts it was not possible to measure fat excretion using the conventional procedures for the determination of fecal fat. The method adopted involved drying the feces in a vacuum oven at 95-100°C, followed by a primary extraction for 24 hours with diethyl ether and a second extraction with a 2:1 chloroform-methanol mixture. The latter extraction was continued until the extract was colourless (about 100 hours required). The amounts of ether and chloroform-methanol extracts were weighed separately.

Calculation of the utilization of the fat added to the diets was as follows:

$$\frac{\text{weight fecal fat derived from 1000 g of fat-supplemented diet}}{\text{minus weight of fecal fat derived from 1000 g of control diet}} = \text{\% of supplementary fat which was excreted.}$$

TABLE III - Average Weights (g); Efficiency of Feed Utilization and Fat Excretion of Chicks in Experiment 2.

Fat supplement to diet	Av.wt. of chicks g	Feed eff. *	% of fat suppl. excreted		Lipid utilized %**	Calc. utilization of total meal lipid %	% Relative to herring oil***
			Ether sol.	Ether insol. $CHCl_3$-CH_3OH sol.			
Test 1 - freshly prepared herring meals							
Lipid suppl.							
None	274	2.93					8
Herring oil	321	2.30	4.8	1.2	94.0		
Hydrog. veg. oil	328	2.41	23.9	-1.0	77.1		
Normal meal ext.	332	2.36	8.8	11.6	79.6	76	81
BHT meal ext.	319	2.24	6.6	7.9	85.5	86	91
-20° meal ext.	303	2.58	10.0	16.2	73.8	74	79
Test 2 - herring meals stored for 2 months							
None	362	2.70					
Herring oil	359	2.30	2.8	0.03	97.2		
Hydrog. veg. oil	363	2.27	9.4	2.6	88.0		
Normal meal ext. stored at 25.5°C	343	2.49	5.4	17.5	77.1	62	64
BHT meal ext. stored at 25.5°C.	346	2.44	4.4	6.0	89.6	88	91
Chilled meal ext. stored at -20°C.	338	2.59	5.5	16.4	78.1	74	76
Test 3 - herring meals stored for 11 months							
None	348	2.84					
Herring oil	371	2.44	6.5	5.6	87.9		
Hydrog. veg. oil	378	2.34	19.6	4.6	75.8		
Normal meal ext. stored at 25.5°C	368	2.62	5.9	22.7	71.4	59	67
BHT meal ext. stored at 25.5°C	344	2.57	6.3	17.6	76.1	72	82
Chilled meal ext. stored at -20°C	344	2.80	7.8	28.9	63.3	59	67
"Old" herring oil	363	2.43	10.2	4.3	85.5		

* Feed consumed/gain in weight.

** Assumed zero utilization of unextractable fat.

*** $\frac{\text{Utilization of total meal fat}}{\text{Utilization of herring oil}} \times 100\%$.

The values for excretion of ether-soluble fat and ether-insoluble, chloroform-methanol-soluble fat were calculated separately. The % utilization of each fat supplement was calculated by subtracting from 100% the sum of the percentages of fat excreted in the two fractions.

Results and Discussion

Experiment 1. The average weights of the chicks in experiment 1 at 5 weeks are shown in Table II. The stimulus to growth obtained with the supplementary vitamins indicated that the basal diet was deficient in one or more vitamins. Any of the fats, when fed without the supplementary vitamins, depressed growth markedly. The extract from the antioxidant-treated meal depressed growth significantly more (P 0.05) than did the extract from the untreated meal stored for the same length of time. The more marked growth-depressing effect of the BHT-treated extract could be interpreted as being due to a longer induction period resulting in marked peroxidation of the lipid after incorporation into the rations with consequent vitamin destruction.

The fat present in the unstabilized meal underwent more rapid oxidation than that in the BHT-treated meal. This was apparent from the marked difference in the fluidity of the fat from the normal and stabilized meal. The fat extracted from the BHT-treated meal was much more fluid and "oily" than the fat from the normal meal. Accordingly, it may be assumed that when the fat extracted from the unstabilized meal was mixed into the diet, oxidation was already further advanced and the fat less reactive than that from the BHT-treated meal. Consequently, vitamin destruction was not so rapid as when the extract from the BHT-treated meal was mixed into the diet.

It was reported previously that addition of herring oil to a diet marginal in vitamins depressed growth but that growth rate was normal if the vitamin content of the diet were adequate. Similarly, in the present experiment, when the diet was fortified with additional vitamins, the rate of growth of the chicks fed the diets with any of the herring meal extracts was similar to that of the chicks fed the diet with cellulose. The slight depression in growth resulting when herring oil was added to the diet fortified with vitamins was not statistically significant.

From the results of this experiment it was concluded that the effect of the herring meal fat was related to the vitamin content of the diet and to the destruction of vitamins when the reactive fat was introduced into the diet.

The fat extracted from the herring meal did not, in itself, appear to be toxic when the vitamin content of the diet was adequate. It has been demonstrated that peroxides are readily absorbed in the animal body and that they interfere with normal metabolic processes. The fact that no evidence of toxicity was observed in the present instance suggests that when the fats were mixed into the diet oxidation proceeded so rapidly that no appreciable amount of peroxide accumulated. The high vitamin intake of the chicks, however, may have mediated against adverse metabolic effects from any peroxide absorbed.

Experiment 2. Upon storage of the herring meals the percentage of fat extractable with chloroform-methanol decreased. The greatest reduction in extractability occurred in the meal stored at 25.5°C. Only 11.7% of fat was extractable from this meal at the end of 11 months. After the same length of time, 13.3 and 13.2% of fat was extracted from the meal with BHT and from the meal stored at -20°C respectively.

The percentage of nitrogen in the chloroform-methanol extracts increased over the storage period. The highest nitrogen content in the extracts was noted in the

meal stored at 25.5°C. If, however, the amount of nitrogen is expressed in terms of the absolute amounts extracted by the solvent mixture the amount remained constant and represented approximately 0.3% of the herring meals.

The amounts of ether-extractable fat in the meals stored for 11 months represent 62, 82 and 70% of the chloroform-methanol extractable fat for the meals stored at 25.5°C, the BHT-treated meal and the meal stored at -20°C respectively. The iodine values of the ether extracts were 78, 121 and 63 respectively.

It was reported previously that BHT-treatment of herring meals retarded the decrease in both ether-extractability and iodine value of the extract. This stabilizing effect of BHT was also apparent in the present test. The extractability of the unstabilized meal stored at 25.5°C decreased more rapidly in the present test, however, than in the earlier experiment. On the other hand, in contrast to findings in the previous experiment, the fat in the meal stored at -20°C showed less change in extractability than did that in the meal stored at the higher temperature. At the end of 11 months storage the iodine value of the ether extract of the unstabilized meal stored at 25.5°C had dropped to 78, indicating more rapid changes in the meal fat than had been noted previously for a similar meal. The iodine value of the extract of the meal stored at -20°C was 63. This value is similar to that noted for extracts of meals stored at low temperature in the earlier test.

It was found in the previous study mentioned above that the herring meals prepared and stored under very similar conditions to those employed in the present study did not differ in their nutritive value as protein or vitamin B complex supplements in chick diets. The diets in which the meals were fed had, however, been formulated so that in no instance was the available energy content of the fishmeal a factor in the response of the chicks. In Experiment 1 of the present study it was shown that herring meal fat had no deleterious effect on chick growth provided the vitamin intake was adequate. In Experiment 2 the diet in which the chloroform-methanol extracts of herring meals were fed was well fortified with vitamins and was so formulated that utilization of the fat would be reflected in improved utilization of the diet as a whole.

The average weight of the chicks and the efficiency of feed utilization are given in Table III. Using either growth rate or efficiency of feed utilization as criterion, none of the extracts from the meals showed any toxicity. The level at which the extracts were fed (10% of the diet) was so high as to be equivalent to the feeding of 75% of herring meal. The effect of dietary energy level is not so critically reflected in growth as in feed efficiency. The weights of the chicks, therefore, must be regarded principally as an indication of the absence of any toxic effect from the herring meal extracts.

In all instances but one the addition of herring meal extracts to the diet improved the efficiency of feed utilization, although the extracts were not so well utilized as ordinary herring oil. Treatment of the herring meal with BHT resulted in the chloroform-methanol extract being slightly better utilized than the extract from the unstabilized meal stored at the same temperature. The extract from the meal stored at -20°C gave the least improvement in feed efficiency and after the meals had been stored for 11 months the extract of the meal stored at the low temperature failed to improve feed efficiency.

TABLE IV - Analytical Data on Herring Meal Extracts

Test 1 - freshly prepared herring meals	Chloro-form methanol extract %	% $CHCL_3$- CH_3OH soluble nitro-gen in meal	Ethyl ether extract %	Acetone extract %	Ether & acetone extracts %	Iodine value of ether extract
Test 1 - freshly prepared herring meals						
Extract of normal meal	13.5	0.31				
Extract of BHT-treated meal	14.1	0.28				
Extract of chilled meal	14.1	0.30				
Test 2 - herring meals stored for 2 months						
Extract of normal meal stored at 25.5°C	11.3	0.25				
Extract of BHT-treated meal stored at 25.5°C	13.8	0.30				
Extract of chilled meal stored at -20°C	13.3	0.29				
Test 3 - herring meals stored for 11 months						
Extract of normal meal stored at 25.5°C	11.7	0.30	7.2	2.9	10.1	78
Extract of BHT-treated meal stored at 25.5°C	13.3	0.32	10.9	2.3	13.2	121
Extract of chilled meal stored at -20°C	13.2	0.30	9.2	2.5	11.7	63
Herring oil						127
Hydrogenated vegetable oil						74

Estimations of the utilization of the herring meal extracts on the basis of the effects on the utilization of the diet as a whole were borne out by the data obtained on fat excretion. It was noted that the feces of the chicks fed the herring meal extracts were normal in appearance. This is contrary to the observations of others who reported that the feces of rats fed polymerized oil were dark and sticky.

Comparisons of the utilization of the different meal extracts have to be made with reference to the utilization of the herring oil and the hydrogenated vegetable oil within the individual tests. The latter, it should be mentioned, was chosen as a second control fat because, being a standardized and more stable product than herring oil, the response which it elicited in the tests could be used for reference to confirm conclusions drawn on the basis of the data obtained with the less stable herring oil. The differences noted in utilization of fat from test to test are the result of variations in composition of the natural ingredients making up the basal diet.

Of the three meals which were extracted after different periods of storage, the meal which had been stabilized with BHT gave the best utilized extract at the time of each test. Storage of the meal at -20°C did not protect the oil content of the meal against oxidative changes. In two of the tests the extract from the meal stored at the low temperature was less well utilized than was the extract of the meal stored at 25.5°C. It has to be considered, however, that the extracts were fed at a level of 10% in the diet regardless of the amount which was extracted from the meal. Thus, for example, in test 3 the extract obtained from the normal meal stored at 25.5°C represented only 11.7% of the meal whereas the other two extracts represented 13.3 and 13.2% of the respective meals from which they were derived. If it may be assumed that the lipid material in the meal which was so altered as to be insoluble in chloroform-methanol had little or no nutritive worth, then the value of 71.4% utilization for the extract of the normal meal is erroneously high on the basis of the total amount of lipids present in the meal and is high also in relation to the utilization of the more extractable lipids in the other two meals.

In Table III of the % utilization of the herring meal, fat has been calculated assuming the total fat content of the meals to be 14.1% and the utilization of the fat unextractable by chloroform-methanol to be zero. In the last column of Table III the utilization of the total meal fat is calculated relative to herring oil for each test. The fat in the freshly prepared normal herring meal was 80% as well utilized as herring oil. After storage of the meals for 11 months the utilization was only 67% of that obtained with herring oil. The fat present in the freshly prepared meal stabilized with BHT was 91% as well utilized as was herring oil and after storage for 11 months utilization dropped only to 82% of the value obtained with herring oil. These values, because of various factors such as vitamin or protein levels and age of the birds, which affect fat utilization and particularly the utilization of poorly utilized fats, cannot be considered as absolute. Nevertheless the values do indicate that the fat contained in freshly prepared herring meal is well utilized. In accord with the effect of BHT treatment on the chemical characteristics of herring meal fat the nutritive value of the fat is likewise protected by the antioxidant.

Despite the improvement in the stability of the fat in herring meals treated with BHT it is not felt that unqualified recommendation for antioxidant treatment of herring meal can be made. In view of the hazards associated with the feeding of the unsaturated fat, e.g. the possibility of vitamin destruction in the diet and of unfavourable effects in flavour and storage stability of fats in poultry meat, the desirability of protecting the unsaturated fat in herring meal is open to question.

Furthermore, the variability in the action of individual antioxidants and of antioxidant mixtures would suggest the advisability of further investigation of the problem.

Summary

The nutritive value for the chick of the lipid fraction of herring meal has been studied. When fed at a level of 10% in a vitamin-deficient diet, chloroform-methanol extracts of herring, in common with herring oil, depressed growth. Most severe growth inhibition resulted from the feeding of fat extracted from a meal which had been stabilized with BHT. The growth depressing effect of the fats was overcome completely when the diet was adequately fortified with vitamins.

Utilization of the lipid fraction of freshly prepared meal was approximately 80% that of herring oil. This value dropped to about 70% after storage of the meal for 11 months. BHT treatment of herring meal protected the fat content against oxidative changes. The rates of decrease in solvent extractability and in the iodine value of the fat were retarded. Utilization of the fat in BHT-treated herring meal was likewise improved. In freshly prepared BHT-treated meal the fat was utilized 91% as well as herring oil and after storage for 11 months the relative utilization dropped only to 82%.

SUMMARY NO. 9

HERRING MEAL LIPID ANALYSIS — F.G. Claggett

This work was initiated in connection with the study on storage effects of fish meal. It was felt to be of interest to determine the extent of the changes in the lipid fraction due to oxidation of the meal during storage.

As an initial step it was decided to follow the changes in free fatty acids and total phospholipids during the storage period. The lipid fraction of the meal was removed by repeatedly slurrying the meal with a 2:1 mixture of chloroform and methanol. The extract was water washed, enough methanol added to redissolve any solids present and a sample placed on a silicic acid column. The free fatty acid fraction was eluted with ethyl ether, taken to dryness in a rotary vacuum evaporator, and then redissolved in ethanol. This solution was then titrated with 0.1 N ethanolic potassium hydroxide.

The phospholipid fraction was then eluted from the silicic acid column with a 1:4 chloroform-methanol mixture. A colorimetric determination of the phosphorous was then made using ammonium molybdate and aminonaphtholsulphonic acid.

Several samples have been analyzed by these procedures with very erratic results. The highly oxidized nature of the lipid fraction in the meal makes the extraction and washing procedures difficult. In the washing procedure, when water is added to the extract, the methanol is removed and part of the lipid comes out of solution. This fraction is very difficult to redissolve and much time has been spent trying to arrive at a standard method for water washing. It has been found that chloroform as well as methanol must be added after washing to redissolve this fraction.

The next problem encountered is in eluting the fraction from the silicic acid column. The sample when placed on the column forms a gummy mass near the top. As a large portion of this sample appears insoluble in ethyl ether, it has been found that a very long time is required to completely elute the free fatty acid fraction

and that a significant amount of the sample is not eluted by the chloroform-methanol mixture during the phospholipid elution. It is felt that part of this may be high-molecular weight polymers that have been formed during the oxidation of the meal.

This project is continuing but has been placed as of secondary importance to other projects currently under study and is carried on as time permits.

SUMMARY NO. 10

FISH FLOUR — F.G. Claggett

This is a continuation of the work described in Summaries Nos. 37 and 49 of this Station's Annual Report for 1959-60 and 1960-61, respectively.

The azeotropic extraction method using n-butanol was abandoned when it was found to be very difficult to remove the final traces of the solvent from the flour.

As solvents useful for this type of extraction are limited, it was decided to concentrate on a liquid extraction method instead. The initial attempts at this type of extraction required investigating the use of low-boiling solvents which are non-toxic to humans and yet give a fairly good extraction of the lipid. We have for several years used a chloroform-methanol mixture to obtain complete oil extraction from meals, but in private communications we learned that the Norwegians have used hot ethanol with about the same success. Ethanol was used in preparation of the German "egg white substitute" - Viking Eiweiss, during the last war. This solvent was therefore chosen.

The meal chosen for extraction was an air-flow dried sample analyzing on the average 14.3% oil (total lipid), by chloroform-methanol extraction, 8.1% moisture, with about 72% protein and the balance as ash.

The meal was extracted for 4 hours in a soxhlet extraction unit, the extracted meal dried for 12 hours under 25" vacuum at 80°C. The resultant meal was ground in a ball mill to pass an 80-mesh screen. The miscella was recovered and the solvent removed in a flash evaporator. The lipid recovered was 13.9% on the wet weight of the meal. No significant further amount of lipid could be recovered by chloroform-methanol extraction.

Samples of this flour were added to bread dough at 10% and 20% levels, and though the dough gave a slight off-odour during baking, and the bread at 20% was thought to be a little heavy, the bread with 10% fish flour was found to be quite palatable.

A further sample of air-flow dried meal weighing 10 lb was extracted in a giant soxhlet and the flour recovered as before. This was added to bread at the levels shown in Table I. The bread was tasted by 20 people from this Station and allowing for individual taste, even the high level was thought to be acceptable to the hungry people of the world.

Flour is at present being made from herring presscake to determine if there is any improvement in flavour if scorching and excessive oxidation is avoided.

Tentative plans are to test the effect of the antioxidant BHT in the presscake, and to test other solvents such as hexane.

Since industrial interest in this product is quite high, bench experiments to allow the use of existing extraction equipment on a large scale will have to be performed.

A dietitian from the Department of Fisheries will probably be testing the use of this flour in other products, as well as determining the optimum levels at which this high-protein source should be used in these products.

TABLE I - Bread with added herring flour.

Test sample	% Wheat protein	% Fish protein	Odour	Taste
1	13	0	Nil	Pleasant
2	13	5	Nil	Pleasant
3	5	10	Fishy	Fishy
4	8	5	Nil	Slight
5	6	5	Slight	Detectable

SUMMARY NO. 11

SOLUBLES PLANT PROBLEMS — F.G. Claggett

This work was undertaken at the request of a local fishing company (Summary No. 48 of this Station's Annual Report for 1960-61) to solve a problem encountered with the scaling of evaporator tubes during solubles production.

On the advice of this section, sulphuric acid is being added to the stick-water before condensing and has been found to alleviate the scaling to a large extent.

Literature investigation revealed that although this will correct the scaling, that tube failure will undoubtedly be encountered eventually. An alternative has been suggested to the company which, although it involves more labour and down time, will eliminate tube failure.

The procedure suggested involves flooding the evaporators after each 24 hours running time with a 10% solution of phosphoric acid containing an inhibitor. The inhibitor suggested is NEP 22, which was used in phosphoric acid solutions for removing boiler scales for locomotives. With this procedure both the used phosphoric acid and the recovered scale could be mixed with the fish meal.

At the present time it has been decided to continue with the sulphuric acid addition to determine the effect on the life of the tube bundles, and if the procedure proves uneconomic the phosphoric acid wash may be adopted as an alternative.

SUMMARY NO. 12

THE NUTRITIVE VALUE OF DIFFERENT COMMERCIAL FISH MEALS AND THE EVALUATION OF VARIOUS CHEMICAL ANALYSES AS ESTIMATES OF NUTRITIVE QUALITY

B.E. March, J. Biely,
(Dept. Poultry Science, U.B.C.)
H.L.A. Tarr

Twenty-eight samples of fish meal have now been subjected to biological and chemical testing under a project initiated in March, 1961. Of these samples, 21 were B.C. herring meals, 5 were South American anchovy meals and 2 were Atlantic coast menhaden meals.

Formulation of the individual diets for the biological tests with the various meals so as to assure as high a degree of uniformity as possible with regard to all of the nutrients necessitated knowledge of the proximate analysis of each meal. The analyses were performed and the results are summarized in Table I.

TABLE I - Description of meals and chemical analysis.

Type of Meal	Production Date	Code No	% Protein	% Ash	% Moisture	% Ether Extract
B.C. Herring Meal	March	14	76.2	10.7	5.1	9.3
	Feb	24	73.2	10.0	7.5	8.6
	Dec	18	72.6	10.5	6.1	12.0
	March	15	72.3	11.1	7.5	10.9
	Dec	23	73.1	10.6	6.7	9.9
	Dec	20	73.2	11.1	5.5	11.1
	Feb	4	74.7	11.9	7.3	8.9
	Jan	19	71.9	11.9	5.6	11.7
	Dec	13	73.1	11.1	6.7	9.2
	Feb	26	73.4	10.6	4.0	13.1
	Nov	1	71.6	11.4	8.7	10.8
	Feb	28	75.8	10.6	5.8	8.3
	Jan	27	73.8	10.4	6.6	9.6
	Feb	5	71.1	11.4	8.7	10.8
	Dec	25	71.8	11.6	6.1	8.5
	Feb	8	71.8	16.3	5.1	10.1
	Oct	10	70.9	13.2	7.8	6.0
	Nov	7	71.9	13.6	7.2	10.1
	Jan	3	73.5	12.4	8.0	7.5
	Feb	11	74.2	10.5	7.5	7.7
	Dec	9	73.4	12.8	7.1	8.6
Anchovy Meal		16	67.5	14.8	5.9	4.6
		12	68.0	14.8	7.2	2.9
		2	61.9	17.8	9.8	4.6
		6	66.6	17.4	8.9	3.7
		22	65.6	16.3	6.6	3.6
Menhaden Meal		21	64.3	16.3	7.0	10.8
		17	60.2	20.7	6.9	10.4

The protein nutritive values of the meals have been compared in two types of chick biological assay. The "Gross Protein Values" of the meals were determined according to the method of Heiman, Carver and Cook (Poultry Science 18, 1934) with some modifications. The reference protein employed was a mixture comprising 44.75

parts vitamin-free casein, 44.75 parts isolated soyabean protein, 4.63 parts l-arginine HCl, 3.11 part dl-methionine and 2.77 parts l-lysine HCl. What has been termed the "Supplementary Protein Value" of each of the meals has also been determined. In this latter assay of protein quality the meals were fed as the sole source of supplementary protein in a practical type chick starting diet. The reference protein employed was isolated soyabean protein supplemented with 3.33 percent dl-methionine. Both "Supplementary Protein Values" and "Gross Protein Values" were calculated on the basis of growth rate of the chicks fed the meal in question relative to the rate of growth of the chicks fed the reference protein. In Table II the values obtained in both types of assay are shown. The meals are listed in decreasing order of the "Supplementary Protein Values" for herring meals, anchovy meals and menhaden meals respectively.

TABLE II - Relative nutritive value of meals as supplements in two types of chick diet.

Type of Meal	Code No	Relative S.P.V.*	Relative G.P.V.**
B.C. Herring Meal	14	141	102
	24	140	97
	18	137	106
	15	137	105
	23	136	99
	20	136	97
	4	135	98
	19	135	98
	13	134	96
	26	132	91
	1	130	108
	28	130	102
	27	130	99
	5	128	101
	25	127	100
	8	126	95
	10	125	100
	7	122	99
	3	121	96
	11	118	98
	9	115	95
Anchovy Meal	16	122	109
	12	119	102
	2	116	100
	6	112	99
	22	107	87
Menhaden Meal	21	134	95
	17	125	107

* S.P.V. - Supplementary protein value.
** G.P.V. - Gross protein value.

Increasing interest in the nutritional properties of the lipids present in fish meals suggested that the studies which we have already reported on the differences in lipid extractability by different solvents should be extended to the present needs. To date, only a very few meals have been subjected to such nutritional evaluation and therefore advantage was taken of the present collection of commercial meals to ascertai

whether appreciable differences existed in the solubility characteristics of the fat present. The data are shown in Table III. The meals are again listed in the same order as in Table II.

TABLE III - Extractability of lipids in meal with different solvents.

Type of Meal	Code No	% diethyl ether extract	% 1st acetone extract	% 2nd acetone extract	% total ether & acetone extract	% chloroform methanol extract	Ether extract x 100 / chloroform methanol extract
B.C. Herring Meal	14	9.3	1.28	1.32	11.9	16.3	57
	24	8.6	2.40	1.38	12.4	16.8	51
	18	12.0	1.08	1.32	14.4	18.5	65
	15	10.9	1.16	1.16	13.2	17.3	63
	23	9.9	1.49	1.95	13.3	17.2	58
	20	11.1	1.56	2.35	15.0	17.5	63
	4	8.9	0.77	1.90	11.6	13.6	65
	19	11.7	2.05	1.96	15.7	18.1	65
	13	9.2	1.07	1.30	11.6	16.8	55
	26	13.1	2.98	1.78	17.9	22.4	58
	1	10.8	0.73	1.52	13.0	17.4	62
	28	8.3	1.18	1.70	11.2	14.2	58
	27	9.6	1.30	1.68	12.6	16.9	57
	5	10.8	0.76	1.04	12.6	15.9	68
	25	8.5	2.09	1.92	12.5	18.3	46
	8	10.1	0.92	1.24	12.3	18.5	55
	10	6.0	0.59	1.67	8.3	10.5	57
	7	10.1	0.73	1.30	12.1	18.0	56
	3	7.5	0.68	1.10	9.3	14.5	52
	11	7.7	0.65	1.82	10.2	13.2	58
	9	7.6	0.92	1.20	10.7	18.0	42
Anchovy Meal	16	11.6	0.97	1.30	13.9	11.5	-
	12	2.9	0.69	2.47	6.1	10.1	29
	2	4.6	1.24	4.90	10.7	11.0	42
	6	3.7	1.28	1.51	6.5	11.7	32
	22	3.6	2.49	3.70	9.8	13.1	27
Menhaden Meal	21	10.8	1.45	2.21	14.5	15.9	68
	17	10.4	1.32	1.15	12.9	14.9	70

In Table IV are listed the results of this study obtained in various chemical analyses completed to date which pertain to the protein in the meals. The meals are listed in the same order as above. The percentage of indigestible nitrogen has been determined on most of the fish meals. Indigestible nitrogen was measured on the insoluble residue remaining following pepsin digestion of the samples. Available lysine was determined on all samples. As lysine is one of the essential amino-acids lacking in cereal protein, it is important that any protein supplement to the cereal portion of the diet supply sufficient lysine in an available form to overcome the deficiency. Furthermore, lysine in fish meal may be rendered unavailable by overheating if the processing conditions are not carefully controlled. It has therefore been suggested that the available lysine content of fish meals might be used as a criterion of the quality of the protein in the meals.

TABLE IV - Protein digestibility, % available lysine, % hot water soluble protein and nitrogen content of chloroform-methanol soluble extracts in fishmeal samples.

Type of Meal	Code No	% total nitrogen not digestible	% available lysine in protein	% total nitrogen in chloroform-methanol extract	% hot water soluble protein in crude proteins
B.C. Herring Meal	14	6.2	6.0	7.0	8.0
	24		5.9	7.2	9.2
	18	7.2	6.5	7.9	8.5
	15	6.9	6.0	7.8	7.8
	23		6.7	8.3	8.5
	20	6.2	6.4	6.9	7.6
	4	5.8	6.4	3.6	5.1
	19	6.7	6.4	7.0	7.5
	13	8.3	6.6	8.7	7.2
	26		6.3	8.7	10.6
	1	6.6	6.6	7.1	8.1
	28		6.1	4.6	7.4
	27		6.2	7.4	8.5
	5	6.0	6.7	4.9	6.2
	25		6.1	10.3	8.7
	8	7.1	6.1	9.8	9.4
	10	7.8	7.5	3.1	5.4
	7	7.2	6.8	8.7	7.7
	3	7.6	6.6	6.8	7.6
	11	8.5	6.7	4.3	4.9
	9	6.4	6.3	10.9	10.9
Anchovy Meal	16	6.9	6.9	4.0	4.9
	12	9.7	6.8	3.9	6.0
	2	14.7	6.0	3.2	4.9
	6	11.9	5.8	6.0	5.1
	22		5.8	7.8	6.2
Menhaden Meal	21	7.0	6.6	5.8	10.3
	17	9.8	6.5	5.5	8.5

The percentages of the total nitrogen which were present in lipid complexes extractable with chloroform-methanol (2:1) in the meals are also tabulated.

The meals were also subjected to treatment whereby the fraction of the crude protein which was solubilized upon boiling with water was determined. The values obtained from this analysis are also shown in Table IV.

The "Supplementary Protein Value" of the herring meals ranged from 115 to 141, of the anchovy meals from 107 to 122 and the two menhaden meals had values of 125 and 134. The average values were 130 and 115 respectively for the herring meals and the anchovy meals. The "Gross Protein Value" of the herring meals ranged from 91 to 108, of the anchovy meals from 87 to 109 and the two menhaden meals had values of 95 and 107. The average value of both the herring and the anchovy meals was 99.

It will be seen that in the case of the anchovy meals the ranking of the meals was the same according to either their "Supplementary Protein Values" or their "Gross

Protein Values". In the case of the herring meals and the menhaden meals the "Supplementary" and the "Gross Protein Values" of the meals did not appear to be so closely related.

The determination of the "Supplementary Protein Value" is considered, for reasons to be expounded in the complete report on this project, to provide the better estimate from the practical point of view, of the quality of fish meals.

The herring meals, on the basis of their "Supplementary Protein Values" were of better average quality than were the anchovy meals. There was, however, considerable variation in the quality of both types of meal. The reasons for the poor performance of certain of the meals are in some cases apparent from the results of the various chemical analyses carried out. Other instances of relatively low quality have more obscure or complex causes which are not apparent without a detailed study and correlation of all the data available on the meals. This is presently being done.

The unused portions of the samples have been stored under warehouse conditions. Further biological tests are to be made of representative meals over the entire range of quality with a view to ascertaining if the nutritive quality of fish meal is altered upon storage. Previous studies have indicated that it is not. However, the fact that meals are available with known initial variations in quality provides an excellent opportunity of obtaining more conclusive information in this regard.

THE FLAVOUR OF FISH

SUMMARY NO. 13

INITIAL STUDIES WITH SPRING SALMON AND OTHER FISH

A.P. Ronald
W.A.B. Thomson

Although the malodorous compounds produced due to the spoilage of fish have been the subject of much investigation, no serious attempt has been made to date to identify those compounds which give a fish its characteristic and desirable flavour. Research designed to elucidate this complex problem has now been initiated.

Flavour is a complex sensation and can be considered to consist of two chemical senses, taste and odour, and in addition psychological and physiological effects. The four primary tastes that can be detected are sweet, sour, salt and bitter, although these tend to overlap one another. For the majority of foods taste provides the background or basic flavour and odour gives the food its characteristic flavour. If the odour is prevented from entering the nose by pinching the latter, then a flavourless sensation is experienced when most foods are eaten. As the compounds contributing to the odours of many foods are known to be present in extremely low concentrations, e.g., less than 1 part per billion, the nose has until recently been the only device capable of detecting them without a great deal of preliminary concentration. Recently problems of this type have become more amenable to solution due to the development of gas chromatography and of ionization detectors for gases. The latter almost rival the human nose in sensitivity to many volatile compounds.

The spring salmon (Oncorhynchus tschawytscha) was selected as the first subject of study because of its commercial importance and because of its availability throughout the year. The initial study was an attempt to determine the relative

be concluded that salmon oil contains a very high proportion of the compounds involved in the flavour of the fish. Although water-soluble compounds are probably also important no attempt has been made to assess their contribution. In fact many highly volatile, low-molecular weight compounds are both fat- and water-soluble. However, as a general rule it can be stated that compounds contributing to odour are fat soluble and those contributing to taste are water soluble.

It is expected that gas chromatography, infrared spectroscopy and mass spectrometry will be widely used in the identification of the compounds contributing to the flavour of fish.

Mrs. Mary Smith, dietitian, Department of Fisheries, Vancouver, made many valuable suggestions, supervised some of the fish baking, and judged the baked salmon and halibut samples to be of excellent quality and flavour.

SUMMARY NO. 14

SYNTHESIS OF UNSATURATED ALDEHYDES

A.P. Ronald
W.A.B. Thomson

Unsaturated aldehydes might be involved in fish flavour for two reasons. First, they could conceivably arise from the breakdown of the highly unsaturated fatty acids which in the free and combined form constitute marine oils. Secondly, several investigators have described the odours of some of these compounds as "fishy". As the only ones commercially available are acrolein and crotonaldehyde (2-propenal and 2-butenal) it was decided to synthesize a series of 2-alkenals and a series of 2,4-alkadienals in order to have them available as reference compounds.

2-Heptenal

This compound was synthesized according to the method of Radlove. Freshly distilled heptanal (46.7 g) was dissolved in dry carbon tetrachloride (30 ml) in a 500-ml flask fitted with a neck for a thermometer and a funnel with a pressure equalizing arm. The mixture was cooled to -8°C and then bromine (63.0 g) was added slowly during the course of two hours while keeping the temperature at -5 to -10°C and stirring vigorously. The mixture was stirred for one hour after the addition of bromine and then the temperature was allowed to rise slowly at 5°C. The mixture was poured into dry absolute ethanol (138 ml) previously cooled to -5°C. The temperature was kept below 10°C during mixing and then the mixture was stirred overnight at room temperature.

The mixture was poured into ice-water (275 ml) and extracted with diethyl ether. After washing with water (2 x) and with dilute sodium bicarbonate (3 x) the ether extract was dried with anhydrous sodium sulphate. After removal of the solvents the product was distilled through a 15-cm Vigreaux column. The product, diethyl acetal of 2-bromoheptanal, b p 106-107°C (8 mm) was obtained in 57.2% yield (62.6 g).

Potassium metal (13 g) was added as small pellets to freshly distilled dry t-butanol in a 500-ml flask and heated under reflux till all the potassium dissolved. To this was added the diethyl acetal of 2-bromoheptanal (52.0 g) and the mixture was heated under reflux for 3 hours. After cooling, the mixture was extracted with diethyl ether and the ether extracts were washed with water (2 x), ice-cold dilute sulphuric acid (1 x), water (2 x) and dilute sodium bicarbonate (3 x).

After drying with anhydrous sodium sulphate and removal of the solvent the product was distilled through a 15-cm Vigreaux column. The product, 2-heptenal, b p 51-54°C (9 mm) was obtained in 47.3 yield (10.3 g). The overall yield was 27.1%.

FISH LIPIDS AS HYPOCHOLESTEROLEMIC AGENTS

SUMMARY NO. 15

HYPOCHOLESTEROLEMIC AGENTS FROM STEROLS OF MARINE ALGAE

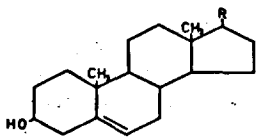
E. Reiner
J. Topliff

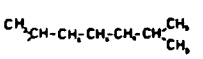
In continuation of the studies on the effects of marine sterols on hypercholesterolemia in chicks (see Summary No. 39 of this Station's Annual Report for 1960-61) we have investigated the sterols of bladder wrack or common rockweed (Fucus gardneri) and Sargassum muticum (Yendo) Fensholt.

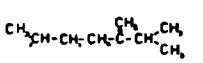
Isolation of Sterols

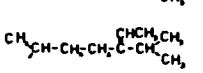
Fucus gardneri, the common rockweed or bladder wrack, was gathered at low tide in August (at Spanish Banks, English Bay, and Stanley Park, Vancouver, B.C.) and Sargassum muticum (Yendo) Fenshold, a species of brown algae accidentally introduced with the Japanese oyster on the east coast of Vancouver Island, B.C. was collected with a rake at Departure Bay, Nanaimo, B.C. Each species was dried and care was exercised to remove contaminating flora and marine fauna. The dried material was ground to a powder and extracted with hot acetone. The acetone solutions were concentrated to an oil and this in turn was saponified by means of 10% ethanolic potassium hydroxide. The saponification mixture was diluted with water and extracted three times with ether. Concentration of the ether solutions and crystallization from boiling methanol afforded reasonably pure sterols, i.e., they were devoid of pigmentation, phospholipids, and gave fairly narrow melting-point ranges. Yields were of the order of 0.08% to 0.25%, based on weight of dried seaweed. The sterols of Fucus and Sargassum in the present study have been established respectively as fucosterol (Fig. 1 c) and tentatively a mixture of Sargasterol (Fig. 1 d) and fucosterol. A report on the chemistry of these sterols will be made in a forthcoming publication.

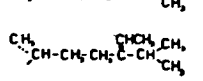
Fig. 1.

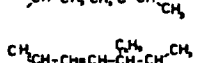


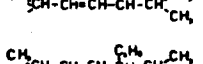
a. 

b. 

c. 

d. 

e. 

f. 

Cholesterol, R=a
24-Methylene cholesterol, R=b
Fucosterol, R=c
Sargosterol, R=d
Stigmosterol, R=e
β-Sitosterol, R=f

Preparation of Diets

A basic feeding formula for chicks known as FRB #1 was fed alone in one group of chicks; a second group's diet was supplemented with 1% cholesterol; a third group received the basic formula to which was added 1% cholesterol and 1% sterol from algae. Each diet was sieved, thoroughly mixed, and stored at 0° during the course of the feeding experiments.

Experimental Procedure

Day-old Leghorn cockerels were fed a basic diet for 7 days. Each chick was then weighed, banded, and randomly selected for one of the three diet groups. In experiment 1, fucosterol was the test sterol and the duration of feeding was 14 days on three groups, each consisting of 16 chicks. The test sterol of experiment 2 was derived from Sargassum and in this case the period of feeding was 13 days on 4 groups of 8 chicks each. Drinking water was available at all times.

On the last day of feeding, for each chick ca 2 ml of blood were collected from the brachial vein into heparinized tubes. Cholesterol levels were measured by the method of Sperry and Webb. The chicks were autopsied and the aortae and livers examined and weighed (Experiment 1) or heart and livers examined and weighed (Experiment 2). Pertinent data and results are shown in Table I.

Discussion and Results

These experiments were designed primarily to test the efficacy of sterols of marine algae in lowering blood cholesterol levels. The effect was measured over a short term, viz, the first three weeks of the animal's life only. A depressant action was observed to the extent of 83% (fucosterol) and 59% (Sargassum sterols).

The cockerels tolerated the diets very well and in experiment 1 the chicks fed fucosterol actually exhibited a slight but significant increase in average body weight over other groups.

Gross examination of the livers enabled one to prejudge the plasma cholesterol level. Thus, the cholesterol-fed chicks had fatty, yellow-coloured livers and the basic diet chick livers were red; those from chicks which had been furnished with sterols from marine algae were red, yellow, or a mottled intermediate between the two. These colorations paralleled the cholesterol concentration of the plasma to a remarkable degree.

Two identical cholesterol-fed groups were tested in experiment 2 as a check on precision of method. The variance of these two groups ($P = 0.80 - 0.70$) amply attests to the uniformity of the procedure.

The liver or liver plus heart weights were higher in the cholesterol-fed chicks than in the other groups. Furthermore, it is to be noted that the cholesterol-fed chicks in experiment 2 showed the lowest average gain in body weight coupled with the highest average combined liver, heart, and gall bladder weight.

TABLE I - Effect of fucosterol on plasma cholesterol levels of chick.

Group	No. in group	Wt 7th day (Group mean values) g	Wt 21st day† g	Wt gain g/chick	Food consumption g/chick/day	Liver g	Aorta g	Plasma Cholesterol mg % ± S.E.
Experiment 1								
Group 1 - Basic Diet	16	63.1	149	85.9	16.2	3.6	0.09	219.6 ± 9.5*
Group 2 - Basic + 1% Cholesterol	16	63.1	154	90.9	17.2	5.2	0.09	692.8 ± 52
Group 3 - Basic + 1% Cholesterol + 1% Fucosterol	13**	63.1	164	100.9	18.6	4.9	0.098	299.4 ± 12
Experiment 2			Sargassum Sterols					
Group 1 - Basic Diet	8	58.3	139	80.7	15.9	5.3***		273 ± 14
Group 2 - Basic + 1% Cholesterol	8	57.9	128	70.1	15.8	5.5		689 ± 40
Group 3 - Basic + 1% Cholesterol	8	58.5	130	71.5	15.6	5.8		712 ± 78
Group 4 - Basic + 1% Cholesterol + 1% Sargassum Sterols	8	58.4	131	72.6	16.0	5.3		449 ± 40

* Analysis of Variance. Experiment 1 - Groups 1 and 2, $P<0.001$; Groups 2 and 3, $P<0.001$; Groups 1 and 3, $P = 0.20-0.10$.

Experiment 2 - Groups 1 and 2, $P<0.001$; Groups 2 and 3, $P = 0.80-0.70$; Groups 3 and 4, $P<0.001$;

Groups 1 and 3, $P<0.001$; Groups 1 and 4, $P = 0.02-0.01$; Groups 2 and 4, $P = 0.005-0.001$.

** Two chicks died first day; one, second day of feeding.

*** Experiment 2 - liver, heart and gall bladder combined weight.

† Experiment 2 - feeding ended on 20th day.

Returning once again to the reasons why or how cholesterol levels are diminished, there appears to be no facile explanation. The mechanism by which cholesterol-lowering takes place could be due to a multitude of factors; prevention of endogenous synthesis of cholesterol (e.g., by vitamin B deficiency), interference with cholesterol clearance or with Co A content, or promotion of conditions leading to deposition of cholesterol, to mention a few. In addition, studies on in vivo esterification and interference of steroids with cholesterol absorption suggested that many steroids inhibit cholesterol absorption at a point between the lumen (of intestine) and site of esterification. The inhibitory step is thought to be stereospecific and probably enzymatic in nature.

The different abilities to depress cholesterol levels shown here by the two sterols might be attributed to a variation in stereochemistry of the side-chain R (Fig 1). The C_{20} methyl group of fucosterol, like that of cholesterol, has the β-configuration. On the other hand, the presumed sargasterol possesses the α-configuration for that particular group.

The question whether a sterol need be absorbed from the intestinal tract or interfere with cholesterol absorption in order to bring about hypocholesterolemia cannot be definitely answered. Apparently, none of the C_{27} sterols other than cholesterol itself is readily absorbed; ergosterol, a C_{29} sterol, is absorbed in some measure and produces hypocholesterolemia. β-sitosterol (Fig 1 f) and stigmasterol (Fig 1 e), on the contrary, are not absorbed themselves, but do prevent hypercholesterolemia and atherosclerosis in chicks.

Of incidental interest in this field is the demonstration that sulphated polymannuronides derived from seaweeds, when administered to humans, exert a rapid correction of hypercholesterolemia.

In conclusion, perhaps we are unable to ascribe the role of hypocholesterolemic agent in lipids solely to components of the unsaponifiable fraction, yet evidence strongly suggests that the latter play an important part in the control and regulation of cholesterol metabolism.

Thanks are due to Dr. Carole A. Reiner, U.H.M. Fagerlund and P.J. Schmidt for help in gathering bladder wrack; D.N. Outram and B. Wildman of the Biological Station, Nanaimo, B.C. for generous assistance in collecting Sargassum; to Drs. R.F. Scagel and Janet Stein, algologists at the University of British Columbia for valuable discussions, and to Dr. Michael Smith for his help and interest.

This investigation was supported (in part) by a PHS Research Grant, A-4490, from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

SUMMARY NO. 16

THE CHEMISTRY OF STEROLS ISOLATED FROM MARINE ALGAE

E. Reiner

In the previous summary mention was made of the sterols obtained, namely fucosterol from Fucus and a presumed mixture of sargasterol and fucosterol from Sargassum.

The former has been characterised by several criteria: melting points, infrared spectra, derivatives, and by thin-layer, reverse-phase paper, and column chromatography (of a colored ester).

The same general treatment was accorded the Sargassum sterols. Data are being completed in order to bring this programme to an end.

SUMMARY NO. 17

LARGE-SCALE PREPARATION OF 24-METHYLENECHOLESTEROL

E. Reiner
D.R. Idler

The synthetic route to 24-methylenecholesterol from methyl-3β-hydroxy-5-cholenate described in Summary No. 39 of this Station's Annual Report for 1960-61 has proved impracticable as a route for large-scale preparations because the reaction of 3β-acetoxy-5-cholenyl chloride with diisopropyl cadmium gave the desired 24-ketocholesterol in low and unreproducible yield. Consequently alternative routes to 24-methylene cholesterol or the 24-ketone have been investigated.

Chromatography of the phenylazobenzoyl esters of clam sterols whilst giving the desired 24-methylene steroid in good yields, was considered too time-consuming a procedure.

An alternative route to 24-methylenecholesterol involves the ozonolysis of fucosterol to yield 24-ketocholesterol followed by conversion of the latter to the desired steroid by procedures already developed in this laboratory. To this end bladder wrack (Fucus gardneri) was collected and air dried. After extraction by the usual procedures fucosterol was obtained in 0.2 - 0.25% yield (based on the dry weight of algae). The processing of this material is now being carried out at the Halifax Technological Station.

SUMMARY NO. 18

HYPOCHOLESTEROLEMIC ACTIVITY OF VITAMIN A AND RELATED COMPOUNDS AS INFLUENCED BY THEIR CHEMICAL STRUCTURE

J.D. Wood

Previous work in this laboratory showed that certain fish oils could prevent the hypercholesterolemia induced in chicks by cholesterol feeding. The substance in the oils responsible for most, if not all, of the activity was later identified as vitamin A. The present investigation was undertaken to ascertain whether the chemical structure of the hypocholesterolemic agent was critical for its activity or whether a general group of compounds was effective. The influence of the group at the terminal carbon atom of the vitamin A sidechain was studied by comparing the hypocholesterolemic action of compounds having acidic, alcoholic (free and esterified) and aldehydic groupings in this position. The activity of different stereoisomers was also compared and the investigation extended to include compounds which may be considered related chemically to vitamin A such as β-carotene, citral and phytol.

Materials and Methods

Birds

The control diet was formulated as shown in Table I. All test compounds were dissolved in the corn oil prior to the mixing of the diets with the exception of β-carotene which was mixed as a slurry in corn oil. The latter procedure became necessary because the carotenoid was not completely soluble under the conditions

employed in the experiment. Cholesterol, where needed, was added directly to the diet. The chicks were fed the diets ad libitum but the feed in the troughs was renewed completely each day from the main bulk of the diet stored at 2°C.

TABLE I - Composition of the control diet.

	%		%
Ground wheat	49.4	Distillers' dried solubles	2.25
Cornmeal	9.0	Dehydrated grass	1.12
Ground oats	4.5	Iodized salt (fine)	0.45
Wheat middlings	4.5	Ground limestone	0.90
Wheat bran	4.5	Manganese sulphate	0.01
Soybean meal	6.8	Vitamin oil (2250A, 300D/g)	0.22
Fishmeal	1.8	Nicarbazin	0.05
Meatmeal	4.5	Corn oil	10.00
	Riboflavin 50 mg/100 lb feed		

Analyses of liver and plasma

Each liver was analyzed separately. After saponification of the tissue with alcoholic KOH, the unsaponifiable material was extracted three times with ether. The extract was washed with several portions of water, made up to 100 ml with ether and suitable aliquots removed for estimation of cholesterol, total unsaponifiable material and vitamin A. The cholesterol determination was carried out by evaporating the sample of ether extract to dryness, redissolving the residue in alcohol-acetone (1:1 v/v) and proceeding thereafter as described by Sperry and Webb. Vitamin A was estimated spectrophotometrically on a small portion of the extracted lipid dissolved in cyclohexane, the correction formulae given by Cama, Collins and Morton being used. The unsaponifiable content was obtained by evaporating a sample of the ether extract to dryness at 50°C under a stream of nitrogen with final drying in vacuo at room temperature.

Plasma cholesterol was determined using the method of Sperry and Webb and the plasma vitamin A was extracted as described by Glover et al. and estimated spectrophotometrically in cyclohexane solution using the correction formulae of Cama, Collins and Morton.

The vitamin A aldehyde content of liver and blood was estimated using the thiobarbituric method of Futterman and Saslaw.

Results

Various vitamin A derivatives were incorporated into the diets of cholesterol-fed chicks. With the exception of vitamin A acid the compounds did not alter the food consumption and growth rate of the birds (Table II). The acid derivative, however, caused a 50% drop in food consumption and a much lower growth rate and at the end of the 14 days test period all birds on this diet showed symptoms of hypervitaminosis A as described by Rodahl, namely pale combs, sore and swollen eyes and drooping tail feathers. The chicks on the other diets appeared normal and in good health. The results in Table II were obtained from the birds used in Experiment 1 of Table III. Food consumption and weight gains in other experiments were similar to the above and are therefore not given in detail here.

TABLE II - Food consumption and weight gain of chicks fed various test diets.

Addition to control diet	Dietary cholesterol (1% level)	Food consumption (g/chick/day)	Weight gain (g)
None	-	14.5	96 ± 3†
None	+	15.1	98 ± 3
Vitamin acetate*	+	14.8	92 ± 2
Vitamin A acid	+	7.6	28 ± 2
Vitamin A alcohol	+	14.9	93 ± 2
Vitamin A aldehyde	+	15.7	97 ± 3

† Mean value ± standard error.
* Concentration in feed was 0.4 m moles/lb.

TABLE III - The influence of the terminal carbon group of vitamin A compounds on hypocholesterolemic activity.

Addition to cholesterol-containing diet	Concentration in diet (m moles/lb)	mg Cholesterol/100 ml plasma Expt. 1	Expt. 2
None	-	824 ± 49*	819 ± 83
Vitamin A acetate	0.4	800 ± 59	727 ± 72
Vitamin A acetate	0.8	-	532 ± 99
Vitamin A acid	0.4	678 ± 57	-
Vitamin A alcohol	0.4	855 ± 60	-
Vitamin A alcohol	0.8	-	411 ± 72
Vitamin A aldehyde	0.4	381 ± 45	377 ± 67

* Mean value ± standard error.
The plasma cholesterol levels for control rats in Experiments 1 and 2 were 136 ± 3 and 140 ± 4 mg/100 ml, respectively.

The effect of the vitamin A derivatives on dietary-induced hypercholesterolemia is shown in Table III. All the components, if they were present in sufficient concentration in the diet, reduced the degree of hypercholesterolemia. Vitamin A alcohol and vitamin A acetate appeared equally effective but vitamin A aldehyde was much more potent. Little significance can be attached to the plasma cholesterol level of vitamin A acid-fed chicks because of their low food consumption and poor state of health.

The three stereoisomers of vitamin A aldehyde tested all possessed hypocholesterolemic activity (Table IV). The presence of a cis double bond in the 9- or 13- position of the molecule did not bring about any noticeable change in the plasma cholesterol level from that observed with the all-trans compound.

β-carotene, phytol and citral were compared with vitamin A aldehyde in regard to hypocholesterolemic activity (Table V). The first two mentioned compounds failed completely to prevent the dietary-induced hypercholesterolemia and although citral lowered the plasma cholesterol level somewhat, the decrease was not statistically significant. The results in Table V also indicate that the ability of vitamin A aldehyde to prevent hypercholesterolemia in cholesterol-fed chicks is proportional to the amount of the aldehyde in the diet.

TABLE IV - Hypocholesterolemic properties of vitamin A aldehyde stereoisomers.

Vitamin A aldehyde isomer in the diet*	1% dietary cholesterol	Plasma cholesterol (mg/100 ml)
None	-	223 ± 10†
None	+	930 ± 74
All-trans	+	637 ± 54
9-mono-cis	+	647 ± 90
13-mono-cis	+	625 ± 61

* Concentration in feed was 0.4 m moles/lb.
† Mean value ± standard error.

TABLE V - The effect of compounds related to vitamin A on the plasma cholesterol levels in cholesterol-fed chicks.

Addition to cholesterol-containing diet	Concentration in diet (m moles/lb)	Plasma cholesterol (mg/100 ml)
None	-	819 ± 83*
Vitamin A aldehyde	0.04	377 ± 67
β carotene	0.08	802 ± 40
None	-	930 ± 74
Vitamin A aldehyde	0.04	637 ± 54
Vitamin A aldehyde	0.08	463 ± 66
Citral	0.08	777 ± 58
Phytol	0.08	943 ± 79

* Mean value ± standard error.

The above results have dealt solely with plasma cholesterol levels but a more extensive study of the effects of dietary cholesterol and vitamin A compounds is reported in Table VI. Although the weights of chicks fed the various diets were approximately the same, the liver weights were significantly different. The addition of cholesterol to the diet increased the weight of the liver and this increase was counteracted by vitamin A acetate and vitamin A aldehyde, the effect of the compounds paralleling their hypocholesterolemic activity. Dietary cholesterol also caused a sharp increase in the amount of unsaponifiable material in the liver and this increase was shown to be due mainly to an increase in liver cholesterol. Changes in liver cholesterol levels of chicks on the various diets reflected closely alterations in plasma cholesterol levels but the changes in the liver lipid were more pronounced.

The vitamin A content of plasma and liver was not influenced by the presence of cholesterol in the diet but increasing amounts of the vitamin were observed in these tissues as the vitamin A acetate concentration in the diet increased. The inclusion of vitamin A aldehyde in the diet increased liver vitamin A levels even more than did equimolar amounts of vitamin A acetate but the increase in plasma vitamin A was only one-third of that observed with the acetate. The liver lipids from vitamin A aldehyde-fed chicks were examined spectrophotometrically and the spectra compared with those of vitamin A aldehyde and vitamin A acetate (Fig. 1).

TABLE VI - Some effects observed in chicks fed cholesterol and vitamin A compounds.

Addition to control diet	1% dietary cholesterol	Body weight (g)	Liver weight (g)	Total liver unsaponifiable material (mg)	Cholesterol		Vitamin A	
					Liver (mg)	Plasma (mg/100mg)	Liver (I.U.)	Plasma (I.U./100 ml)
None	-	157±4*	4.55±0.16	31± 2	13± 1	140± 4	755±27	184
None	+	154±3	5.95±0.18	418±23	393±21	819±83	872±68	217
Vitamin A acetate (0.8)†	+	145±4	4.63±0.18	203±27	147±24	532±99	107,700±7,700	4,079
Vitamin A acetate (0.4)	+	148±3	5.07±0.12	294±18	263±20	727±72	34,200±1,800	2,771
Vitamin A aldehyde (0.4)	+	147±4	4.54±0.09	145±21	120±20	377±67	43,600±2,100	1,035

* Each value in the table is the mean ± standard error except for plasma vitamin A levels in which case the values are the means for two groups of plasma, each group containing samples from 8 birds.

† Value in parenthesis indicates concentration in m moles/lb feed.

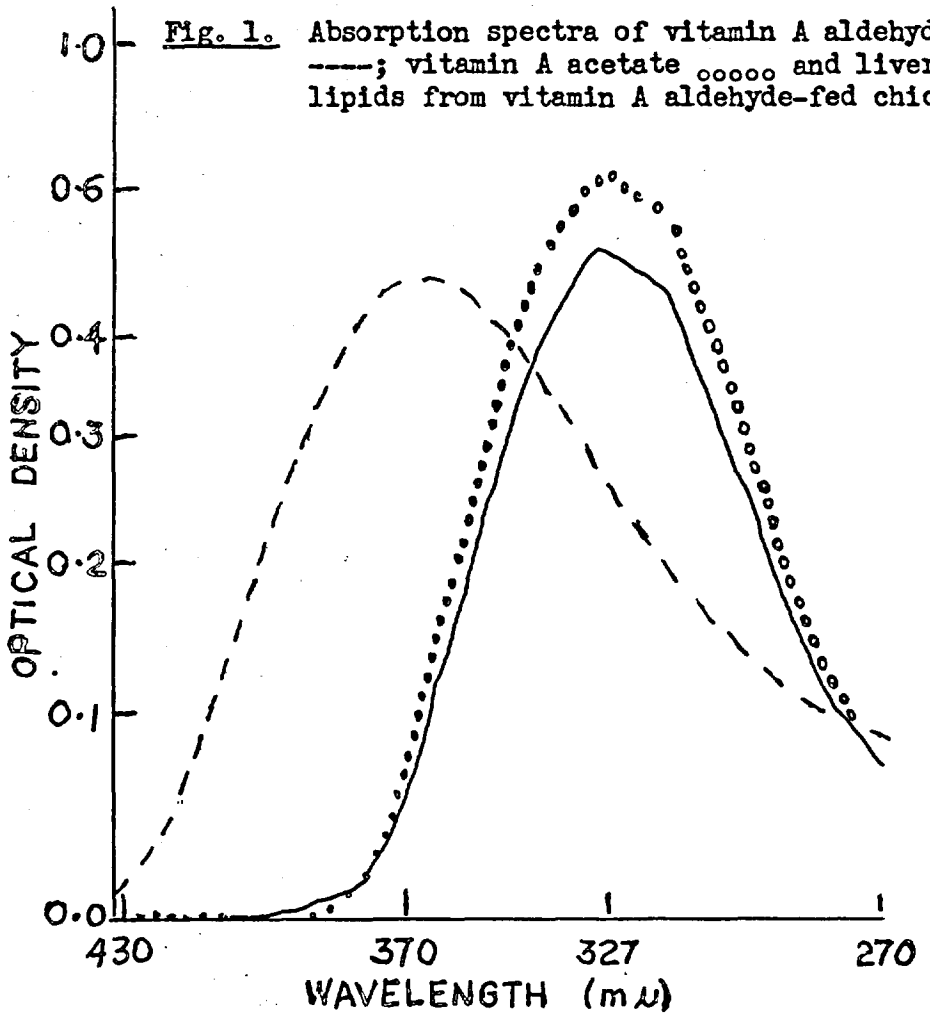


Fig. 1. Absorption spectra of vitamin A aldehyde ----; vitamin A acetate ooooo and liver lipids from vitamin A aldehyde-fed chicks ——.

It is evident from the results that there was little if any of the aldehyde in the liver after feeding the compound. Further evidence for this phenomenon was obtained when the liver lipids were analyzed for vitamin A aldehyde by the procedure of Futterman and Saslaw. No trace of the aldehyde was detected in the liver lipids in spite of the sensitivity of the method employed. Similar studies on the plasma lipids indicated that here too no vitamin A aldehyde was present after feeding the compound in the diet.

It would appear from the work presented here that the vitamin A type structure is necessary for hypocholesterolemic activity and that compounds related chemically but having a different general structure were inactive in this respect. For example, β-carotene possesses a structure closely resembling two molecules of vitamin A in which the terminal carbon atoms are joined and no longer exposed but the carotenoid was unable to lower cholesterol levels. In view of this, the possibility was considered that the sidechain of vitamin A was necessary for activity. However, when citral was tested it produced no significant decrease in plasma cholesterol in spite of the fact that its structure closely resembles that of the sidechain of vitamin A aldehyde, itself a very active compound. In addition, phytol, although containing no ring structure, may be considered related chemically to vitamin A since both compounds are derived from 4 isoprene units, yet it possessed no hypercholesterolemic properties.

The nature of the terminal carbon grouping influenced the activity of the vitamin A compounds to some extent but the type of group did not appear to be too critical as the alcohol (free and esterified), the aldehyde, and possibly the acid all possessed activity. However, vitamin A aldehyde was considerably more potent in this respect than were the other derivatives.

Ames et al. reported that the geometrical isomers of vitamin A aldehyde differed in their biopotency as judged by growth and liver storage in rats, the 9-mono-cis compound in particular having a low biopotency. The present studies indicate that no such stereoisomeric differences occurred with respect to hypocholesterolemic activity in chicks when the all-trans, 9-mono-cis and 13-mono-cis aldehydes were tested.

The question now arises as to where in the chick body the vitamin A compounds exert their hypocholesterolemic action. Two possible sites are the liver, by inhibition of cholesterol biosynthesis, and the intestine, by interference with cholesterol absorption. An examination of the results in Table VI shows that there was no correlation between the amount of vitamin A in the liver and plasma and the levels of cholesterol in these tissues, thus rendering unlikely the former possibility. Moreover, in vitamin A aldehyde-fed chicks none of the aldehyde was detected in the liver and plasma and since the amount of vitamin A in these tissues could not account for the greater activity of vitamin A aldehyde, it is reasonable to conclude that the hypocholesterolemic action was occurring before or during absorption through the intestinal wall. The absence of the aldehyde in the plasma and liver is in agreement with the report of Glover, Goodwin and Morton that the intestinal wall is very efficient in converting the aldehyde to vitamin A itself. The mechanism whereby vitamin A aldehyde is more active than other derivatives remains unexplained.

Although a study of the effect of dietary cholesterol on liver vitamin A stored was not a primary object of the present investigation, the results reported here indicate that the liver vitamin A content of cholesterol-fed immature cockerels is somewhat higher than that of control birds. This finding contrasts sharply with the results obtained by Green et al. using rats which showed that dietary cholesterol lowered vitamin A stored in the immature male animal.

SUMMARY NO. 19

THE EFFECT OF EXCESSIVE AMOUNTS OF DIETARY VITAMIN A ON EGG PRODUCTION IN WHITE LEGHORN HENS

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

Although adequate amounts of vitamin A are necessary for the normal metabolism of animals, and humans, it is well known that excessive quantities of the vitamin can prove harmful. It has been reported that large amounts of dietary vitamin A adversely affected the reproductive ability of the rat and a similar conclusion was reached in 1961 in studies with mink. During the course of an investigation by Wood, Biely and Topliff on cholesterol metabolism in White Leghorn chickens, it was observed that the presence of dogfish liver oil in the diet resulted in a cessation of egg production. As the oil was rich in vitamin A it was thought that perhaps this was due to the massive doses of vitamin A ingested by the birds. The present investigation was carried out to ascertain whether vitamin A did interfere with egg production, and if so, at what level of the vitamin in the diet the effect became evident.

Experiment

White Leghorn hens, 14 months old, were used in the study and they were kept in groups of 20 in colony houses. The dietary supplements are shown in Table I. The basal diet consisted of an all mash laying ration containing 5266 I.U./lb of vitamin A and 3.5 mg/lb carotene. The synthetic vitamin A acetate was dissolved in corn oil prior to mixing with the basal diet. The lingcod liver oil had a vitamin A potency of 97,000 I.U./g and the salmon oil a potency of 162 I.U./g. Two groups of birds were placed on each of the diets and were fed ad libitum, fresh feed being offered every day to avoid the ingestion by the birds of any oxidation products of vitamin A which might occur in feed exposed to light and warm temperature. The main bulk of the diets was kept in a dark storage room at 2°C and was renewed each week.

TABLE I - The effect of dietary supplements on food consumption and body weight of White Leghorn hens.

Diet	Supplement	Supplementary Vit A (I.U./lb feed)	Food consumption (oz/hen/day)	Mean change in body wt (lb)
1	None	None	3.84	-0.05
2	1.0% corn oil + vit A acetate	440,000	3.21	-0.25
3	1.5% corn oil + vit A acetate	220,000	3.95	-0.05
4	1.0% lingcod liver oil	440,000	3.27	-0.15
5	0.5% lingcod liver oil	220,000	3.45	-0.15
6	0.1% lingcod liver oil	44,000	3.91	-0.10
7	0.05% lingcod liver oil	22,000	3.90	-0.20
8	1.0% salmon oil	700	3.90	-0.05

Results

The food consumption and change in body weight of the hens during the 5-week experimental period are shown in Table I. The food consumption varied from 3.21 to 3.95 oz/bird/day, being lowest with the diets which caused loss of egg production. The changes in body weight were small, none amounting to more than 5% of the initial weight.

The effects of the test diets on egg production are indicated in Table II. It is obvious that vitamin A per se caused a striking decrease in egg production but the effect was not so marked as with lingcod liver oil containing equivalent amounts of the vitamin. Approximately twice as much synthetic vitamin A added to the corn oil was required to bring about the same effect as the vitamin A in the fish oil. It was thought that perhaps the greater degree of unsaturation of fish oil might itself be causing a lowered rate of egg production but this theory was dispelled by the results obtained with low vitamin A potency salmon oil which caused no cessation of egg production in spite of the unsaturated nature of the oil (Iodine absorption number = 129). The effect of lingcod liver oil was evident only at the higher levels in the diet and the addition of 44,000 I.U. vitamin A/lb feed produced no loss in production. It is obvious that normal amounts of vitamin A, as used in commercial rations, will not result in a reduced egg production and that this effect is produced only when massive amounts of the vitamin are ingested by the birds.

TABLE II - Effect of dietary vitamin A and fish oil on egg production.

Diet	Percent egg production				
	1st week	2nd week	3rd week	4th week	5th week
1†	70.4, 61.4*	74.3, 74.3	66.4, 61.4	67.8, 60.7	59.3, 59.3
2	71.4, 62.1	65.0, 64.2	39.3, 38.6	26.4, 22.9	12.9, 13.6
3	66.4, 72.1	73.6, 73.6	71.4, 65.7	51.4, 52.9	44.3, 43.6
4	72.1, 70.7	60.9, 52.9	23.6, 31.1	12.1, 7.5	2.1, 0.0
5	64.3, 65.0	51.4, 65.0	38.6, 41.4	24.3, 30.0	12.1, 12.9
6	75.7, 62.8	68.6, 67.1	72.8, 67.1	63.6, 67.8	67.1, 64.3
7	71.4, 62.8	77.8, 68.6	72.1, 68.6	75.0, 63.6	69.3, 60.0
8	67.8, 71.4	72.1, 72.8	70.0, 60.7	70.7, 69.3	68.6, 62.1

† Composition of the diets is given in Table I.
* Each value in the table is the percent egg production for a group of 20 birds.

Summary

The effect of dietary vitamin A on the egg production of White Leghorn hens was investigated. Dietary vitamin A in the form of lingcod liver oil caused a greater loss in egg production than did corn oil to which an equivalent amount of the vitamin A acetate had been added. A decrease in egg production occurred only when massive amounts of vitamin A (44,000 I.U./lb feed) were included in the diet.

SUMMARY NO. 20

AN INVESTIGATION INTO A POSSIBLE ENHANCEMENT OF VITAMIN-A-INDUCED-HYPOCHOLESTEROLEMIA IN CHICKS BY THE FEEDING OF GLYCERYL ETHERS

J.E. Topliff

The effectiveness of the unsaponifiable portion of lingcod liver oil as a hypocholesterolemic agent was studied by Wood and Biely. Unsaponifiable material was separated into three fractions, characterized by vitamin A, cholesterol, and the glyceryl ethers. The vitamin A fraction prevented hypercholesterolemia induced by cholesterol feeding. Pure crystalline vitamin A acetate had the same effect. Wood found, however, that only 73 to 85% of the activity of lingcod liver oil unsaponifiable material can be attributed to its vitamin A content. An experiment was designed to ascertain whether glyceryl ethers, ineffective in themselves as hypocholesterolemic agents, might not, when added along with vitamin A acetate, help vitamin A exert its activity in lowering plasma cholesterol. This experiment is now in progress here.

PITUITARY HORMONES OF FISH

SUMMARY NO. 21

GENERAL INTRODUCTION TO PROGRAMME

P.J. Schmidt, B.S. Findlay, H. Tsuyuki and M. Smith

The pituitary gland in vertebrates is the source of at least eight hormones which control such important bodily functions as growth, water regulation and (in the mammals) lactation and uterine contraction. Other functions of the pituitary hormones include regulation of the thyroid and adrenal glands as well as gonad development. Mammalian hormones have been extensively purified and their chemical and physical properties defined. As a direct consequence of the availability of purified mammalian hormones, many physiological conditions in humans, which are caused by endocrine malfunctions, can now be corrected medically. Physiological experiments have demonstrated the presence of pituitary hormones in fish but no extensive programme of isolation and characterization of piscine hormones has been attempted. The present study is specifically directed toward the isolation and characterization of these hormones. It is believed that the results will provide the pisciculturist with the means to control the development of his fish to a much greater extent that is at present possible.

SUMMARY NO. 22

COLLECTION AND PRESERVATION OF SALMON PITUITARY GLANDS

H. Tsuyuki, P.J. Schmidt, B. Findlay, A. Birnie and M. Smith

Some preliminary work on the collection of sockeye salmon pituitary glands was described in Summary No. 16 of this Station's Annual Report for 1960-61.

The method commonly employed by other investigators for the removal of the pituitary gland from fishes consisted of cutting a series of slices from the top of the head until the brain is exposed. The brain is then lifted out and the pituitary gland located just under it is removed. We have found this method applicable for the removal of a few glands but when large numbers (thousands) are concerned, the method was tedious and time-consuming. It was found more convenient to take advantage of the cartilaginous structure of the salmon head to remove a core of about 1" in diameter extending from the top of the head through the brain and to the bony structure in the roof of the mouth. The diameter of the core was selected to allow sufficient leeway to avoid missing the gland. The core may be split through the region of the brain cavity by hand or by knife. The brain is lifted over and the pituitary gland picked out by a probe conveniently looped on the end. The gland may be picked out immediately or the entire core frozen in a container of dry ice and returned to the laboratory. The cores may then be partially thawed and the glands removed.

The various types of coring equipment developed here are shown in Fig. 1. The corers (A and D) are fashioned from a relatively hard grade of tubular stainless steel and the two types of core ejection shafts (C and E) are likewise made of stainless steel rods. The hand-operated prototype (A) constructed by Messrs. F. Freeman and K. McLean of this Station was used for our initial operations. It is operated

like a cork borer and the core is punched out by means of a rod of convenient size. This borer is still used exclusively by some workers by preference and is useful for handling relatively small numbers of samples. Considerable improvement was achieved through the construction of the cutter D (made to specification by Modern Engineering of Vancouver). The two types of core ejector shafts (C and E) are machined to fit snugly inside the cutter. In their cutting positions the ejector shafts are drawn up into the collar of the cutter and locked in position by depressing the lever G to which is attached a pin which locks into the hole F drilled into the ejector shaft. When the core is drilled through the head to the roof of the mouth the cutter and the core within are removed from the head and the core is ejected by depressing the lever G and pushing the ejector shaft through the cutter. The smaller (C) of the two ejector shafts is constructed to use with the rachet screw-driver (B). The core is drilled with the rachet in the clockwise turn position to impart a turning motion to the cutter while pressure is applied to the screw-driver. The core is ejected by setting the rachet on the lock position, depressing the lever G and simply pushing the screw-driver through the cutter as far as it will go. This equipment was found to be very satisfactory for use in areas where electric power was not available. The operation is fast and one man could hold the fish and drill simultaneously. However, for larger fish (spring and chum salmon) considerable pressure is required and when a source of electric power is available we prefer to use a heavy duty ½-inch drill attached to the longer shaft, E. For this operation a second man is required to hold the fish in a holder as shown in Fig. 2. As shown clearly in the photograph the core is drilled at a 90° angle to the curvature of the head at a position just behind the eye. From this position the pituitary gland is found on or near the centre of the core. Where electric power was not available a Black & Decker battery-operated ¼" drill was used to advantage. With the power-driven cutters an average time of 15 seconds is required for each core.

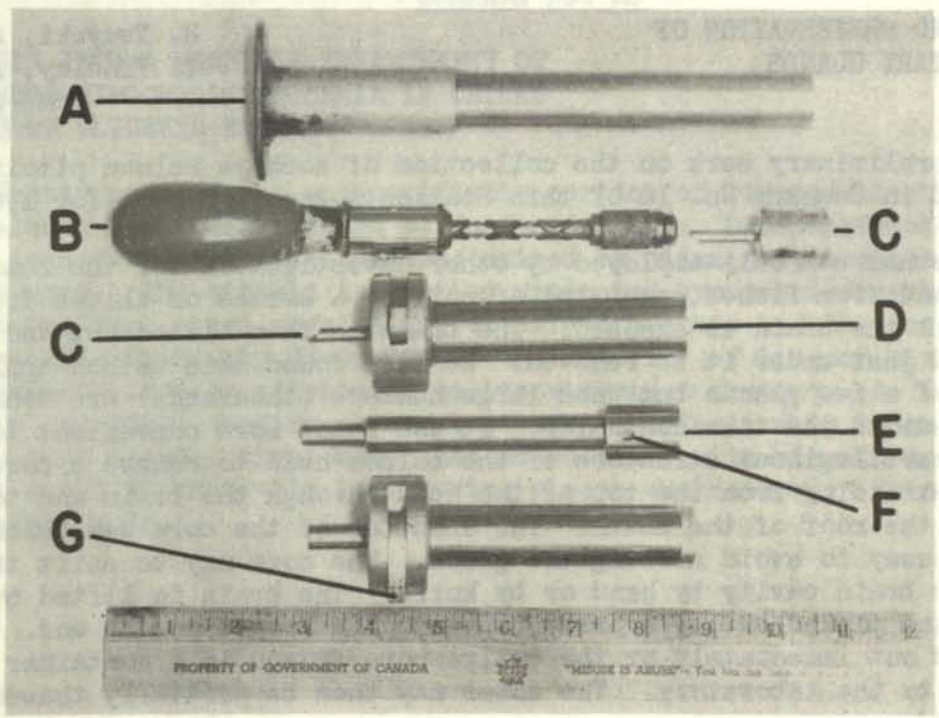


Fig. 1.

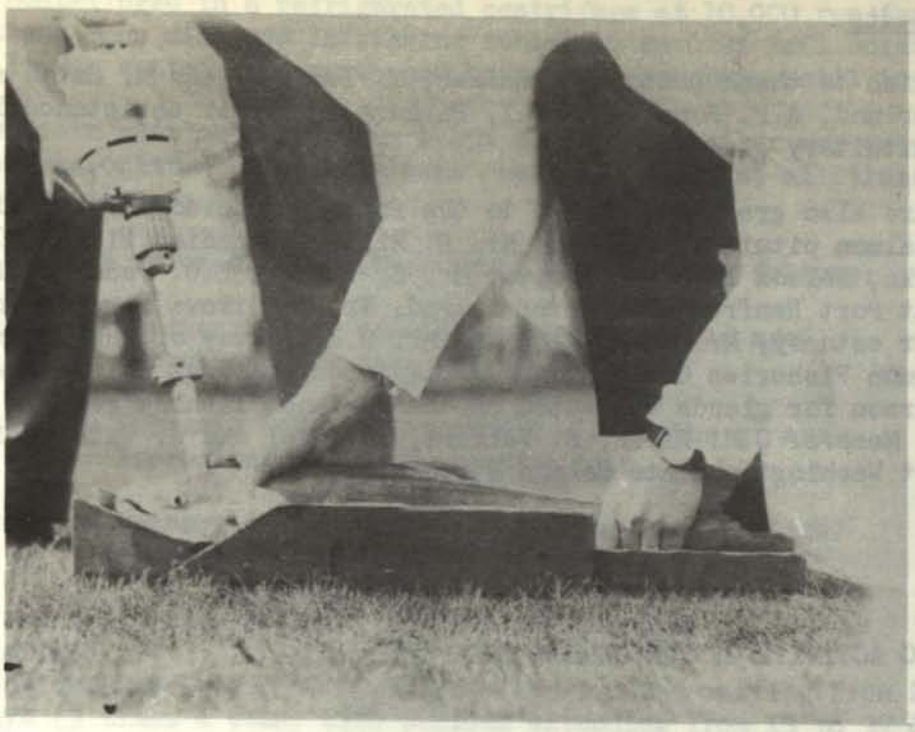


Fig. 2.

Salmon pituitary glands were collected from a number of fishing areas starting from the last week of July and continuing to the end of November. Samples were taken from fish at various physiological states, time after death, and by a variety of fishing operations as summarized below.

Location	Type salmon	No. of glands	Fishing operation	Estimated physiological state of fish	Time interval after death (in hours)
Port Renfrew	Sockeye	500	Seine net	2-3 months to spawning	0-2
" "	"	1000	Fish camp	"	3-10
Fraser River	"	600	Gill net	1 month to spawning	0-1
Lummi Island	Coho	1711	Reef net	1½ months to spawning	0-1
" "	Chum	360	" "	"	0-1
" "	Pink	453	" "	"	0-1
Green River and Issaquah Hatcheries	Spring	4000	-	Ripe	2-3
Cultus Lake	Sockeye	100	Beach net	Ripe precocious males and post-spawned fish	0-1

Acknowledgments

We wish to thank Doctors E. Reiner, T. Todoroff and M. Smith and Messrs. U.H.M. Fagerlund, A.P. Ronald and I.I. Bitners for their assistance in collection of salmon pituitary glands.

We are also greatly indebted to the following persons for their help in obtaining salmon pituitary glands: Mr. D. Miller, Canadian Fishing Company; Mr. J. Clarke, Nelson Bros. Fisheries; Mr. S. Furney, B.C. Packers, for glands collected at Port Renfrew, Vancouver Island; Mr. J. Oikawa for collection in the Fraser River estuary; Mr. S. Killick and Mr. H.H. Harvey of the Internation Pacific Salmon Fisheries Commission for glands obtained at Cultus Lake, B.C.; Mr. G. Anderson for glands collected on the reef-net fishing gear at Lummi Is., Washington; Messrs. C.H. Ellis, S. Fallert, R. Engel and G. Watkins for glands collected at Washington State Salmon Hatcheries.

SUMMARY NO. 23

GONADOTROPIC ACTIVITY OF THE SALMON PITUITARY

Barbara S. Findlay
P.J. Schmidt
H. Tsuyuki
M. Smith

Gonadotropic hormones are involved in the processes controlling the physiology of reproduction and regulate the maturation of the gonads.

To the fish culturist, preparations of fish pituitary glands have found important use in inducing the reproduction of many captive fishes which would otherwise not reproduce. A few species of fish will yield ripe eggs or sperm when treated with mammalian and bird pituitary preparations but in general their degrees of effectiveness do not warrant their use in fish. On the other hand, although there is some evidence of species specificity of these hormones, preparations from any species of fish or other cold blooded vertebrates have marked gonadotropic activity in other fishes. While mammalian gonadotropic activities result from the influence of the interstitial cell-stimulating hormone (ICSH) and the follicle-stimulating hormone (FSH), the corresponding activities in the case of fish are not as clearly defined. Although the presence of ICSH-like activity in fish pituitary preparations are sufficiently well documented, the presence of an FSH activity is not on such firm grounds.

Because of the evidence for species specificity, we are attempting to develop suitable bioassays in fish and amphibians which are known to respond to fish pituitary gonadotropins. We are indebted to Dr. O.H. Robertson, Stanford University, for helpful discussions about fish gonadotropic assays.

1. Assay Using Immature Trout

The pituitaries used in this experiment were obtained from sockeye salmon at Port Renfrew in July, 1961 and from mature spring salmon taken at the Green River Hatchery, Washington, in October and November 1961. The sockeye salmon can be considered "green" as they were in their early stage of migration, not as yet having entered fresh water. The spring salmon were "ripe" just having been stripped for eggs and milt for hatchery spawning. The glands were extracted with a 2% NaCl solution using 2 ml per gram of pituitaries. The glands (equal number of both sexes) were pulverized in the salt solution in a glass homogeniser. The

suspension was centrifuged in a refrigerated centrifuge at 10,000 rpm for 20 minutes. The supernatant was then dialized in visking tubing in an 0.9% NaCl solution overnight at 0°C. It was centrifuged again and kept in an ice bath until injected.

Other extracts were made by using 1.25% NaCl in a similar way except that the dialization step was omitted. The solutions were kept cold at all times both during and after extraction.

Three different batches of immature trout were used as follows:

Batch 1 - Capilano River steelhead - 18 months of age.
Approximate average weight - 40 gm.

Batch 2 - Skamania Hatchery steelhead - 8 months of age.
Approximate average weight - 6 gm.

Batch 3 - Rainbow trout from a trout farm - unknown age.
Approximate average weight - 35 gm.

Both Batches 1 and 3 had some sexually precocious male fish.

The injections were made intraperitoneally in doses varying from 20 to 100 µl every 2 or 3 days (3 times a week) over periods extending from 13 to 37 days. With each group of test fish there was a similar group of control fish receiving equal injections of saline solution. Before each injection the needle was dipped into a chloromycetin solution. The fish were fed daily, except where otherwise noted. Just prior to injection the fish were anaesthetized using MS222 (85 mg/liter of water). In all experiments except one the fish were kept in running water with no temperature control. In the last experiment (as indicated under results, Table I) the water temperature was controlled at 15°C using 150-watt heaters and a thermoregulator in 50-gallon tanks with no incoming fresh water. Water circulation was provided by bubbling air. The water was changed every 2 days.

No attempt was made to determine the sex of the live immature fish, as this can be done only by laparotomy. On killing the fish the sex was established by a microscopic examination of the gonads.

After killing the fish the weight of the fish and the length were obtained. The gonads were removed and weighed. The adrenals and thyroid were also taken. Histological studies made on these organs are discussed in section 4 of this Summary.

Results

In every experiment the pituitary extracts had a smaller effect on the gonads of the female fish. Experiment 3 appears to show that when the dosage injected is too high the gonadotropic effect decreases as the 480-mg dosage in the male fish showed a smaller % gain in weight of gonads than the 240-mg dosage. Experiments 3 and 4 showed some discrepancies that could not be explained. It was thus decided to do experiment 5 which compares the results at 2 different water temperatures. This experiment showed that the response was much higher at the higher temperature of 15°C as compared to 5°C. All future experiments will thus be conducted at a controlled higher temperature. The dosage can be calculated in terms of the number of glands by using the following data:

1 sockeye gland weighs approximately 25 mg;
1 spring " " " 78 mg.

TABLE I - Gonadotropic effect of salmon pituitary extracts on the gonads of rainbow and steelhead trout.

Expt No	Sex	Total wt pituitary* (in mgs)	Water temp °C	Source of pituitaries	No. of fish used in test	Batch no. of test fish	Total time period of injections (days)	% gain in wt gonads
1	Male	156	14-14.5	Sockeye	3	1	37	1325
	Female	156	"	"	3	1		none
2	Male	128	12-13	Sockeye	10	1	23	600
	Female	128	"	"	9	1		74.8
3	Male	120	8.5-9.5	Spring	9	3	14	192
	Male	240	"	"	6	3		560
	Male	480	"	"	9	3		185
	Female	120	"	"	7	3		44.8
	Female	240	"	"	11	3		62.8
	Female	480	"	"	6	3		87.8
4	Male	24	7-7.5	Spring	7	3	14	235
	Male	80	"	"	3	3		381
	Female	24	"	"	10	3		19.3
	Female	80	"	"	7	3		24.7
5	Male	117	5	Spring	11	2	14	150
	Male	117	15	"	9	2		464
	Female	117	5	"	7	2		57.3
	Female	117	15	"	9	2		26.2

* Extracted and injected per fish.

2. Attempted Assay Using Tropical Fish

Tropical fish have a short spawning cycle (e.g. Betta splendens spawns once every three weeks at 25°C). Some attempts have been therefore made to utilize this property to provide a rapid assay of gonadotropic activity. With Betta splendens, injections of salmon pituitary extracts appeared to stimulate ovary development. However, in practice, it was impossible to obtain sufficient fish at one time in the same stage of sexual maturity as is required for a useful bioassay and this approach has been dropped.

3. Frog Bioassay for Pituitary Gonadotropins

The Galli-Mainini frog pregnancy test has been considered as an assay method for fish gonadotropins. This test was introduced in 1947 for determining pregnancy in women. It involves the injection of urine into the dorsal lymph sac of the frog causing the release of sperm. During early pregnancy urine contains high concentrations of human chorionic gonadotropin (HCG) and this gives the gametokinetic response. Workers have shown that the pituitaries from mammals and some fish will also give this response. It was hoped, therefore, that we could use this quick method for determining the relative gonadotropin activities of salmon pituitaries by establishing a dose-time response using crude pituitary preparations.

Methods and Materials

Male frogs, Rana pipiens were used as the assay animals. They were weighed individually, placed in separate containers without water and allowed to come to room temperature before any injections were made. Following injection, the cloacal fluid was sampled every 10 minutes and examined microscopically for the first appearance of sperm.

Before assaying any of the pituitary extracts, varying doses of standard HCG were prepared and injected to determine whether a dose-time response could be established. HCG is standardized in International Units (IU) of gonadotropin. The injected doses were calculated in terms of IU per gram-weight of frog and all doses were made up to a volume of 1 ml with isotonic frog saline (NaCl - 0.6%, KCl - 0.0075%, $CaCl_2$ - 0.01% and $NaHCO_3$ - 0.01% in distilled water).

Crude pituitary extracts were prepared by homogenizing whole frozen glands with 0.5% NaCl, centrifuging and removing the supernate for immediate injection. Portions of the extracts were frozen for future assay. The injected doses represented from 10 mg to 150 mg of whole frozen glands and were made up to a volume of 1 ml. Dose levels were chosen to correspond to levels used by other investigators. Extracts were also prepared from glands which had been in absolute ethanol for 18 hours prior to homogenizing in 0.5% NaCl. Otsuka's method for preparing gonadotropic extracts was used to obtain purer material for injection. All assays were carried out at a room temperature of 25°C; however, to observe the effect of temperature on the response, assays were set up at 11°C and 20°C.

Results

The results of the HCG assay are shown in Table II. A dose-time response was noted. At first some inconsistencies did arise. However, by omitting the water in the test containers and keeping the volume of the injected dose constant, reproducible results were obtained.

The assays with the pituitary extracts were disappointing as the results were most inconsistent. A dose-time response could not be established. It was thought that the dose level was in the wrong range; however, after decreasing and increasing the range it was found that the results still could not be correlated. Although the activities of the preparations could not be established in terms of IU of HCG, the extracts did evoke a positive gametokinetic response.

On examining the extracts that had been frozen, it was found that the time required for a positive response to occur had increased considerably, indicating that the activity of the frozen extract had decreased. The assays that were carried out at temperatures other than room temperature, were set up to observe whether temperature had a controlling influence on the frog response. At 11°C, using a range of doses from 0.5 to 1.9 mg of pituitary per gram-weight of frog, the response was very slow and many of the frogs gave a negative response after 3 hours. At 20°C, there was a much faster response than at 11°C; however, there was still no relationship between dose and time.

Conclusion

A relationship between dose and time response in the frog bioassay for gonadotropins was not established. Further investigation of this assay method is planned in hope that the method will be of use in determining the relative activities of salmon pituitary extracts.

TABLE II - Gametokinetic dose-time response in frogs injected with human chorionic gonadotropin.

IU of HCG injected	Wt of frog (grams)	Dosage - HCG IU/g wt frog	Time to positive response (min)
200	23.6	8.47	10
200	20.3	9.85	10
150	19.6	7.65	20
150	20.9	7.17	20
100	27.8	3.60	25
100	23.0	4.35	25
50	24.5	2.04	35
50	28.9	1.73	30

Acknowledgment

The authors wish to acknowledge the kind help and advice of Dr. W.S. Hoar, Department of Zoology, University of B.C.

4. Histological Changes in the Gonads, Thyroid and Adrenal Cortical Tissue of Rainbow and Steelhead Trout Injected with Pituitary Extract

Histological techniques were employed in conjunction with the bioassay to observe the effect of injections of pituitary extract on the gonads, thyroid and adrenal cortical tissue of immature rainbow and steelhead trout.

In the testes of the immature trout, the germ cells or spermatogonia are found in closely packed cysts or nests of cells which are separated into lobules containing 2 to 6 cysts. Increase in the numbers of cells in the cysts due to mitosis is evidence of spermatogenesis. The spermatogonia divide forming smaller cells called primary spermatocytes and on staining, these cells show densely packed chromatin in the nucleus. The chromatin disperses throughout the nucleus and the gland becomes lobulated. Secondary spermatocytes are formed on further division and the cells develop into spermatozoa, the last stage of development in the maturing testes. Throughout this process, the size of the tissue becomes greatly enlarged. The development of the ovaries is characterized by the increasing presence of granular yolk in the oocytes.

In the trout, the cells of the adrenal cortical tissue are dispersed in the lymphatic tissue of the head kidney, in the narrow zone at the cross arms of that tissue. The cells occur in small clumps surrounding the cardinal vein or as discrete islets. On nuclear staining, the granules of the inactive or immature cells stain less heavily than do those of the active cells and the nucleolus is not as prominant. The size and number of cells increase upon maturity.

In the thyroid of the immature trout, the follicles are well filled with colloid and they show considerable vacuolization. The shape of the cells is low and cuboidal. In the sexually maturing fish, the cells become enlarged and the colloid disappears.

Methods and Materials

Two days after the last injection of pituitary extract, the fish were killed and the gonads, thyroids and head kidneys were removed and immediately placed in Bouin's fixing solution. When necessary the tissues were cut to the proper size of 2-3 mm before putting them into the fixative. The tissues were fixed for 12 to 24 hours and then transferred through several changes of 70% ethanol to wash out the excess picric acid. A pinch of $LiCO_3$ or a few drops of 2% NH_4OH may be added to the alcohol to remove all traces of the fixative colour. For the dehydrating and embedding processes, the tissues were individually wrapped in gauze, labelled and placed in an "autotechnicon". This instrument passes the tissues through a series of graded ethanols, 70% to 100%, chloroform and melted wax, allowing the tissues to remain in each solution for 2 hours with constant vibration. After this 24-hour procedure, the tissues are removed from the wax and embedded in molten wax that is poured into individual embedding containers. The wax blocks containing the tissue are then sectioned at 6 to 8 μ, mounted and stained with haematoxylin and eosin dyes. Depending upon the age of the dyes, the time required to produce a good stain varies. Therefore, the slides were examined microscopically immediately upon removal from the stain, before proceeding to the next step in the procedure. The nuclear material is stained blue by haematoxylin and should not be too dark in colour; the counterstain, eosin, should faintly colour the cytoplasm pink.

Results and Discussion

At the time of reporting only two sets of histological data were available, and these data were based on somewhat unsuccessful experiments as far as gonad stimulation was concerned. Most of the gonads from these experiments were immature or only slightly developed. The tissues from the fish injected with pituitary extracts appeared no different from those of the control or saline injected fish. The testes were composed of spermatogonia and a few primary spermatocytes. The ovaries were slightly more mature than the testes and a few showed development of the yolk and the presence of secondary oocytes. There were a few precocious males in both experiments and the examination of the testes from these fish showed progression in the developmental stages of spermatogenesis from secondary spermatocytes to spermatozoa. The interstitial cells in these tissues were well differentiated, indicating full maturity.

The adrenal cortical cells were little differentiated between mature or precocious fish and immature fish. Examination of the thyroid tissue has not yet been completed.

Conclusion

A satisfactory technique has been established for histological studies of various fish tissues. These studies will continue on future bioassay fish.

Acknowledgment

The authors wish to acknowledge the kind help of Dr. W.S. Hoar and Mr. Nazar Ahsan, Department of Zoology, University of B.C., with the interpretation of the experimental data.

SUMMARY NO. 24

ELECTROPHORESIS OF PITUITARY EXTRACTS

H. Tsuyuki
P.J. Schmidt
A. Birnie

Since the hormones present in the pituitary gland are proteinaceous in nature a method suitable for their separation was investigated. For this purpose a starch gel electrophoretic method (described in Summary No. 28 of this Annual Report) was used in view of the fact that it is one of the most sensitive procedures known for the separation of proteins.

Conditions required for the optimal separation of the proteins present in a crude 2% NaCl extract of sockeye salmon pituitary glands were investigated. A pH of 8.5 was found to be the most suitable on the basis of the number of discrete protein bands obtained. At this pH value 9 components were distinguishable on the cathode side and 5 on the anode (Fig. 1). At a pH of 8.0 the separation on the anode side was poor, while at 9.0 most of the proteins were negatively charged and trailed badly towards the anode. The stained bands in the starch gel does not necessarily indicate the presence of protein hormones but may represent any non-hormone proteins as well. These hormones may be present in amounts not detectable by the stain and may lie anywhere along the gel. Biological activity values of the various sections of the gel should be determined to find out if correlation could be obtained between hormonal activity and the protein bands. Purification of the hormones could be undertaken as soon as the biological activity is located on the gel.

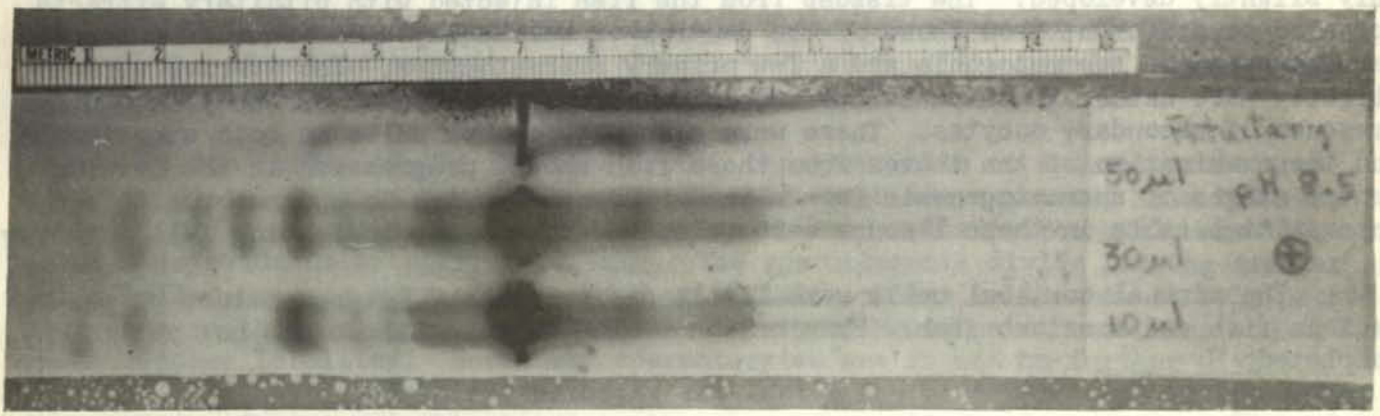


Fig. 1.

SALMON ATTRACTANTS AND REPELLENTS

SUMMARY NO. 25

OLFACTORY PERCEPTION IN SALMON:
CHEMICAL ATTRACTANTS AND REPELLENTS

U.H.M. Fagerlund, J.R. McBride,
M. Smith, N. Tomlinson,
H.S. Sehdev

Response of Great Central Lake and Cultus Lake Sockeye Migrants to Various Waters

This report is a continuation of the work reported in Summary No. 1 of this

Station's Annual Report for 1960-61. The mode of capture of the migrant sockeye and transport of the fish to the Station from the field sites, fish-holding procedures, treatment of disease and experimental bioassay procedures used to determine the responses of the fish towards various waters, were essentially the same in this investigation as those outlined in the 1960-61 report. In the past, fish diagnosed as suffering from furunculosis were discarded. This year, however, it was found that weekly intramuscular injections of 0.5 cc terramycin (25 mg) initiated immediately after capture of the fish and continued for a period of 6 to 10 weeks, not only removed all external symptoms of this disease in infected fish but also prevented the appearance of the disease in healthy fish held in the same tank as the infected ones. The completion of the new fish-holding facilities at this Station allowed the transfer of the project from the Vancouver Public Aquarium. Instead of the experimental 366x91.5x91.5 cm cement tank used in the 1960-61 study, a fibreglas tank of identical size was installed. Also, 1350-litre capacity, self-cleaning, circular fibreglas tanks were used instead of the oblong cement fish-holding tanks described in the previous report. The use of smooth fibreglas in place of rough cement reduced the incidence of injury to the fish during periods of disease treatment, transfer of the fish in and out of tanks and during the acclimatizing period immediately following the transfer of fresh fish to the tanks.

In brief review of the assay procedure, a test water sample injected into the lighted end of the experimental aquarium was said to evoke a positive response when: (1) the school dispersed; (2) at least three of the four fish present left the shaded end of the tank and entered the lighted area of the aquarium; (3) the swimming speed of the fish increased. In some instances this activity took the form of what appeared to be an escape behaviour pattern in that the fish moved up and down one side of the tank with their snouts pressed directly against the side of the tank.

The tests described in the previous report on the responses of Great Central Lake and Cultus Lake migrant sockeye to their respective homestream waters and to waters other than their home streams were relatively few in number. Positive responses were obtained, however, in all cases where known homestream waters were presented to fish migrating to the corresponding area and no detectable response to waters taken from areas other than the known migrant streams. In Table I are given the results of additional tests carried out this year on the responses of Great Central Lake and Cultus Lake migrant sockeye to whole untreated waters. Prior to injection the water to be tested was brought to within ± 4°C of the aquarium water temperature. The amount of water being used in each test was 63 litres and this was injected within a period of approximately 4 minutes into the observation tank which holds 1980 litres.

The Great Central Lake sockeye responded positively to the following waters: (1) to 14 (out of 15) tests with Great Central Lake water; (2) to one test of Forest Camp Creek water; (3) to one (out of two tests) with McBride Creek; (4) to one test with Fawn Creek water; (5) and to one (out of two tests) with Cultus Lake water. With the same fish no responses were observed to: (1) one sample of Great Central Lake water; (2) 11 samples of aquarium water; (3) one sample of Cultus Lake water; (4) one sample of Drinkwater Creek water; (5) and one sample of McBride Creek water. The positive responses of Great Central Lake sockeye to water samples from Forest Camp and McBride Creeks, both of which empty into Great Central Lake, and from Cultus Lakes, were not expected as none of these waters is known to contain or feed any Great Central Lake sockeye spawning sites. The positive response to Cultus Lake water, a water system located on the mainland of British Columbia several hundred miles from Great Central Lake, could possibly have been due to the fine mud suspension present in this particular sample (excitation by muddy water has been noted previously) but not present in any of the other samples. The lack of response to the Drinkwater

Creek samples is interesting in that this creek contains a spawning bed. Reasons for the differences in the results between this year's and last year's testing of water from creeks flowing into Great Central Lake are not clear. Several possibilities can be suggested (e.g. several races of fish might spawn in the system, one race responding only to water from the creek in which it spawns, or fish might respond to water from a particular creek only at a certain period in the spawning cycle), but only further investigation can help to clarify the situation. This year's results with McBride Creek water, of course, cannot be explained by either of the suggested possibilities, but since very few tests of this water were made, an occasional deviation from the expected results, as has been observed in the more extensive series of tests with outlet water from the lake, could have been responsible.

TABLE I - Response of Great Central Lake and Cultus Lake sockeye to various waters.

Fish	Water tested	Response	No response
Great	Great Central Lake	15	1
Central	Forest Camp Creek	1	
Lake	McBride Creek	1	1
Migrants	Drinkwater Creek		1
	Fawn Creek	1*	
	Cultus Lake	1	1
	Aquarium water		11
Cultus Lake	Cultus Lake	2	
Migrants	Aquarium water	1	7
	Great Central Lake		1

* Trace response - considerable activity was noted in the shaded area of the tank and at least one fish entered the lighted area of the tank

The Cultus Lake fish responded positively to two samples of Cultus Lake water, positively to one out of eight samples of aquarium water, and failed to respond to one sample of Great Central Lake water. Thus the only response obtained that was not expected was the single positive response by these fish to aquarium water. To our knowledge no changes in procedure were apparent in this latter experiment that could account for this result.

Bull and his co-workers have demonstrated the ability of certain fish (sockeye were not examined) to detect temperature differences of $\pm 0.5^{\circ}C$. While no temperature difference could be detected in the experimental aquarium immediately following the injection of 72 litres of test water which was $4^{\circ}C$ above that of the aquarium water, the possibility nevertheless existed that local temperature differences could be detected by the fish and their response could be misinterpreted. In Table II are listed the responses of Great Central Lake sockeye to waters in 72-litre test samples which were either chilled or heated so as to vary by more than $5^{\circ}C$ from that of the aquarium water temperatures.

It is evident from the results obtained that waters other than those from the migrant stream when heated or chilled so as to differ from the temperature of the aquarium water can elicit a positive response from Great Central Lake fish.

TABLE II- Reaction of Great Central Lake sockeye migrants to different water temperatures.

Water	Temp Test Water	Temp Aquarium Water	Response	No Response
Aquarium	0°C	14°C	1	-
"	0°C	14°C	1*	-
Great Central Lake	19°C	13.5°C	1	-
Aquarium	19°C	13.5°C	1	-
"	19.5°C	13.5°C	1*	-

* Trace response - as in Table I.

One major difficulty encountered in the attempt to fractionate the sockeye attractant has been the manipulation of large volumes of test waters. In an attempt to concentrate the active component(s) present in the homestream waters, two different procedures were investigated. In the first, nitrogen gas was bubbled through the test water at room temperature for periods of from 3 to 16 hours in an attempt to displace the active component(s) and concentrate them in a suitable trap. The trap consisted of a flash evaporator, the two rotating flasks of which were filled with glass wool and cooled in dry ice acetone baths. The trapped materials were then eluted at 5°C by rinsing the glass wool with aquarium water for subsequent testing. In the second method the test water was allowed to freeze slowly in 4-gallon cans held at -20°C to a point where only a small unfrozen core remained. The unfrozen core, which presumably contained all the substances in solution in the whole water, was removed and the remainder melted at 5°C. As noted in Table III, certain samples were stirred continually and other samples were not stirred during the freezing process. All fractions were made up to the volume of the original whole water with aquarium water prior to testing.

It is apparent from the limited number of results obtained with the Great Central Lake and Cultus Lake waters that attempts to concentrate the active component(s) of these homestream waters by nitrogen displacement have been partially successful. With the Great Central Lake fish, all six Great Central Lake water samples through which nitrogen gas was bubbled, and which were tested separately, failed to evoke any response. Of six corresponding displaced eluates, however, four eluates combined into two pairs evoked a trace response while two tested as a pair evoked a positive response. With the Cultus Lake sockeye (Table IV) a positive response was evoked by the residue of 72 litres of water through which nitrogen gas had been bubbled for three hours. The displaced fraction from the same volume of water through which N_2 had been bubbled for 4 hours gave no response in one test and a trace response in a second test. The recombined samples of Great Central Lake water (i.e., displaced fraction plus residue) evoked positive responses in two tests out of three made with Great Central Lake fish and a positive response in a single test of Cultus Lake water and fish. The data suggest that the active component(s) is removed by bubbling nitrogen gas through the homestream waters but in some instances at least it is either escaping through the trap or is not being completely eluted from the trap. A third possibility, as suggested by the results of tests with combined fractions, is that the attractant(s) consists of two components, one of which is displaced by the nitrogen.

TABLE III - Response of Great Central Lake migrant sockeye to fractions of Great Central Lake (GCL) water.

Water treatment	Fraction	Response	No Response
1. 54 litres GCL water, N_2 bubbled through water for 16 hrs.	B. Residue		1
2. 72 litres GCL water, N_2 bubbled through water for 3 hrs. Process repeated with an additional 16 gal GCL water. Displaced fractions caught in same trap.	A. Displaced fraction	1*	
	B. Residue		2
3. 72 litres GCL water, N_2 bubbled through water for 6 hrs. N_2 bubbled through an additional 72 litres for 3 hrs. Displaced fractions from both lots of water caught in same trap.	A. Displaced fraction.		1
	B. Residue		2
4. Repeat No. 2 above.	A. Displaced fraction	1*	
	B. Residue		2
5. 72 litres GCL water, N_2 bubbled through water for 6 hrs.	Fractions combined	1	
6. 72 litres GCL water, N_2 bubbled through water for 5 hrs.	do	1	
7. 72 litres GCL water, N_2 bubbled through water for 6 hrs.	do		1
8. 72 litres GCL water frozen without stirring to yield 18 litres water plus ice.	A. Ice		1
	B. Unfrozen water	1*	
9. 72 litres GCL water frozen without stirring to yield 12 litres water plus ice.	A. Ice		1
	B. Unfrozen water		1
10. 144 litres GCL water frozen without stirring to yield 36 litres water plus ice.	A. Ice	1	
	B. Unfrozen water	1	
11. As in No. 10.	A. Ice	1	
	B. Unfrozen water	1	
12. 144 litres GCL water frozen with stirring to yield 72 litres water plus ice.	A. Ice	1*	
	B. Unfrozen water	1	
13. 72 litres GCL water frozen with stirring to yield 9 litres water plus ice.	A. Ice	1*	
	B. Unfrozen water	1	
14. 180 litres GCL water frozen without stirring to yield 27 litres water plus ice. Ice discarded, water divided into 2 samples as follows: A. 18 litres; B. 9 litres.	A. 18 litres unfrozen water	1	
	B. 9 litres unfrozen water	1	

* Trace response - see Table I.

TABLE IV - Responses of Cultus Lake sockeye to fractions of Cultus Lake water.

Water treatment	Fraction	Response	No Response
(1) 72 litres Cultus Lake water, N_2 bubbled through water for 5 hrs.	Fractions combined	1	
(2) 72 litres Cultus Lake water, N_2 bubbled through water for 3 hrs.	Residue	1	
(3) 72 litres Cultus Lake water, N_2 bubbled through water for 4 hrs.	Displaced fraction		1
(4) 72 litres Cultus Lake water, N_2 bubbled through water for 4 hrs.	Displaced fraction	1*	

* Trace response - see Table I.

Attempts to concentrate the active substance(s) present in Great Central Lake water by a freezing-out technique were also partially successful. In one test (No. 8) no response was obtained with the frozen fraction but only a trace response was evoked by the unfrozen portion of the whole water. Of tests No. 10 to 14 inclusive, five positive responses and one trace response were obtained from the unfrozen fractions, and two positive and two trace responses from the corresponding frozen fractions. The remaining test, Sample No. 9, did not evoke a positive response to either the frozen or the unfrozen fraction. Stirring the water samples during freezing did have some apparent effect in that the unfrozen portion of Samples Nos. 12 and 13 were positive while the corresponding frozen fractions showed only trace activity. The freezing-out technique is partially effective in concentrating the salmon attractant, but the degree of concentration is not established through the experiments. Stirring of the freezing water samples improves the concentration.

Speculation as to the source of the active component(s) in Great Central Lake water led to the consideration of the flora in this area. Hasler has reported that salmon fry can be readily trained not only to recognize but also to differentiate between odours of plants. As a preliminary step, a few of the plants common to the creeks emptying into Great Central Lake were taken, with due care to avoid crushing, from the banks of these creeks or from islands in the creeks and placed in individual plastic bags. The plants were cleaned of all debris and then each individual species of plant was placed in a large volume of aquarium water and allowed to soak for two hours at 5°C. The sample was then filtered through cheese-cloth, the filtrate diluted to 36 litres with aquarium water for subsequent testing and the plant residue discarded. The responses from the Great Central Lake sockeye to these aqueous plant extracts are listed in Table V.

TABLE V - Response of Great Central Lake fish to aqueous extracts of various plants.

Plant	Response	No Response
Maiden hair fern	-	1
Aquilegia formosa fisher	-	1
Algae	-	1
Currant	-	1

None of the plant extracts tested evoked a response from the Great Central Lake fish. It should be noted, however, that the plant species tested would in all probability represent only a fraction of the total number present.

Cultus Lake fish used in the experiments were in all cases at least four weeks from sexual maturity and Great Central Lake fish eight to ten weeks from spawning. When sexually mature but unspawned fish were taken directly from the spawning beds of Weaver Creek, a system that empties into Harrison Lake, the behaviour pattern of these fish in the test system differed from that of the sexually immature fish. The sexually mature fish were extremely excitable, the slightest noise or movement resulted in sudden spurts of movement in all directions in the tank. These sexually mature fish showed no inhibition to light and seldom remained stationary in one area for more than 20 minutes. The fish were found to be unsatisfactory as a test animal in this study.

Summary

Further evidence in support of the homestream theory for migrating sockeye (Oncorhynchus nerka) has been presented. The effect of temperature differences between the aquarium water and the test water on the response of adult migrant sockeye are described. Attempts to concentrate the active component(s) present in the homestream waters by partial freezing and nitrogen gas displacement has given encouraging results.

Acknowledgments

The authors wish to thank Dr. J.R. Brett, Nanaimo Biological Station of the Fisheries Research Board of Canada, Mr. R. MacLaren and Mr. F. Boyd of the Federal Department of Fisheries, and members of the International Pacific Salmon Fisheries Commission for their assistance in trapping fish used in this investigation.

SUMMARY NO. 26

OLFACTORY PERCEPTION IN SALMON:
CHEMICAL ATTRACTANTS AND REPELLENTS

J.R. McBride, U.H.M. Fagerlund,
N. Tomlinson, M. Smith,
H.S. Sehdev

Response of Juvenile Sockeye Salmon to Extracts of Foods

A report on the initial stages of this project was included in Summary No. 2 of this Station's Annual Report for 1960-61. The following is a comprehensive report of the experimental procedures and the results to date.

Methods and Materials

Sockeye smolts (O. nerka) were obtained during March, 1961 from traps located at the outlet of Great Central Lake. This water system, located in the Alberni area of Vancouver Island, B.C., supports a well-established population of sockeye.

The transport tank used to transfer the fish from the field site to the Vancouver Station was an 1800-litre capacity circular insulated aluminum tank, 4 ft in diameter and 4 ft in height. Continuous water circulation was maintained during transport at the rate of 14 litres/min through the use of a bilge pump, and in addition the water was aerated with oxygen supplied by a pressure cylinder. The temperature of the water in the transport tank increased from 7°C at the field site to 9°C upon arrival at Vancouver. The fish were held in fibreglas holding tanks supplied with a continual flow of water. The water inlets in these tanks were so placed as to create a slow current. All water used was dechlorinated. To minimize the possibility of the fish becoming conditioned to light, all holding tanks were covered with black plastic covers.

The smolts were maintained in good health on a diet of Clark's fish pellets (J.R. Clark Co., Salt Lake City, Utah), supplemented twice weekly with a meat ration consisting of 55% canned salmon, 20% beef liver, 20% beef heart, 5% pablum and a tablespoonful of iodized salt for every 5 kg of feed. The fork lengths of the fish varied between 115 mm and 147 mm.

Experiments were carried out in an area from which extraneous noise, light and movement were eliminated. The bioassay method used to assess the attractiveness of various aqueous extracts of foods and commercial bait oils for juvenile sockeye were patterned closely after that of Steven. One modification of Steven's system was made, however, in order to take advantage of the photonegative behaviour of sockeye smolts. The end quarter of each aquarium opposite to the water inlet was darkened by a black plastic sheet which extended across the top and down the side of each tank. The remaining 3/4 of the tank was illuminated by a single 300-watt photo flood lamp placed 45 cm above the exposed end of the tank. Only on very rare occasions apart from experimentation periods were sockeye observed in the lighted area of the tanks. The serum bottles used to hold the test or control solutions were fitted with fine bore stopcocks and the system arranged to deliver between 80 and 85 ml to each aquarium through the water supply inlet in 60 sec. All extracts tested were introduced into aquaria in 250-ml aliquots. The bioassay system was checked continually during test periods to insure minimum variations in illumination, water flow and sample delivery rates between the two tanks.

Tests with potassium permanganate showed that the time interval for distribution of the compound throughout the whole of each tank varied from 2 min and 14 sec to 2 min and 55 sec from the time of injection. It required between 4 hr and 20 min and 4 hr and 45 min to clear all visible traces of permanganate from each tank. The variation in timing reflects the small changes which occur in the water delivery rates. No back flow into the sample bottles was noted during the distribution tests.

The portion of the room containing the two experimental tanks was partitioned off with black plastic curtain extending from the floor to the ceiling and running in a semi-circle around each aquarium. A peephole large enough to accommodate a camera lens was cut into the curtain at a point directly opposite the centre of each tank. The distance from the centre of the outer glass side of the tank to the camera was 2 metres. The curtain enabled the investigator to record the responses of the fish with a 16 mm ciné camera without being seen by them. One additional peephole was provided for each tank by mounting a 10 cm square piece of transparent mirror in

the end plywood partition 25 cm above the water level of each tank.

The aqueous extracts of the various foods tested were prepared by grinding samples and then suspending weighed amounts in a known volume of distilled water. The suspension was thoroughly mixed for 10 to 20 min and then filtered through Whatman No. 1 filter paper. Preparation and storage of extracts before testing was carried out at 5°C. All the aqueous extracts tested were colourless and only the squid extracts had any detectable odour to the experimenter. Certain commercial bait oils also tested were dispersed at the desired concentration in distilled water.

The responses of the fish to the aqueous extracts of the food were studied by introducing 250-ml aliquots of each sample into one aquarium via the water supply while an equal amount of distilled water was injected by the same manner into the other aquarium. As the fish are congregated behind the black plastic curtain at the start of the test, any change in behaviour leading to their movement into the open was readily observed. The behaviour pattern of the fish was watched for 10 min before and 20 min after the completion of each injection. If a response was obtained, it usually occurred 2 to 4 min after the start of the injection. A 24-hr period was allowed between successive tests. Each of the serum bottles was washed exhaustively with dechlorinated water before the next test. All extracts were tested in duplicate, the school in each tank being used in turn as control for the other. All extracts evoking a positive response were tested several times with different schools of sockeye. The tests were carried out either in the early forenoon or early afternoon before the fish received their daily feeding.

A sample was said to elicit a positive response when (1) the school dispersed; (2) the fish left the shaded area and entered the lighted portion of the tank; (3) the swimming speed of the fish increased. In certain instances the fish sampled objects on the bottom of the tank or broke through the surface of the water in chase of some small object floating on the water surface. If the response obtained showed only the first three of these criteria, the behaviour was labelled as "exploratory", but if a distinct feeding activity was observed as evidenced by the picking up of objects from either the top or bottom of the tank, the behaviour of the fish was termed "feeding" behaviour. In every case where feeding behaviour was evoked, an exploratory response was also evident. A trace response was characterized by the appearance of from 3 to 6 fish in the lighted area of the tank.

Pilot experiments indicated that a group of from 6 to 12 fish in each aquarium was an adequate number for visual observation and to provide a consistent response. Larger numbers of fish in each tank tended to produce an overcrowding effect and at times made the interpretation of behaviour questionable. In view of these considerations, 10 fish were held in each tank during testing periods. To minimize conditioned responses, schools were discarded and replaced with fresh ones after each series of experiments, or at the end of 3 weeks, whichever came first. To determine the effect of the introduction of test samples on the temperature and pH of the aquaria water, 250-ml aliquots of distilled water, 1% zooplankton, and 1% brine shrimp aqueous extracts were introduced into the aquaria in the usual manner. No apparent differences in the pH or temperature of the aquaria water were detected immediately following the completion of the introduction of these samples.

Results

Presently available information indicates that adult sockeye are plankton feeders in their natural habitat. As zooplankton is difficult to obtain in uncontaminated form and as the supply is uncertain during the winter months, several

natural foods in addition to zooplankton were tested initially to determine their ability to evoke exploratory and feeding behaviour in the juvenile sockeye. The extracts tested and the responses obtained are shown in Table I.

TABLE I - Response of juvenile sockeye to aqueous extracts of several natural products.

Natural product	Concentration of aqueous extract g %	Number of tests	Response	
			Exploratory	Feeding
Beef liver	1.0	2	2	2
Beef liver	0.1	2	2	2
Beef heart	1.0	4	4	4
Beef heart	0.1	2	2	2
Zooplankton	1.0	6	-	-
Zooplankton	0.1	4	-	-
Brine shrimp	1.0	4	-	-
Brine shrimp	0.1	2	-	-
B.C. shrimp	1.0	2	-	-
B.C. shrimp	0.1	3	-	-
Squid	1.0	4	-	-
Squid	0.1	2	-	-

It is apparent that exploratory and feeding behaviour were evoked only by aqueous extracts of foods to which the fish had presumably been conditioned by eating, in this case beef liver and beef heart. These fish had not eaten the substances to which they failed to respond of which two, zooplankton and squid, are known major constituents of the diet of adult sockeye. Brine shrimp are not known to exist off the B.C. coast or to contribute to the diet of sockeye homing in this area, but are abundant along the California coast and conceivably could constitute part of the diet of those races of sockeye frequenting the latter area. Little is known of the diet of wild, juvenile sockeye, but evidence has been presented that insects and lake plankton form a significant portion of their diet. As it appeared that the smolts were responding only to extracts of those foods to which they had been conditioned by previous eating, an attempt was made to condition groups of smolts to zooplankton or to brine shrimp. Conditioning was carried out by adding small amounts of the substance to the meat supplement for one week followed by daily dispersal of small amounts of the food on the water surface of the tank. It was found that the fish usually became conditioned by the middle of the second week of feeding.

The responses of conditioned fish to serial dilutions of zooplankton and brine shrimp aqueous extracts are given in Table II and III.

It is apparent that once the fish had been conditioned by eating either brine shrimp or zooplankton, aqueous extracts of these organisms evoked both exploratory and feeding responses in the appropriately conditioned fish. Although not recorded in the tables, it was found that fish conditioned to zooplankton but not to brine shrimp did not respond to the extract of the latter, and vice versa.

TABLE II - Response of conditioned juvenile sockeye to aqueous extracts of brine shrimp.

Concentration g %	Number of tests	Response	
		Exploratory	Feeding
1.0	2	2	2
0.1	2	2	2
0.01	2	2	2
0.005	2	2	2
0.0025	2	2	2
0.001	4	4	4
0.0004	2	2	-
0.0002	2	2	-
0.00013	4	4	-
0.00010	2	-	-

TABLE III - Response of juvenile sockeye conditioned to whole zooplankton to aqueous extracts of zooplankton.

Concentration g %	Number of tests	Response	
		Exploratory	Feeding
1.0	2	2	2
0.1	6	6	6
0.01	2	2	2
0.001	6	6	6
0.0004	4	4	-
0.0001*	4	4	-

* Trace response obtained in 3 out of 4 tests.

Numerous commercial bait attractants and countless home recipes for attractants are readily available to the sport fisherman. Generally, the attractant is an oil or a mixture with an oil base and is used as a bait dip. While few of the commercial attractants are promoted as attractants for specific fish, several have been advanced as attractants for a wide variety of fish including trout and salmon. As considerable support exists for these commercial and home-prepared bait attractants, several were selected to determine their effect on the behaviour of juvenile sockeye. For testing, the oils were suspended in 250 ml distilled water and an equal volume of distilled water was used for the control.

Of the five commercial bait oils tested (Table IV), only menhaden oil produced an apparent change in the behaviour of the fish. The response to this oil while consistent at both concentrations tested, was weak in that not all the fish responded. It was characterized by a breaking away of 3 to 4 fish from the school and by rapid spurts of swimming for short periods of time.

Some of the physical characteristics of the attractant present in zooplankton have been elucidated (Table V). A zooplankton extract which was passed through both anionic and cationic resins retained its activity. Tap water that was passed through the same resins had no activity. Heating of a 1-gram % extract at 99°C for 30 minutes did not destroy the activity. When a 1-gram % extract was distilled on a hot plate at atmospheric pressure to leave only 1-2 ml of solution in the distillation

flask, the distillate was active. The distillation residue when made up to appropriate volume with distilled water was also active. The possibility that the activity in both distillate and distillation residue is caused by incomplete distillation of the active volatile component has as yet not been investigated. An alternative explanation, however, is conceivable, namely that more than one compound is able to evoke a response in the conditioned fish is present in the zooplankton extract. The same phenomenon was observed when 250 ml of a 0.1-gram % zooplankton extract was lyophilized to dryness in a flash evaporator at 25-30°C, while the receiver was kept in a dry-ice acetone bath. In three preparations of this type, three responses were evoked with the volatile fraction and two responses evoked with the non-volatile fraction, when each fraction was made up to 250 ml with tap water before being tested.

TABLE IV - Response of juvenile sockeye to commercial bait oils.

Oil	Concentration g %	Number of tests	Response Exploratory	Feeding
Cumin	0.04	2	-	-
"	0.40	2	-	-
Fish liver oil	0.04	2	-	-
" " "	0.40	2	-	-
Patchouli	0.04	2	-	-
"	0.40	2	-	-
Anise	0.04	2	-	-
"	0.40	2	-	-
Menhaden	0.04	2	2	-
"	0.40	2	2	-

TABLE V - Response of conditioned juvenile sockeye to zooplankton extracts which had undergone various treatments.

Treatment	Number of tests	Response
Passing through IR120 and IR 400 resins	1	1
Heating at 99°C for 30 min	2	2
Distillation		
Distillate	2	2*
Residue	2	2
Lyophilization		
Volatile	3	3
Non-volatile	3	2*

* One trace response included in this number.

To examine the possibility that sensory mechanisms other than the olfactory were involved in perception of the test substances, a group of ten conditioned sockeye, which had responded positively to duplicate tests with 0.1% and 0.01% zooplankton aqueous extracts, were anaesthetized with the methane sulphonic acid salt of ethyl m-aminobenzoate(MS222) at a concentration of 1 part in 20,000 and their nasal sacs were plugged with cotton soaked in vaseline. Each sac was then closed with a double suture. At the end of 48 hours 4 of the fish had died, the 6 remaining

fish, all normal in external appearance, were eating at the normal rate. During the next 24 hours these fish were given two feedings of whole zooplankton and then transferred to the glass experimental aquarium where they were left undisturbed except for feeding for an additional 24 hours to enable them to become accustomed to their new surroundings. Duplicate tests carried out over the succeeding 48 hours using 250-ml samples of 0.1% zooplankton aqueous extracts failed to evoke any response. The 6 fish were then transferred to a holding tank, anaesthetized as before, and the nasal plugs and sutures removed. Once again the fish were transferred to the glass experimental aquarium and following a 72-hour period during which they were fed in the normal manner, they were retested for their response to duplicate tests with 250-ml samples of 0.1% zooplankton aqueous extracts. A positive response was obtained in both tests.

During the course of this study sockeye smolts were obtained that had been reared at the salmon hatchery at Hoodsport, Wash. After conditioning to zooplankton the fish were placed in the observation tanks. It was then noted that these fish would not retreat to the darkened area behind the curtain for any length of time but would rather disperse themselves throughout the tank. In order to establish whether this was a persistent behaviour pattern, four observation tanks were supplied with ten smolts each (Table VI), two tanks with Hoodsport fish and two tanks with Great Central Lake "wild" fish. The fish were left undisturbed except for a feeding between 8:30 a.m. and 9:30 a.m. daily. After the fish had been allowed to settle down for 48 hours, the two tanks were observed two or three times a day for six days. During the observation period the hatchery-reared fish were predominantly found dispersed throughout the tank while the "wild" fish stayed in the darkened area behind the curtain.

The same experiment was repeated with sockeye smolts reared at the Biological Station in Nanaimo from eggs obtained from the salmon hatchery at Babine Lake. These fish, too, were found to show no preference for the dark area of the tank. It can be concluded from the results that fish reared in captivity, in contrast to "wild" fish, do not avoid light.

Discussion

Steven reported that both silversides (Hepsitia stipes) and tomtates (Bathystoma rimator) displayed exploratory feeding when exposed to dilute seawater extracts of a number of natural food substances. Some of these natural food substances such as plankton, formed part of the diet of the fish before capture, and others, such as beef liver and clam, were fed to them during captivity. Beef muscle and cod muscle, however, were not fed and would not form part of their diet in nature, yet dilute extracts of these muscles evoked exploratory responses.

The sockeye smolts with which we have worked did not respond to any of the food extracts tested except when they had previously eaten the foods, but they did show some response to menhaden oil, although this oil had not formed part of their diet. Other fish oils and meals (but not liver oils) were contained in the pellets with which they were fed. It is clear that in the present work the fish became conditioned to some component(s) of their food but there is evidence that they may be attracted by substances in foods that they have not previously ingested. This appears also in some of Steven's and Tester's results. The reason for this response is not known but perhaps some cross-conditioning between different foods may occur, particularly when relatively high concentrations of extracts are used.

TABLE VI - Response of "wild" and hatchery-reared smolts to light. Number of fish observed outside shaded area.[1]

Observation date	Time of day	Hoodsport fish Tank 1	Gt. Central Lake fish Tank 2	Gt. Central Lake fish Tank 3	Hoodsport fish Tank 4
Jan 29	4:00 pm	4	0	2	0
	5:30 pm	3	0	3	0
Jan 30	8:00 am	10	0	2	10
	12:45 pm	10	0	6	0
	3:00 pm	10	0	5	9
Feb 1	8:00 am	10	0	1	10
	1:00 pm	5	0	0	4
	3:30 pm	5	0	2	10
Feb 2	8:00 am	10	0	1	10
	3:00 pm	10	0	2	4
Feb 3	8:00 am	6	0	3	7
	1:00 pm	5	0	2	8
Feb 4	8:00 am	8	1	3	8
	1:00 pm	5	0	2	0

Observation date	Time of day	Nanaimo fish Tank 5	Nanaimo fish Tank 6
Feb 9	8:00 am	8	6
	12:00 pm	10	10
	1:00 pm	4	9
	4:00 pm	8	10
Feb 10	8:00 am	10	7
	11:00 am	8	8
	2:00 pm	10	8
Feb 11	8:00 am	10	9
	11:00 am	10	9
	2:00 pm	10	8

[1] Ten fish in each tank.

It appears that sockeye, like other fish, have a very keen sense of smell, as evidenced by their ready response to the introduction into a 200-litre aquarium of an extract equivalent to 2.5 mg wet weight of either brine shrimp or zooplankton.

Acknowledgments

The authors wish to thank Dr. H.S. Hoar, Department of Zoology, University of British Columbia, for his helpful suggestions throughout the course of this investigation; Dr. B. McK. Bary, Department of Zoology, University of British Columbia for kindly supplying zooplankton; Dr. J.R. Brett, Biological Station, Nanaimo, Mr. R. McLaren

and Mr. F. Boyd of the Department of Fisheries and members of the International Pacific Salmon Fisheries Commission for their assistance in trapping fish used in this study, and Dr. J.R. Brett, Nanaimo, and Mr. C.H. Ellis and Mr. Richard Noble, Washington State Department of Fisheries, Seattle, for supplying hatchery-reared fish.

PROTEINS OF SALMON MUSCLE

SUMMARY NO. 27

AN INVESTIGATION OF MUSCLE PROTEIN AND OTHER SUBSTANCES SOLUBLE IN SALT SOLUTIONS OF LOW IONIC STRENGTH BY COLUMN CHROMATOGRAPHY

H. Tsuyuki
E. Roberts
R.E.A. Gadd

A series of investigations was initiated the previous year (see Summary No. 29 of this Station's Annual Report for 1960-61) for the purpose of obtaining some fundamental information on the muscle protein framework of fishes commercially important here on the west coast. These studies have so far been confined to that portion of the muscle proteins soluble in low ionic strength salt solutions. As these investigations represent a new series of studies the subject matter is introduced in somewhat more detail.

The literature dealing with the mammalian sarcoplasmic protein fraction, comprising some 20-30 percent of the total muscle protein, has been the subject of numerous reviews (Czok, 1960; Dyer and Dingle, 1960; Perry, 1956, 1960). This protein fraction is characterised by its solubility in water or salt solutions of low ionic strength and has been referred to collectively as 'myogen' by Weber in 1933. Two-thirds of this myogen have been reported to be comprised of crystallizable enzyme proteins and indeed as many as 50 have been described (Czok, 1960; Engel' Hardt, 1941). Rabbit muscle has been the chief target for most of these studies. As many as 11 distinguishable components have been described from the myogen fraction of mammalian sources by means of moving boundary electrophoresis. Some attempts have been made to correlate the electrophoretic components with purified enzymes with some degree of success.

Somewhat similar approaches to the study of fish myogens have been carried out by several groups of investigators. A total of about 25 different fishes (Connell, 1953; Nikkila and Linko, 1955) have been surveyed by electrophoretic means. Hamoir and Henrotte have studied the carp muscle proteins in some detail and Dingle et al, the Atlantic cod and the sturgeon by electrophoretic methods. Preliminary studies on the separation of the myogen from one of the Pacific salmon species by the use of DEAE cellulose columns have been reported by Tsuyuki and Roberts.

In the present study the use of column chromatography to investigate the myogens and other components was extended to cover four of the five Pacific salmon species as well as other fishes.

Methods

Sampling of Fish

In most instances fillets from freshly-killed salmon were used. When live salmon were not readily available those held in ice 1-2 hours after death were used.

On occasions muscle tissue frozen at -30°C for various periods of time was used, as freezing has been shown to have little effect on the myogen fraction.

Adult cohoes and spring salmon were captured alive by commercial trolling in the Howe Sound area and held in salt water tanks at the Vancouver Public Aquarium. As samples were required the fish were anaesthetized with tricainemethanesulfonate (MS-222), transported to the laboratory, and killed. Adult sockeye salmon were obtained fresh frozen in dry ice from Port Renfrew, B.C. and also live from Great Central Lake, B.C. and held in this Station's fresh water aquarium facilities until used. Chum salmon were obtained from reef-net operations at Lummi Island*. Lingcod were captured by drag-net operation and held live at the Vancouver Public Aquarium.

Preparation of Muscle Protein Extract

Freshly-killed fish were filletted and sliced into 100 gm samples and those not used immediately were wrapped in polyethylene bags and frozen at -30°C. The frozen fish were sliced into approximately 100 gm pieces and the muscle tissue removed while still frozen.

The protein extracts used for column chromatography were prepared by chopping the muscle tissues into small pieces and homogenizing in a Waring blendor with 7 volumes of an 0.05 ionic strength phosphate buffer of pH 7.5 by a procedure previously described (Tsuyuki and Roberts, 1961). All operations were carried out at 0°C and all glassware was pre-chilled. A blendor jar of one-litre capacity was loosely fitted with a 1/16" plexiglass baffle plate extending just below the surface of the liquid to eliminate the fortex (Dyer et al, 1950). The baffle plate was attached to a 1/2" plexiglass rod which extended through a snugly-fitting one-hole rubber stopper attached to a hole in the centre of the jar lid. The depth of the baffle plate could thus be adjusted to any volume of liquid by sliding the rod up and down through the rubber stopper.

Column Chromatography

DEAE cellulose type 20, of 0.85 meq/g capacity (Carl Schleicher and Schuell Co., Keene, New Hampshire) was washed 8 times with distilled water, decanting the fine particles after each washing. This procedure was repeated 3 more times with 0.001 M tris (tris(hydroxymethyl)aminomethane). It was possible to remove the fine particles by this washing procedure and to avoid plugging the column. The DEAE-cellulose prepared in this way was packed under 3.5 lbs air pressure into a 1.4 cm inside diameter jacketed column to heights of 40, 60, and 90 cms. On a dry weight basis, 8.7, 13.0 and 19.5 gm of DEAE-cellulose were required to prepare these three columns. The protein extracts were dialyzed 24 hours against several changes of 0.001 M tris, pH 8.5. A total of 4 litres of buffer was used. The dialysate was clarified by centrifugation at 30,900 x g and between 300-575 mg of protein in 100 ml of the original extract were put on the column. A concave gradient elution of the type used by Pontis and Blumson (1958) was applied to deliver a solution of increasing KCl and tris concentration. Two nearly parallel-sided vessels connected across their bases by an 0.07" polyethylene tubing were selected to obtain a suitable concave gradient. The reservoir consisted of a 250-ml polypropylene graduated cylinder of 3.3 cm inside diameter. The mixing chamber consisted of a 2.0-litre aspirator bottle of approximately 12.9 cm inside diameter. The heights of the menisci of the two liquids of different density were arranged according to the method of Bock and Nan-Sing Ling (1954). Thus 950 mls

---

* Arrangements were made through the co-operation of Mr. Gerry Anderson, representative of the reef-net fishermen at Lummi Island, Washington.

of 0.001 M tris pH 8.5 in the mixing chamber and 62 mls of a mixture of 2.5 M KCl and 0.1 M tris of pH 8.5 were found to give a satisfactory gradient for these two vessels. In later columns the volumes were increased to 1250 ml in the mixing chamber and to 83 ml in the reservoir to delay the rapid increase in the KCl concentration near the end of the elution process. The theoretical gradient elution curves for these two sets of liquid volumes were calculated. Of the many different salt concentrations and gradients used, these two offered the best resolution of the components in the muscle extracts. For the total volume of eluants used, either the 40 or the 60-cm columns were the most effective. The 90-cm column produced a poorer resolution under these conditions. A correspondingly larger volume of total eluants should be used for longer columns to effect greater resolution. Otherwise the earlier components by virtue of the longer path of movement will not be eluted before the later components, which elute with higher KCl concentration, begin to mask the earlier ones.

Most of the proteins were not retained on the column at pH 7.5, while considerable precipitation was encountered at pH 5.0. At pH 8.5 all the proteins were retained on the column and over 90% of the total protein was invariably recovered from the columns.

Column chromatography was conducted at 4°C and 5 ml fractions were collected by a GME Model 10 fraction collector equipped with a 280 mμ interference filter for proteins and a 265 mμ filter for nucleotides.

Diethylaminoethyl (DEAE) Sephadex A-50 (medium), obtained from Pharmacia, Uppsala, Sweden, and DEAE-cellulose columns were used under the same conditions.

Paper Electrophoresis

The nucleotides were identified by electrophoresis on Whatman #3 paper in a buffer system described by Crestfield and Allen which consisted of 0.1 M ammonium formate at pH 3.5. A potential gradient of 30 volts/cm was applied for 90 minutes.

Fractions from the DEAE-cellulose column showing ultraviolet absorption maximum at 248 mμ characteristic of hypoxanthine derivatives were lyophilized to a small volume and the proteins precipitated with acid. The hypoxanthine derivatives were separated from the KCl and tris which were used for eluting the DEAE cellulose column by passage through Amberlite IR-120 and identified with appropriate reference standards by paper electrophoresis. The ultraviolet absorbing areas were located in a Chromato-Vue cabinet (manufactured by Ultra-Violet Products, Inc., San Gabriel, California).

Paper Chromatography

Nucleotides and nucleosides were hydrolyzed to their free bases by heating for 2 hours at 175°C with 90% formic acid. The bases were then identified by descending chromatography on Whatman #1 paper for 18 hours in water-saturated n-butanol containing ammonia in the bottom of the chromatographic tank. To confirm the results from the first chromatogram, the samples were also chromatographed by ascending development for 6 hours in water at pH 10.0.

Protein Determinations

The protein concentration of the muscle extract was measured by the method of Snow (1950). For analysis of protein fractions from the columns, the more sensitive method of Lowry et al (1951) was employed. The values from the two methods were

standardized against micro-Kjeldahl values obtained for crystalline bovine plasma albumin. All readings were determined with the Beckman DU instrument.

Results

The Use of pH Gradients in DEAE Cellulose Columns

Attempts to improve the separation of fish myogens by DEAE cellulose column by using a suitable pH gradient rather than a salt gradient proved unsuccessful. Experiments were carried out by introducing the protein extract into the column at pH 8.5 and then dropping the pH to 7.5 by a suitable gradient. Other experiments were also carried out by dropping the pH to 7.0. No attempts were made to lower the pH much below 7.0, as the proteins were not retained on the DEAE-cellulose below this value. A more extensive investigation of conditions for pH and gradient control are necessary before a full evaluation can be made of the effectiveness of pH gradient elutions in the separation of fish muscle proteins.

DEAE-Sephadex A-50 Column Chromatography

Attempts were made using DEAE-sephadex A-50 in columns of 1.4 x 40 cms to separate sockeye salmon muscle proteins. The columns were eluted under the conditions of the smaller total volumes referred to earlier in the methods section. Under these conditions the proteins were eluted in one large peak. No further protein was eluted by flushing the column directly with a mixture of 2 M KCl and 0.1 M tris. A possible explanation may be that DEAE-sephadex A-50, which is suitable for the separation of proteins of molecular weight around 35,000, has an exchange capacity approximately 5-fold greater than DEAE-cellulose per gram of exchanger. An exchanger of such high capacity will have a tendency to retain the proteins more tenaciously and may not be suitable for the separation of fish myogens whose physical characteristics appear to be too closely related. Further experiments are in progress in an attempt to select conditions suitable for the use of this exchanger.

DEAE-Cellulose Column Separations of Fish Muscle Myogens and Other Components

The myogen fraction from spring salmon separated by DEAE-cellulose column is shown in Fig. 1. The presence of a number of protein fractions as well as large amounts of non-protein components is evident. The ultra-violet absorption of the non-protein fraction A is almost completely free of protein, while that of B is a composite of both the protein and the non-protein fraction. Upon further examination fractions A and B showed maximum absorption in the 248-250 mμ region in 0.01 N HCl, characteristic of hypoxanthine derivatives. Formic acid hydrolysis followed by paper chromatography showed that hypoxanthine was the base involved in both fractions. No other bases were present in detectable quantities. Paper electrophoresis and chromatography showed that the unhydrolyzed fractions A and B were inosine and inosinic acid, respectively. Other minor ultraviolet absorbing non-protein components were not investigated any further. These two hypoxanthine derivatives also served as natural internal markers for the comparison of protein fractions from other fishes. The two hypoxanthine derivatives have been shown to be only very slowly dialyzable under the conditions of preparing the protein extracts.

When protein fractionation of a whole extract is followed by ultraviolet absorption at 280 mu, false protein peaks are observed, chiefly due to the contribution to absorption displayed by nucleosides and nucleotides. The results of any method used for following protein fractionation that is non-specific, such as the change in refractive indices used commonly in the moving boundary electrophoresis or ultraviolet absorption, must be re-interpreted where other non-protein components

or non-protein ultraviolet absorbing components are present.

The spring salmon myogen fraction (Fig. 1) has been arbitrarily divided into seven components for the purpose of comparison with other fishes. Each of these components is probably a mixture of several different proteins which are too closely related in their physical characteristics to be separated under the conditions of the column used. A more sensitive method is required to evaluate the mixtures fully. Components II to VII are not completely separated.

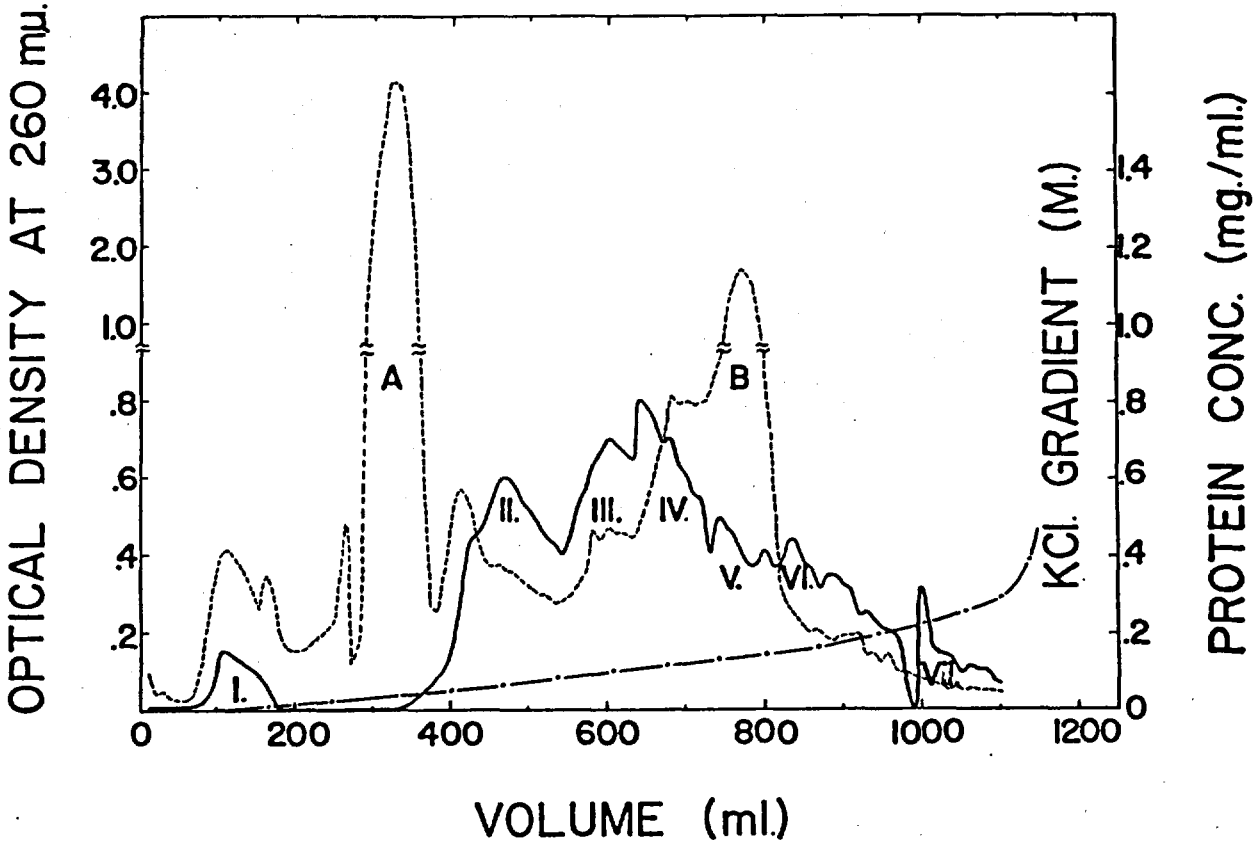


Fig. 1. DEAE-cellulose column separation of muscle myogen and other non-protein constituents from spring salmon. The column size was 1.4x60 cm containing 13.0 gm dry weight of DEAE-cellulose. The broken line represents ultraviolet absorption at 260 mμ; the solid line, protein concentration; the dotted line, potassium chloride gradient, which is the same in Fig. 1 to 5. Components designated A and B refer to inosine and inosinic acid, respectively. The Roman numerals indicate the various protein fractions.

The myogen patterns of chum (Fig. 2), sockeye (Fig. 3), coho (Fig. 4) and lingcod (Fig. 5) show considerable differences. The coho myogen fraction is not readily comparable since the absorption values at 260 mμ and hence the internal markers, are not available. In the other samples studied the inosine and inosinic acid have been characterized and appear in varying concentration ratios to each other, depending on the physiological condition of the fish prior to the preparation of the protein extract. The various protein fractions have been divided into seven components for comparison with the spring salmon and based on the inosine and

inosinic acid internal markers as well as on the actual separation achieved by the column. Fraction I clearly represents the same group of proteins in every instance. The quantity, however, varies considerably between fishes. Of the salmon species, the spring and coho differ from the chum and sockeye in having a relatively small percentage of their total proteins in this fraction. Fractions corresponding to II to VI in the spring salmon appear to be present in varying amounts in all the salmon species studied. An exception is evident in the case of coho where a fraction corresponding to II is not present but another large fraction appears which does not correspond to any of the protein fractions in the other species. The position of this fraction in the column pattern appears to be directly under that of inosine, which is not recorded in this particular case. The small fraction VII does not appear in the other salmon species as clearly defined as in the spring.

The overall pattern of the lingcod myogens are qualitatively different from the salmon species. Fractions I, III, V, VI and VII appear to be similar on the basis of their markers. However, the position of the markers themselves are also quite different from the salmon. The fractionation of the lingcod myogens is the most clearly defined of any studied so far. The components of the myogen fraction in this case show physical characteristics that are not as closely related as in the salmon species. In the lingcod, additional fractions designated Ia and Ib appear which are totally absent in the salmon species except for the coho.

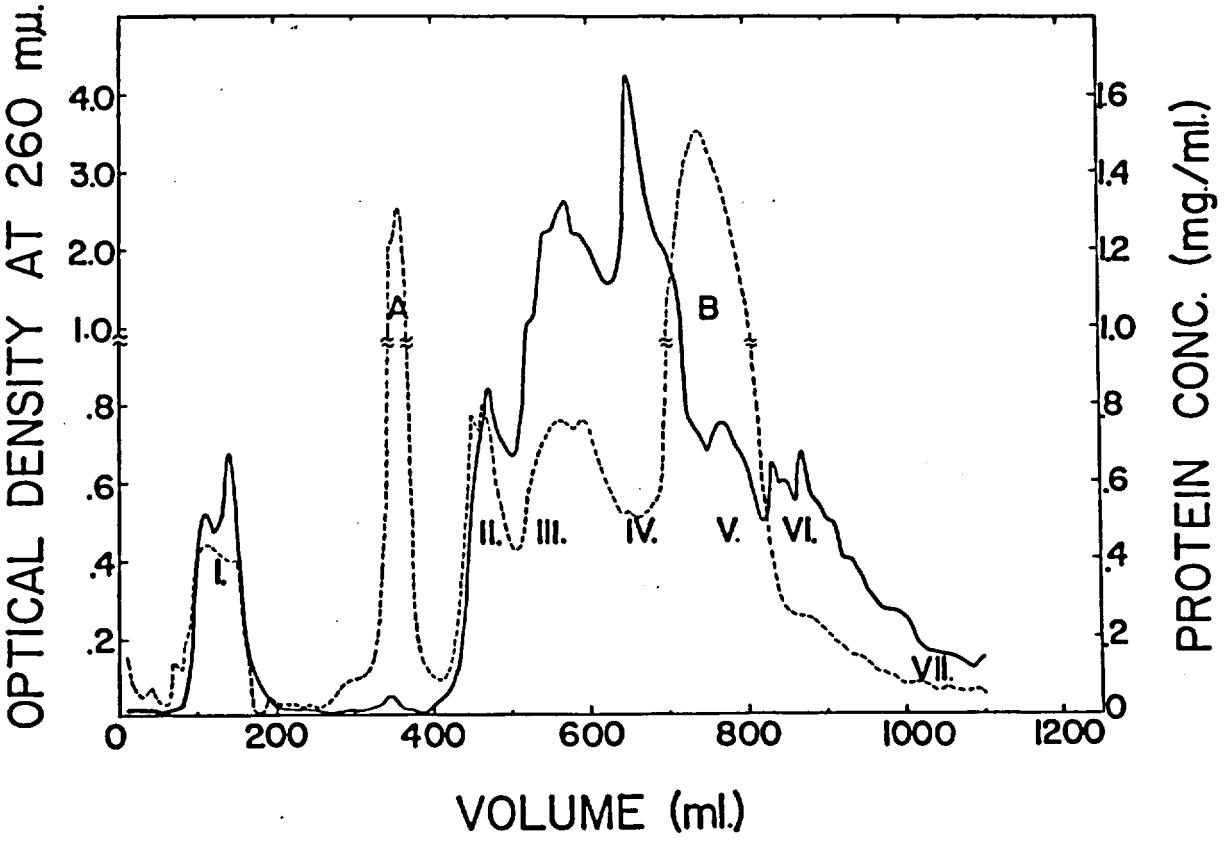


Fig. 2. DEAE-cellulose column separation of muscle myogen and other non-protein constituents from chum salmon. The column specifications and all descriptions are similar to Fig. 1.

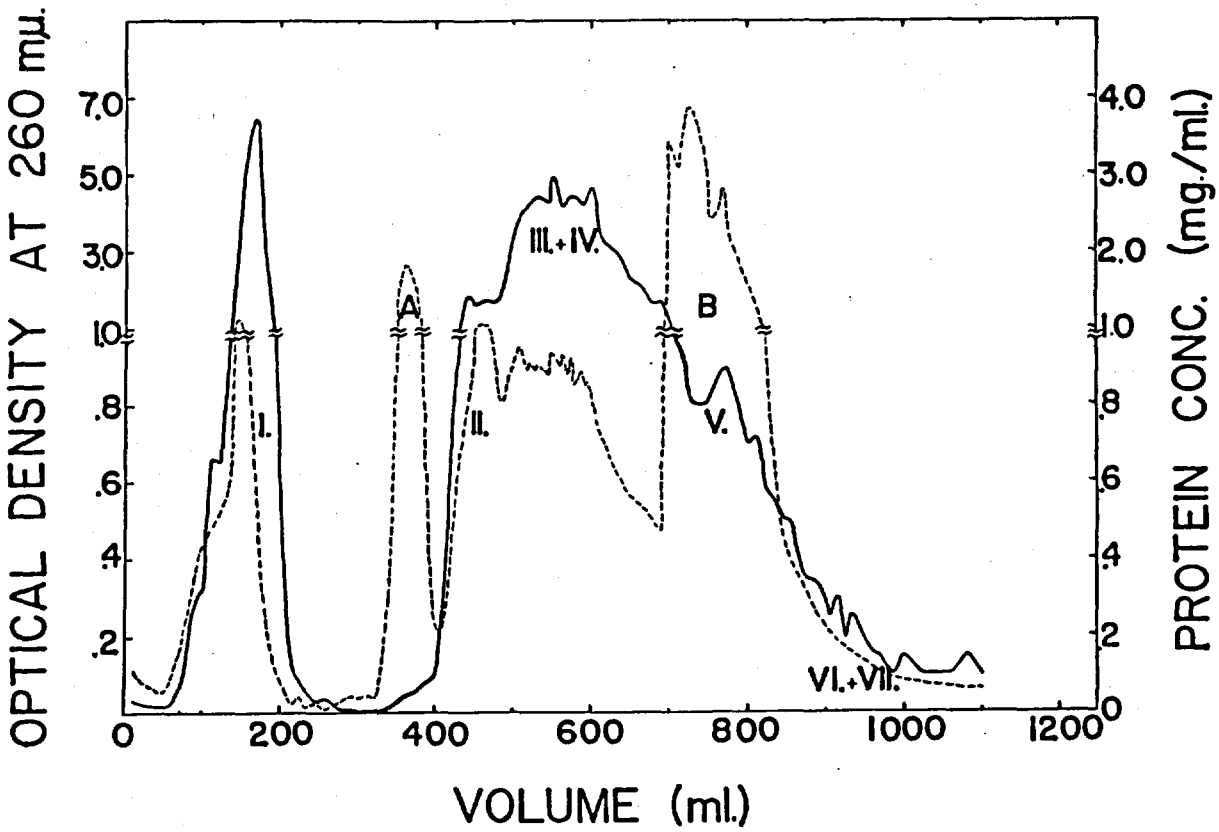


Fig. 3. DEAE-cellulose column separation of muscle myogen and other non-protein constituents from sockeye salmon. The column specifications and all descriptions are similar to Fig. 1.

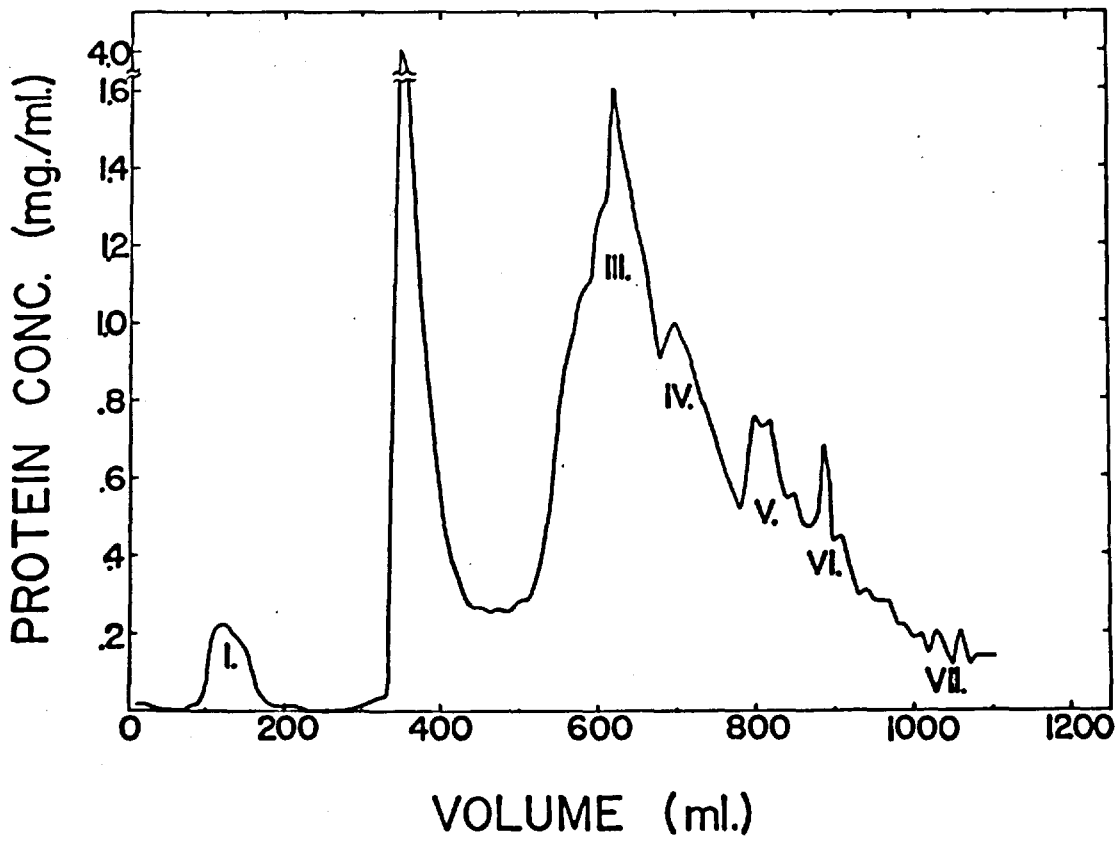


Fig. 4. DEAE-cellulose column separation of muscle myogens from coho salmon. Column specifications are similar to Fig. 1. Only the protein curve is shown as the values for ultraviolet absorption are not available. Protein fractions indicated by Roman numerals are only approximate as the internal markers of inosine and inosinic acid are not available.

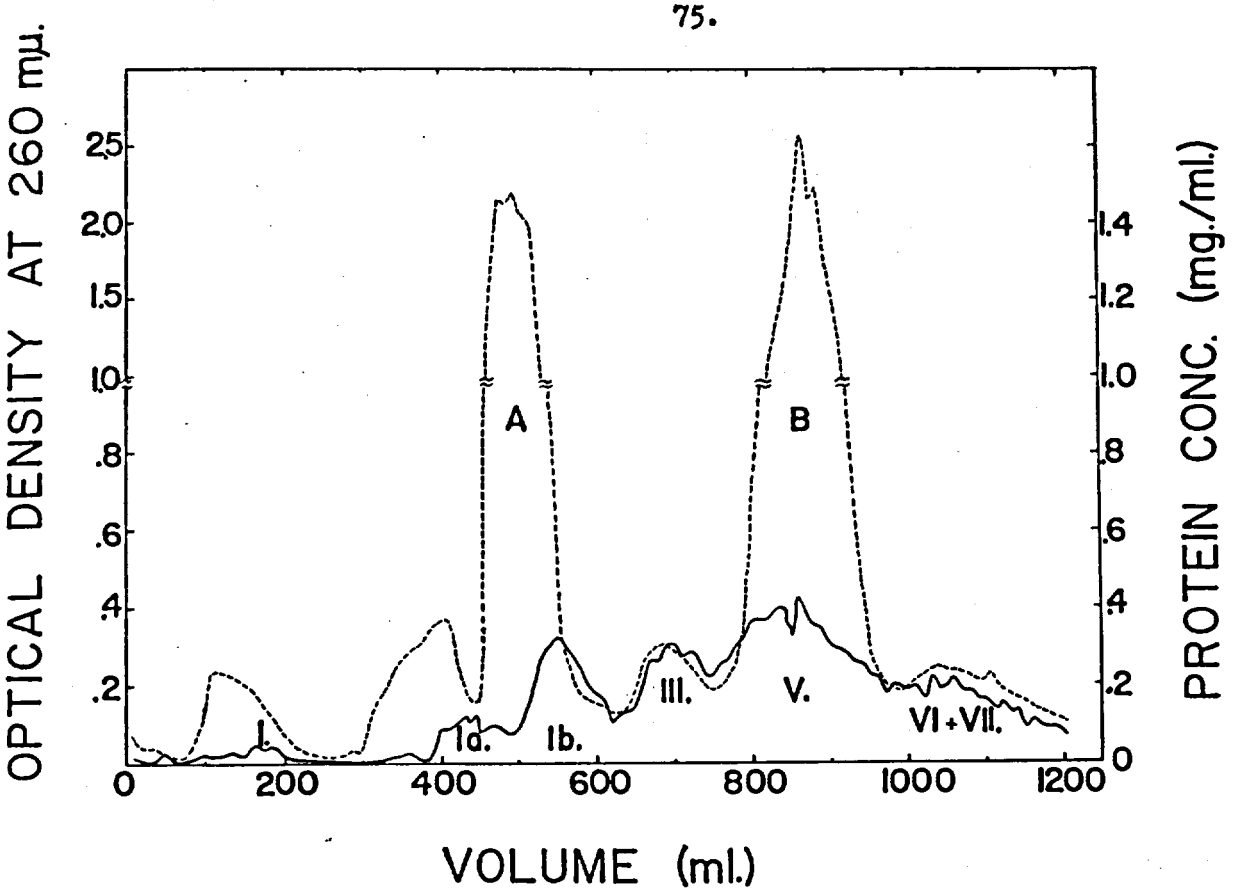


Fig. 5. DEAE-cellulose column separation of muscle myogens and non-protein constituents from lingcod. The column specifications and all descriptions are similar to Fig. 1.

Discussion

The DEAE-cellulose column separation studies of fish muscle myogens show an overall difference in the protein patterns in the various species. However, for a better definition of the similarities and differences a more precise method is required. Such a method involving starch gel electrophoresis will be described in a later communication.

Column chromatographic studies of fish myogens lend themselves more to the analysis of components separated. The bulk of the fish muscle myogen studies by moving boundary electrophoretic methods by the very nature of the instrument which follows the fractions separated by the change in refractive indices, make it difficult to detect the presence of non-protein components in the extract. Components such as inosine and inosinic acid, which are only very slowly dialyzable under the conditions of preparing the protein extracts, could very well lead to false minor protein peaks in the electrophoretic patterns. A brief account of an observation of a nucleotide-like substance migrating with a high mobility has been mentioned by Dyer and Dingle (1960). The results of Creelman and Tomlinson (1960) show that upwards of 4 μmoles of inosine and over 5 μmoles of inosinic acid per gram wet weight are present in salmon muscle tissue immediately after killing. The data of Tarr (unpublished) also indicate a level of inosinic acid as high as 7.75 μmoles per gram wet weight of muscle tissue in the fishes investigated. Inosine in concentration of 0.40 and 1.17 μmoles per gram wet weight of muscle was recovered from columns in the case of chum and spring salmon, respectively. These reported values are within the lower concentration limits of some moving boundary electrophoresis instruments.

By moving boundary electrophoresis of salmon muscle myogens at pH 8.0 (Tsuyuki and Roberts, 1961), seven components were identified. However, the mobilities of the individual components were so close together that a clean-cut fractionation could not be achieved by any single run at a variety of pH values. On the other hand, Atlantic cod muscle myogens (Connell, 1953; Dingle et al., 1955) generally show good separation at pH 6.5. Column techniques show a similar difference in the ease of separation between the salmon and lingcod. Whereas the bulk of the salmon myogens are not too well resolved, the lingcod components differ sufficiently in their physical characteristics to be readily separable. Although the column method of separating salmon muscle myogens still leaves much to be desired, it offers certain advantages over that which could be achieved by moving boundary electrophoresis.

SUMMARY NO. 28

THE SEPARATION OF MUSCLE PROTEINS SOLUBLE IN LOW IONIC STRENGTH SALT SOLUTIONS BY STARCH GEL ELECTROPHORESIS

H. Tsuyuki
E. Roberts
R.E.A. Gadd

The need for a method sensitive enough to observe the finer details in the muscle myogen pattern superior to that which could be achieved by moving boundary electrophoresis, paper electrophoresis, or by column methods was pointed out in the preceding report. To this end the possibility of the use of the starch gel electrophoretic method originally described by Smithies (1955, 1959) was investigated. As an example of the sensitivity of this method more than 30 components have been demonstrated in human blood sera by two dimensional gel electrophoresis (Poulick and Smithies, 1958). During a study of muscle myogens of cod, perch, haddock, whiting, pollock, halibut, and flounder by gel electrophoresis, Thompson (1960) found differences sufficient to identify the fishes by their protein patterns.

In the present report the muscle myogens of the five members of the west coast Pacific salmon, the spring (O. tshawytscha), sockeye (O. nerka), coho (O. kisutch), pink (O. gorbuscha), and chum (O. keta), as well as other fishes, have been investigated by means of starch gel electrophoresis.

Methods

Collection of Fish

Most of the fish used were obtained as described in the preceding report. In addition, spring and coho salmon in spawning condition were obtained from the Green River Hatchery in Washington.* Sockeye salmon in approximately the same state of maturity were from the same stock of adult fish obtained at Great Central Lake, B.C., and held in this Station's aquarium facilities until the gonads were in a near spawning condition. The sexually immature adult steelhead were also obtained from the same location. The Atlantic cod was shipped frozen in dry ice from Halifax**. Pink salmon were obtained from the commercial gill-net operations

* Obtained through the co-operation of Mr. Steve Fallert, Supervisor of Green River Hatchery in Washington.
** We wish to thank Dr. D.R. Idler, Director of the Fisheries Research Board of Canada Technological Station at Halifax, N.S., for the sample.

on the Fraser River.

The muscle fillets were obtained either from fish immediately after killing or from those stored in the frozen state.

Preparation of Muscle Protein Extract

The protein extracts were homogenized and prepared as described in the preceding report. Extracts used for starch gel electrophoresis were prepared in the same way except that 2 volumes of the low ionic strength phosphate buffer instead of 7 were used. The protein concentration of the 1:2 muscle to buffer extract was optimal for direct application to the starch gel. Protein concentration was determined by the methods of Snow (1950) and of Lowry (1951).

Starch Gel Electrophoresis

Starch gel electrophoresis experiments were carried out at 0°C both with the apparatus described by Smithies (1955, 1959) and also with an E-C zone electrophoresis apparatus equipped with a starch gel tray 45 cm in length and 1 cm in depth. Electrophoresis was carried out horizontally for 18 hours at a field strength of 6.25 volts per cm across the gel. For salt bridges Whatman No. 17 filter paper was used.

The gel tray for the E-C apparatus was fitted with a sample slot cover similar in design but larger than the one described by Smithies. Whatman No. 17 filter papers used as wicks for salt bridges were placed so that the distance between them measured 39 cms. A potential gradient of 300 volts was applied across the gel for 18 hours.

Protein extracts were introduced carefully into the sample slots by means of a 100 µl Hamilton microlitre syringe. The slots in the Smithies' apparatus were filled with 40 µl of extract while the larger E-C apparatus required 100 µl. The sample slots were layered with a few drops of mineral oil and the entire gel surface was covered carefully with a film of polyethylene or saran wrap. After completion of electrophoresis the gel was sliced either with a fine gauge wire or a single-edged cutter provided with the apparatus. The cutter was modified slightly by grinding to a width of 5 mm, reducing the tendency of the blade to stick to the gel while slicing. The horizontally sliced gel was stained with the cut surface up in the usual manner (Smithies, 1955) with Amido black 10 B. The stained gel was then photographed, wrapped in saran wrap and stored in the refrigerator. The remaining half of the gel was stained for lipoproteins by the method of Talluto et al (1958) using Oil Red O.

Column Chromatography

The diethylaminoethyl (DEAE) cellulose column chromatography was carried out as previously described. The proteins eluted from the column were arbitrarily pooled into 11 fractions, lyophilized to smaller volumes, dialyzed overnight against the borate buffer used for electrophoresis, and centrifuged at 30,900 x g for 15 minutes. One hundred µl of solution from each of the 11 fractions containing 0.014, 0.466, 1.82, 1.82, 0.035, 1.06, 1.63, 0.895, 0.155, 0.218, and 0.921 mg of protein, respectively, were introduced into the gel. The amount of protein put in the gel was not constant since some of the fractions contained very little protein.

Results

The Effect of pH on Gel Electrophoresis

The borate buffer system in both the electrode compartment and the gel was adjusted to various pH values with NaOH. No attempts were made in these studies to adjust the ionic strengths of the two solutions at the various pH values as the end desired was adequate separation of the protein components at whatever pH deemed necessary.

In Fig. 1 are shown the results of the effect of pH on the separation of muscle proteins from various species of fish. At pH 8.5 the best overall separation was achieved and subsequent experiments were conducted at this value. At pH 7.5 some of the proteins migrated to the cathode, a few to the anode, and a portion remained at the origin. By raising the value to 8.0 some of the proteins migrated towards the cathode and others to the anode.

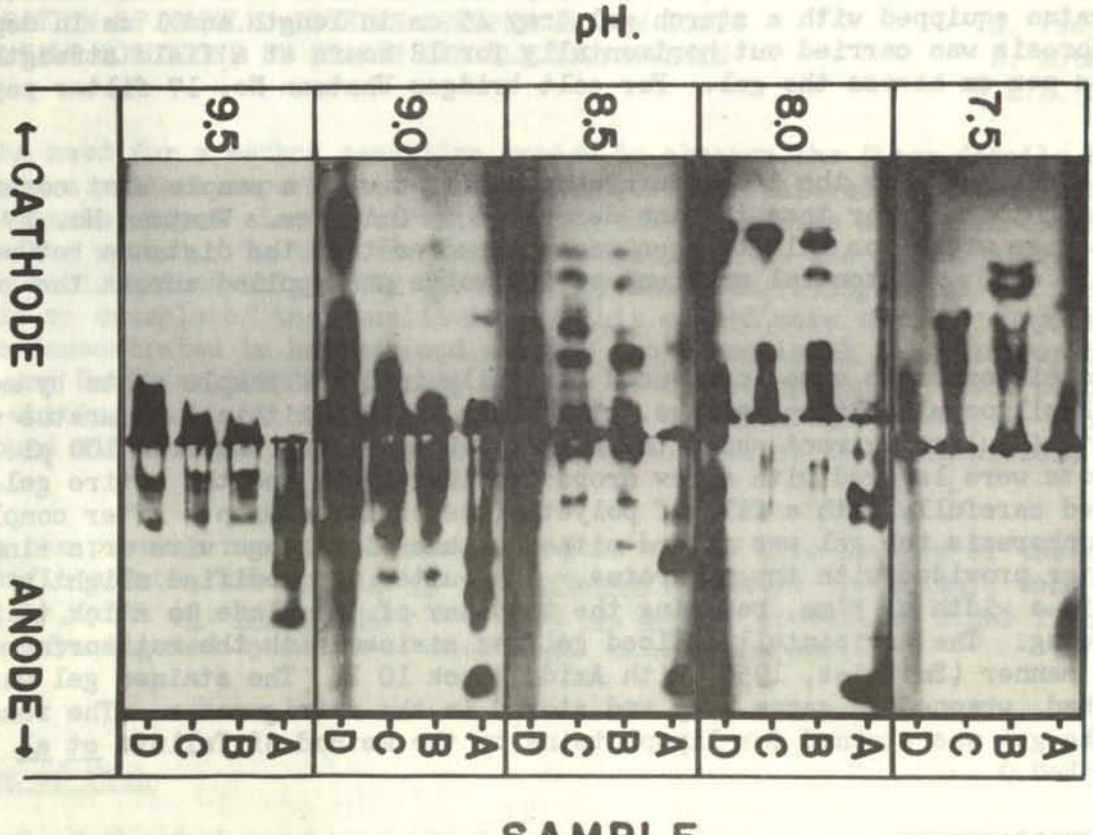


Fig. 1. The effect of pH on the separation of low ionic strength soluble muscle proteins of lingcod (A), coho (B), spring(C), and sockeye (D) salmon by starch gel electrophoresis.

The lingcod proteins differ strikingly from those of the salmon species in that they begin to migrate towards the anode at a lower pH. At pH's 9.0 and 9.5 separations are generally characterized by excessive streaking and heavy concentration at the origin. Even at the optimal pH value of 8.5 considerable protein remains at the origin. Some may be at their isoelectric point since proteins of the

salmon particularly are distributed at either side of the origin. Simple precipitation cannot be ignored. It is possible that high molecular weight actomyosins may be extracted at an ionic strength of 0.05 since at 0.1 the results of Connell (1958) show that some of this protein is extracted from fresh cod muscle tissue containing ATP. In one experiment proteins soluble in high ionic strength solutions failed to migrate from the origin under the conditions of the gel electrophoresis. This lack of movement of high molecular weight proteins through the gel is discussed in detail by Smithies. Proteins whose molecular weights are high enough to hinder migration through the gel may also be present in the myogen fraction since ultracentrifugal studies of cod muscle extracts at an ionic strength of 0.05 have shown the presence of 3 main components with sedimentation constants of 6.4 S, 4.4 S, and 1.3 S and also a small amount of a heavy component with a value as high as 93 S which is thought to be actomyosin (Connell, 1958). Extraction at ionic strengths of 0.1 to 0.2 showed increasing percentages of the heavy component. Hamoir (1955) has also described the presence of these three main ultracentrifugal components of approximately the same sedimentation constants in the carp. The intermediate component has a molecular weight of 67,000 (Henrotte, 1954), while the most slowly sedimenting component had a value between 10,000 to 20,000 (Hamoir, 1955).

Lingcod muscle differs from salmon in that there are fewer components, 4 at pH 8.5 and 8 at the higher pH of 9.0. The coho and spring show 12-14 components and are very similar in their protein patterns at pH 8.5. However, clear-cut differences between the two appear at the lower pH values especially towards the cathode side of the origin. Differences may also be present in the anode side at pH 8.0 but poor separation of the negatively charged proteins at this pH makes comparisons difficult. The sockeye proteins differ clearly from the spring and coho at all pH values.

The Separation of Muscle Myogens

Fig. 2 shows the comparison of the steelhead trout and spring salmon. At pH 8.5 most of the steelhead proteins migrate to the anode. However, in the overall picture, the two patterns show certain similarities. To facilitate description, the various protein bands migrating for the indicated distances from the origin to the cathode are assigned positive values, and those migrating to the anode negative values. The two bands at +1.5 and +2.0 cm present in the spring salmon are faint or absent in the steelhead, while the -1.5 cm band in the spring salmon is very faint. The numerous other faint bands differ to varying degrees in the two fishes. In these and subsequent experiments, the remaining half of the gel did not show any detectable quantities of lipid substance when stained with Oil Red O.

Fig. 3a shows a comparison of the proteins of sexually mature and immature coho and spring salmon. The two species of fishes have very similar protein patterns. The major differences lie in the presence in the spring salmon of an additional strong band at +2.0 cm and the relatively stronger band at -4.5 cm in the coho salmon. Another difference is the appearance of a faint band at -3.0 cm in the coho salmon. There is practically no qualitative difference between the sexually mature and immature fishes of both species except for the slightly stronger band at -2.5 and -3.5 cm in the mature spring salmon.

A similar relationship exists between the sexually mature and immature sockeye salmon, though in this case the -3.5 cm band is considerably stronger in the mature fish of both sexes (Fig. 3b). The positions of the protein bands in Fig. 3a and b are not directly comparable since the two electrophoretic runs were carried out at different times. There is no difference between the male and female sockeye. Frequent comparisons made in these studies between the protein patterns of fresh salmon and those stored at -30°C for as long as 4 months showed no significant differences. By gel electrophoretic means Hughes (1959) also found no qualitative difference in the sarcoplasmic protein pattern of human muscle before and after storage at -25°C for one month.

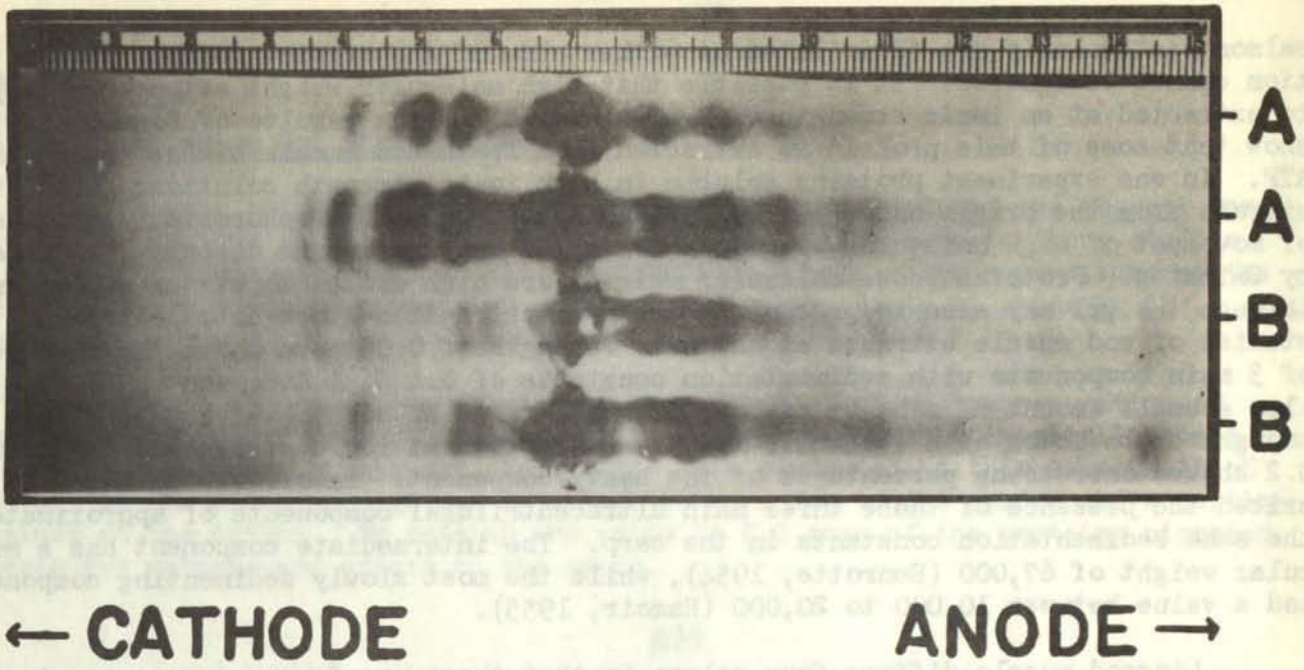


Fig. 2. The comparison of low ionic strength soluble muscle proteins of adult spring salmon (A) and steelhead trout (B) at pH 8.5. The scale represents actual migration in cm of the proteins through the starch gel.

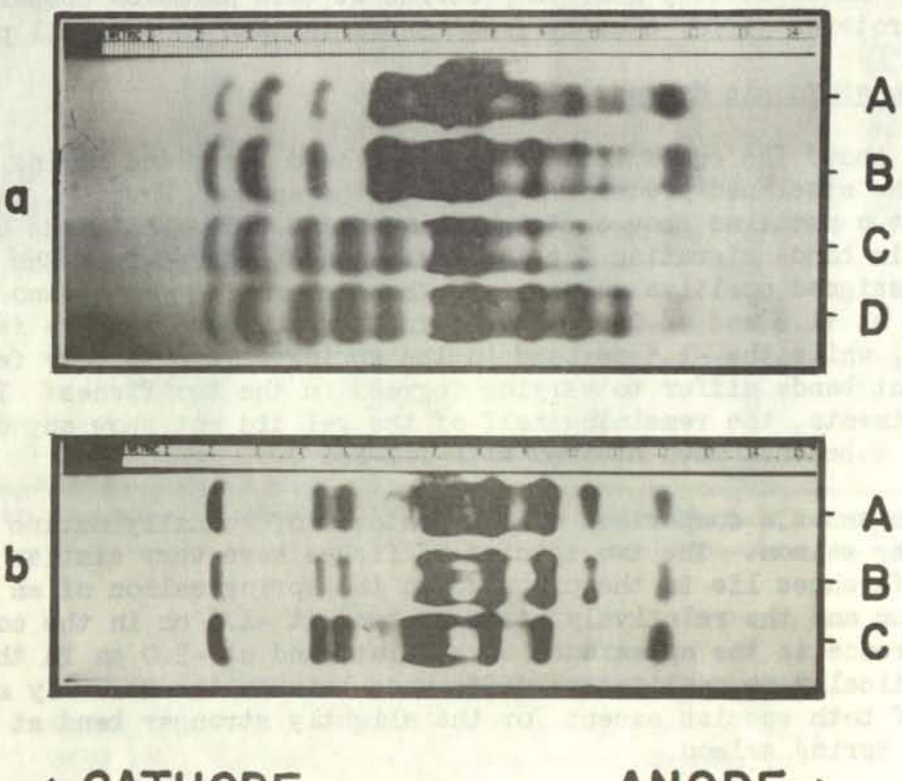


Fig. 3a. Comparison of low ionic strength soluble muscle proteins of sexually immature (A) and mature (B) coho and sexually immature (C) and mature (D) spring salmon at pH 8.5.

Fig. 3b. Comparison of low ionic strength soluble muscle proteins of sexually mature male (A), female (B), and immature (C) sockeye salmon at pH 8.5.

A simultaneous starch gel electrophoretic run of chum salmon and mature and immature samples of sockeye, spring and coho salmon is shown in Fig. 4. This direct comparison clearly shows that in the 4 species of salmon there are many proteins of similar mobilities under these conditions as well as many that are completely different. The differences between each species of salmon are characteristic. However, the similarities are also striking in that some proteins are present in all four species and others are present in some but not all. The protein pattern of chum salmon is qualitatively fairly close to that of sockeye. Although pink salmon is not shown in the same run due to sampling difficulties, comparison with another electrophoretic run (Fig. 5a, b) shows obvious similarities between the pink, sockeye and chum.

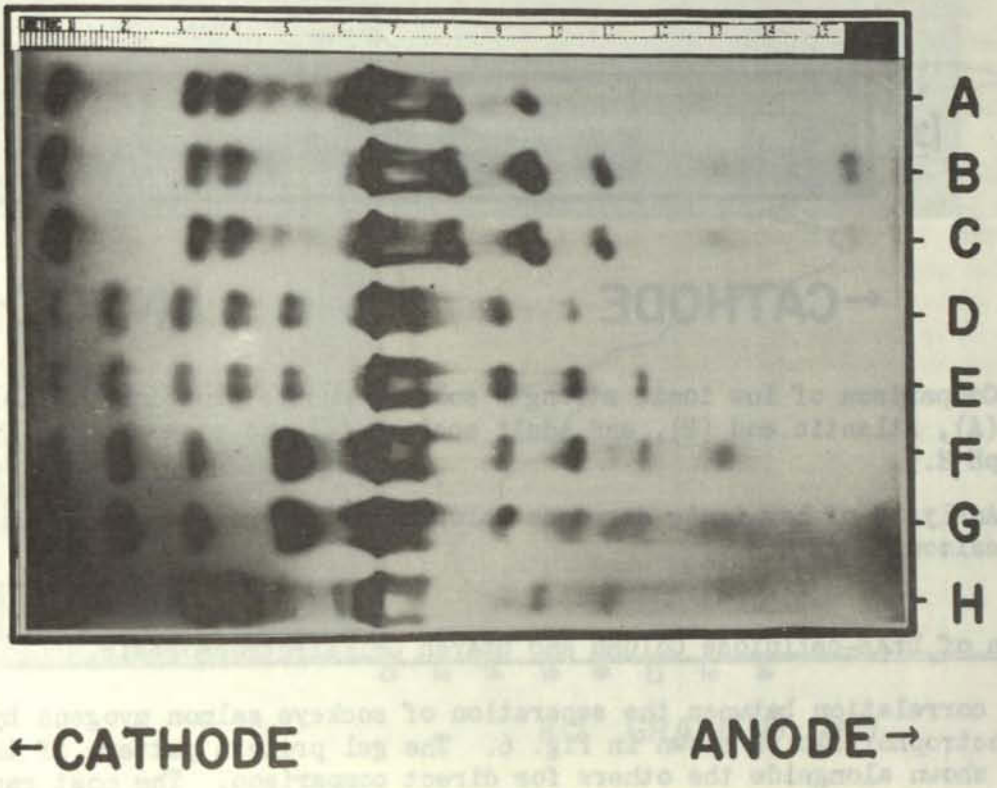


Fig. 4. A simultaneous analysis of low ionic strength soluble muscle proteins at pH 8.5 of sexually immature (A) and mature male (B) and female (C) sockeye salmon, sexually immature (D) and mature (E) spring salmon, sexually immature (F) and mature (G) coho salmon, and chum salmon (H) of intermediate sexual maturity.

Two Pacific salmon species are compared with the lingcod and the Atlantic cod in Fig. 5a. The lingcod and the Atlantic cod show some similarity but the two are readily distinguishable from each other and are also completely different from the salmon. The pink and the sockeye salmon (Fig. 5a and b) are almost identical. However, in a clearer pattern (Fig. 5b) the pink salmon shows an additional faint band at +6.0 cms.

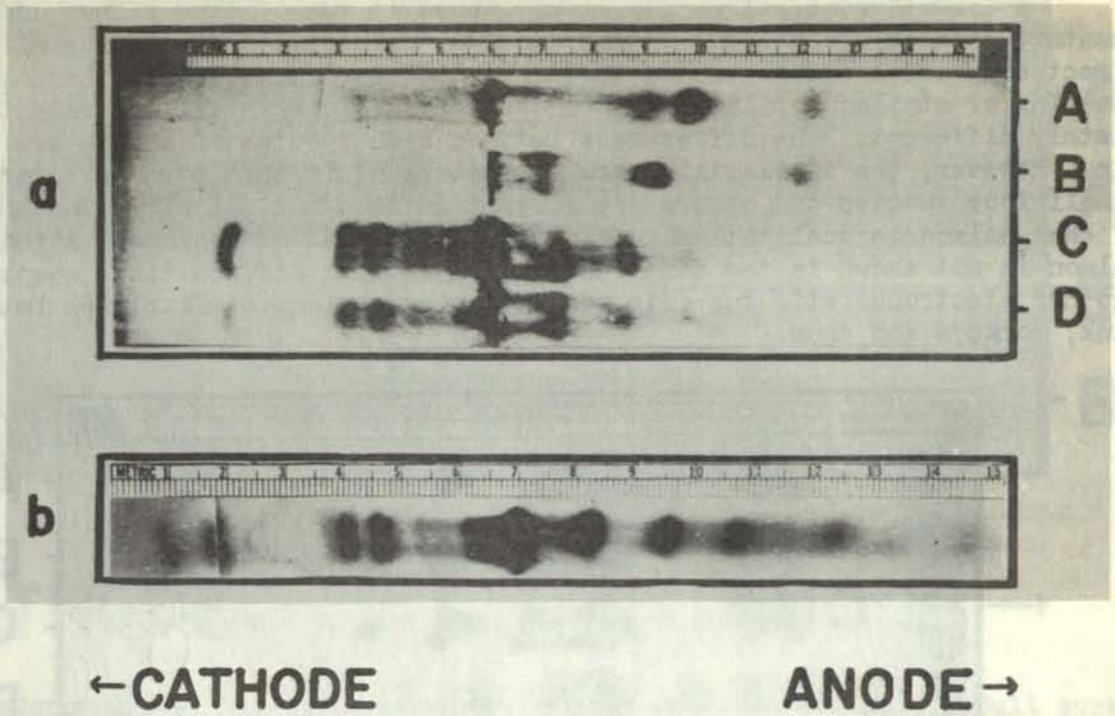


Fig. 5a. Comparison of low ionic strength soluble muscle proteins of lingcod (A), Atlantic cod (B), and adult sockeye (C) and pink salmon (D) at pH 8.5.

Fig. 5b. Analysis of low ionic strength soluble muscle proteins of adult pink salmon at pH 8.5.

Correlation of DEAE-cellulose Column and Starch Gel Electrophoresis.

The correlation between the separation of sockeye salmon myogens by column and gel electrophoresis is shown in Fig. 6. The gel protein pattern of the whole extract is shown alongside the others for direct comparison. The most rapidly migrating protein fraction corresponding to the +5.5 cm band moving to the cathode in the gel and to the completely separated fast-eluting fraction off the column appears to consist chiefly of a single component. Fractions 2, 3, and 4 cover all areas of this peak. However, faint bands at +3.6 and +4.3 cm appear in fraction 2 while in 3 a large amount of protein streaks from the origin towards the cathode, indicative of precipitation rather than further components. The bulk of the positively charged and traces of negatively charged proteins appear in fractions 6 and 7. The separation of the proteins in this region of the column is therefore ill-defined and a technique such as gel electrophoresis which separates proteins by a combination of net charge and molecular sieving effects is required for optimal resolution. This is also the region on the cathode side of the origin where most of the finer differences between the salmon species are displayed. The reappearance of the fast-moving +5.5 cm band in these fractions indicates that one (or more) of these proteins is unstable and is giving rise to part or all of the first peak of the column. Part of the concentration differences observed in the column separations of fraction I referred to in the previous report, as well as in the +5.5 cm band in the gel protein patterns may be a reflection of this instability. However, the unstable protein(s) in fraction 6 and 7 must have remained intact during column chromatography to elute in the positions shown. The possibility still remains that the +5.5 cm bands may be proteins differing in net charge

and molecular size and shape but migrating at the same rate in the gel. The -1.5 cm band also appears in these two fractions. In fraction 8 are found the -2.7 and -3.8 cm bands. The faint band migrating most rapidly to the anode appears in the remaining three fractions. In general the proteins emerging during the early stages of column chromatography migrate more rapidly to the cathode upon electrophoresis while those emerging later migrate to the anode. Those eluting in the intermediate zone are more nearly isoelectric at this pH.

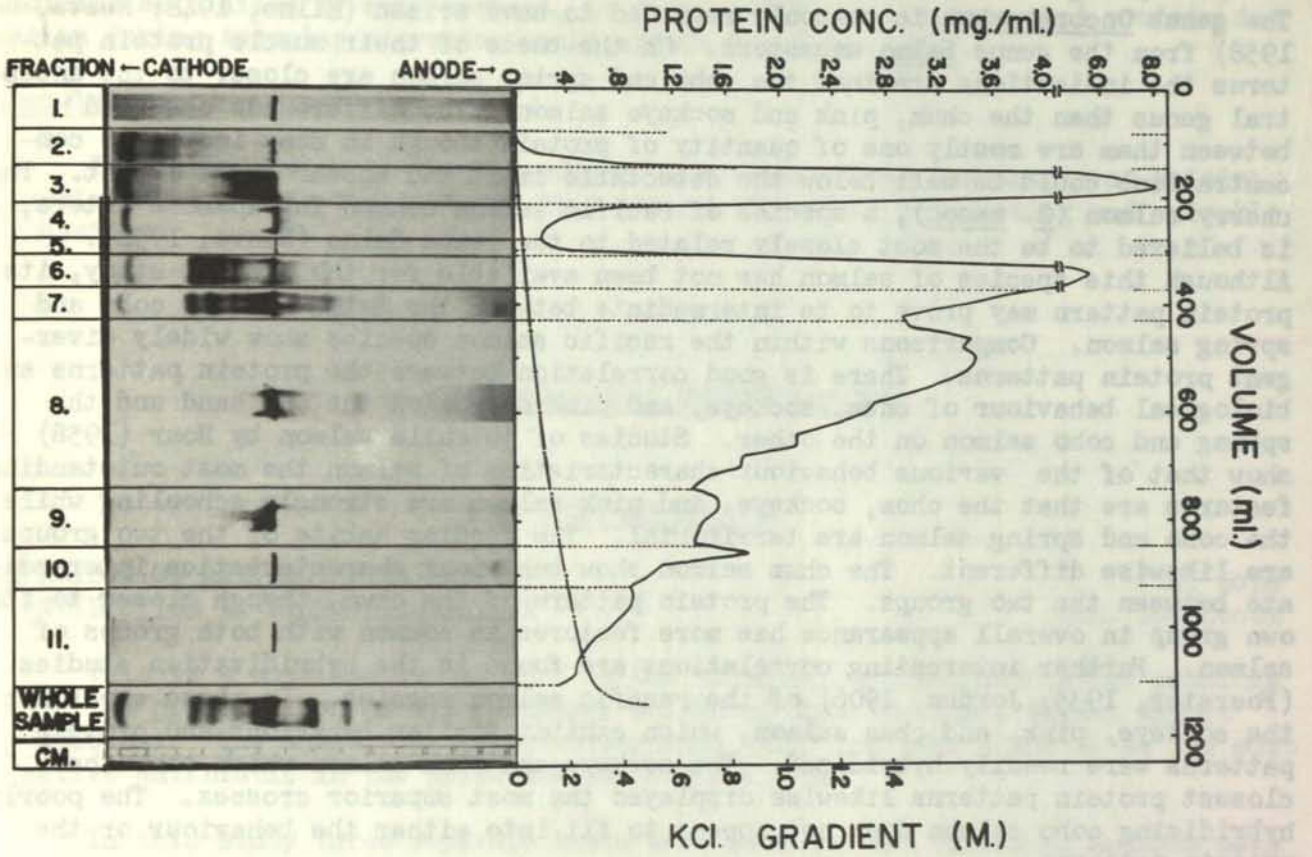


Fig. 6. Correlation of DEAE cellulose (upper) and starch gel electrophoretic (lower) separations of low ionic strength soluble muscle proteins of adult sockeye salmon. The solid curve represents the protein concentration and the dot-dash curve the KCl gradient used for the column. Fractions numbered 1 to 11 inclusive represent the various cuts from the column (indicated by dashed lines) analysed by starch gel electrophoresis at pH 8.5. An electrophoretic analysis of the whole extract of sockeye salmon is shown to the right of fraction 11.

Discussion

It must be understood that the term 'protein' is used in a broad general sense, including glyco- and lipo-proteins, for example, and is not to be construed as pure protein. Any proteinaceous or possibly other substances with an affinity for the dye Amido Black 10 B has been considered a protein band for the purpose of discussion.

Starch gel electrophoresis provides a rapid and sensitive method for the separation of fish muscle myogens. For the purpose of showing species differences

of various fishes, including such closely related ones as the west coast Pacific salmon, the technique offers many advantages over the classical moving boundary method employed by Connell (1953) to show differences in the myogen fraction of a number of fishes. A simultaneous comparison can be made of any number of fishes with the number of sample slots the only practical limitation.

It was possible to show many areas of similarities in the protein patterns between spring and coho salmon and steelhead trout (Salmo gairdnerii gairdnerii). The genus Oncorhynchus is commonly regarded to have arisen (Milne, 1948; Neave, 1958) from the genus Salmo ancestors. On the basis of their muscle protein patterns the indications are that the coho and spring salmon are closer to the ancestral genus than the chum, pink and sockeye salmon. The differences observed between them are mostly one of quantity of protein though in many instances concentrations could be well below the detectable limit and appear to be absent. The cherry salmon (O. masou), a species of Pacific salmon common in Japanese waters, is believed to be the most closely related to the genus Salmo (Neave, 1958). Although this species of salmon has not been available for the present study, its protein pattern may prove to be intermediate between the Salmo and the coho and spring salmon. Comparisons within the Pacific salmon species show widely divergent protein patterns. There is good correlation between the protein patterns and biological behaviour of chum, sockeye, and pink salmon on the one hand and the spring and coho salmon on the other. Studies of juvenile salmon by Hoar (1958) show that of the various behaviour characteristics of salmon the most outstanding features are that the chum, sockeye, and pink salmon are strongly schooling while the coho and spring salmon are territorial. The feeding habits of the two groups are likewise different. The chum salmon show behaviour characteristics intermediate between the two groups. The protein pattern of the chum, though closer to its own group in overall appearance has more features in common with both groups of salmon. Further interesting correlations are found in the hybridization studies (Foerster, 1935; Jordan, 1906) of the Pacific salmon species. In these experiments the sockeye, pink, and chum salmon, which exhibit similar behaviour and protein patterns were readily hybridized. The sockeye and pink salmon which show the closest protein patterns likewise displayed the most superior crosses. The poorly hybridizing coho salmon does not appear to fit into either the behaviour or the protein patterns.

An extension of these studies to the various geographic races of a single species of Pacific salmon has not been attempted at this time. Ridgway et al. (1958, 1960) have made some progress along these lines by the use of serological or immunochemical methods. Some differences were found in the strength of reaction of the cells of individual sockeye salmon when tested against pig anti-sera. Because certain experimental difficulties were encountered in the transporting of fresh whole blood from wide areas of the Pacific ocean, the technique of using soluble antigens from fresh frozen blood was used for later studies. By diffusion precipitin analyses, sera of most Alaskan sockeye salmon possessed at least 14 antigenic components, two of which were missing in the sera of most sockeye salmon from Asian waters.

Similarity of protein patterns also exists between the lingcod and Atlantic cod. The physical characteristics of the proteins of these fishes are clearly distinctly different from the Salmo and Oncorhynchus genera.

During spawning migration sockeye salmon lost a considerable amount of their body proteins (Idler and Bitners, 1958, 1959). Direct analyses of the changes occurring in the muscle proteins of these fishes soluble in both low and high ionic strength salt solutions (Duncan and Tarr, 1958) indicated that the fraction corresponding to the high ionic strength soluble and stroma proteins are most seriously

depleted. The low ionic strength soluble proteins are not depleted to this extent. By gel electrophoretic means it was possible to follow the changes in the various protein components of the muscle myogen fractions during spawning migration of the salmon. In the three species of Pacific salmon studied, the sockeye, spring, and coho salmon, the protein pattern of the sexually mature and of the immature fishes are qualitatively the same. Quantitatively, the only difference lies in the relative proportion of some minor component, rather than a complete absence of any one component. If the proteins of this fraction are involved in 50 odd enzymatic functions as claimed by many workers, it is reasonable to suppose that protein depletion during spawning migration would affect these the least.

Acknowledgment

The authors wish to thank Professor W.S. Hoar of the University of British Columbia and Dr. Neil Tomlinson of this Station for their stimulating and valuable discussions.

MICROBIOLOGICAL INVESTIGATIONS

SUMMARY NO. 29

COMPARISON OF ANTIBACTERIAL ACTIVITY OF ANTIBIOTICS IN FISH PRESERVATION

J.W. Boyd
B.A. Southcott

This is a continuation of work on the screening of antibiotics for possible use in the preservation of fish (Summary No. 18 of this Station's Annual Report for 1960-61). The antibiotic chlortetracycline (CTC) has been found to be the most effective antibiotic in the preservation of fresh fish.

In this study three separate tests were made and the following methods were used for each test.

Treatment and Storage

Fillets of lingcod (Ophiodon elongatus) and gray cod (Gadus macrocephalus) which were obtained from a commercial fish plant were aseptically cut into portions approximately 2x2x0.5 inches in size. The portions were treated by dipping for 1 minute in the antibiotic solution under test, and allowed to drain for 15 minutes at room temperature. Subsequent to draining the treated portions were packed in polyethylene bags and stored at 0° and 4°C. At intervals samples were removed from storage for viable bacterial count and organoleptic evaluation.

Viable Bacterial Count

For viable bacterial counts of samples, the portions of muscle were aseptically cut into sections approximately 0.5x0.5x0.5 inches in size. A 40-g sample of sections was shaken for 5 minutes at 260 oscillations per minute on a reciprocal shaker in 360 ml of 3% (w/v) sodium chloride solution contained in a 1-quart wide-mouthed jar. Appropriate dilutions were prepared from the shaken sample and 3% sodium chloride-Bacto nutrient agar plates were made from the dilutions. Plates were incubated at 25°C for 72 hours.

In the first test lingcod fillets (post mortem age of fish - 1 day) were used for comparing the effectiveness of colistin sulfate (Warner-Chilcott Laboratories) with that of CTC in fish preservation. Colistin is a white basic polypeptide whose salts are soluble in water. Colistin sulfate has a bacteriostatic and bactericidal effect against a wide variety of gram negative bacteria in vitro.

The lingcod portions were dipped "by the method previously described" in one of the following solutions:

1. Control - water
2. Chlortetracycline - 10 ppm
3. Colistin sulfate - 10 ppm

From the results as presented in Table I it is evident that colistin is ineffective in retarding the development of spoilage organisms in lingcod muscle under conditions of the test, while CTC was very effective.

TABLE I - Evaluation of colistin sulfate in lingcod preservation.

Days stored	Storage temp °C	Bacterial count x 10^6 per g of muscle — Treatment: None	CTC	Colistin sulfate
2	0	0.50 (9)*	0.003 (9)	0.039 (9)
4	0	8.0 (9)	0.01 (9)	0.35 (9)
	4	70.0 (8)	0.20 (9)	33.0 (8)
5	0	23.0 (8)	0.08 (9)	5.0 (7)
	4	250.0 (6)	6.3 (8)	400.0 (5)
6	0	160.0 (6)	1.7 (8)	160.0 (6)
	4	300.0 (3)	37.0 (7)	400.0 (3)
7	0	260.0 (2)	4.0 (8)	115.0 (5)
	4	- (2)	156.0 (7)	- (2)

* ( ) = Organoleptic scores
10-6 = Fresh - off-odours
5-1 = Sour - putrid

Gray cod portions (post-mortem age of fish approximately 3 days) were treated in the second test with the following antibiotic solutions:

1. Control - water
2. Chlortetracycline - 10 ppm
3. Colistin sulfate - 20 ppm
4. Neomycin sulfate (Upjohn Company) - 20 ppm
5. Albamycin-sodium (Upjohn Company) - 20 ppm
6. Streptolydigin (Upjohn Company) - 20 ppm

The results of this second test are summarized in Table II. Under the conditions of this test it is once again apparent that only CTC was effective in retarding bacterial development in fish muscle. The other four antibiotics had no appreciable preservative effect.

TABLE II - Antibacterial activity of antibiotics in gray cod preservation.

Days Stored	Storage temp °C	Bacterial count x 10^6 per g of muscle — Treatment: None	CTC	Colistin	Neomycin	Albamycin	Streptolydigin
2	0	0.042	0.020	0.027	0.032	0.170	0.90
	4	0.250	0.013	0.150	1.0	0.50	0.40
4	0	1.5 (7)*	0.006 (8)	22.0 (7)	20.0 (7)	3.0 (7)	16.0 (7)
	4	47.0 (7)	0.08 (8)	12.0 (7)	120.0 (7)	26.0 (7)	30.0 (7)
6	0	30.0 (6)	0.05 (8)	25.0 (6)	250.0 (4)	32.0 (6)	130.0 (4)
	4	- (2)	28.0 (7)	- (2)	- (2)	- (2)	- (2)
7	0	- (2)	1.0 (7)	- (2)	- (2)	- (5)	- (2)

* ( ) = Organoleptic scores
10-6 = Fresh - off-odours
5-1 = Sour - putrid

To study the possibility of synergism developing with combined antibiotics, gray cod portions (post-mortem age of fish - 2 to 3 days) were dipped in the following solutions:

1. Control - water
2. Chlortetracycline - 10 ppm
3. Neomycin sulfate - 20 ppm
   plus
   Albamycin - 20 ppm
4. Neomycin sulfate - 20 ppm
   plus
   Streptolydigin - 20 ppm

Although Neomycin sulfate was combined with Albamycin and Streptolydigin, and the concentration of each antibiotic in solution was twice the concentration of that used for the CTC dip, the results reveal, as given in Table III, that the antibacterial activity of the combined antibiotics was negligible in comparison with CTC.

TABLE III - Evaluation of synergistic mixtures of antibiotics in gray cod preservation.

Days stored	Storage temp °C	Bacterial count x 10^6 per g of muscle — Treatment: None	CTC	Neomycin + Albamycin	Neomycin + Streptolydigin
3	0	2.0 (7)*	0.008 (9)	1.2 (9)	0.16 (9)
	4	55.0 (5)	0.50 (8)	220.0 (4)	20.0 (5)
4	4	150.0 (4)	3.0 (5)	170.0 (3)	124.0 (4)
5	0	120.0 (3)	0.11 (6)	184.0 (4)	43.0 (4)
7	0	-	23.0 (5)	-	-

* ( ) = Organoleptic scores
10-6 = Fresh - off-odours
5-1 = Sour - putrid

SUMMARY NO. 30

EFFECT OF CHLORTETRACYCLINE AND OXYTETRACYCLINE ON OBJECTIVE AND SUBJECTIVE FISH QUALITY TESTS

J.W. Boyd
B.A. Southcott

This investigation was initiated for the purpose of studying the effect of the antibiotics chlortetracycline (American Cyanamid Co.) and oxytetracycline (Chas. Pfizer and Co.) on the formation of trimethylamine in lingcod muscle, and also to ascertain whether any correlation existed between viable bacterial count, trimethylamine content and organoleptic score.

During the storage of fresh fish at chill temperatures, various biochemical changes occur in the muscle, many of these being mediated by bacterial action. The methods employed to study these changes have been developed by many investigators and a number of these methods are used to assess the quality of fish. The determination of viable bacterial counts is used in some laboratories as a measure of quality. One chemical method which is employed to ascertain the degree of spoilage of fish is the determination of the amount of trimethylamine formed by bacterial reduction of trimethylamine oxide. A common subjective test which is used as an index of quality is the organoleptic evaluation of odour development.

Investigators have shown that certain preservatives which have been used in the experimental or commercial preservation of fresh fish modify the spoilage pattern and therefore may make the results of tests for quality invalid.

Lingcod (Ophiodon elongatus) were obtained from a commercial fish plant in the Vancouver area. The post-mortem age of the fish was from 2 to 3 days.

Portions of muscle approximately 2x2x1 inches were cut from fillets and treated with the antibiotics chlortetracycline (CTC) and oxytetracycline (OTC). The muscle portions were dipped for 1 minute in a 10 ppm solution of the antibiotic and control samples were dipped similarly in water. All samples were allowed to drain for 15 minutes after the dipping process. Subsequent to treatment, each lot of muscle was minced in a sterile meat grinder. The minced muscle was packed in polyethylene bags and then stored at 0° and 4°C for storage tests. At intervals samples were taken for viable bacterial counts, trimethylamine nitrogen determinations and organoleptic evaluations.

For the determination of the viable bacterial count, 40 g of minced muscle was added to 360 ml of sterile 3% (w/v) sodium chloride solution contained in a 1-quart wide-mouthed jar and shaken for 3 minutes on a reciprocal shaker at 260 oscillations per minute. Appropriate dilutions were made from the shaken sample and 3% sodium chloride-Bacto nutrient agar plates were prepared. The plates were incubated at 25°C for a period of 72 hours. A Quebec colony counter was used in the determination of the number of colonies per plate.

The trimethylamine nitrogen content was determined by the method of Dyer. A slight modification of this method was used in the extraction process; the extraction was carried out by shaking the trichloracetic acid and sample mixture on a rotary shaker at 260 oscillations per minute for 15 minutes rather than by manual shaking over a half-hour period.

The extent of odour development in the samples was used as an index for organoleptic evaluation. In this study, a sample was classed as acceptable when assigned, within a scoring range of 10 to 0, a score between 10 and 5.5, and unacceptable when it scored less than 5.5.

The results of four separate tests which were made on antibiotic treated and untreated lingcod are presented in Table I and II. It should be noted from the tables that chlortetracycline and oxytetracycline significantly suppress the reduction of trimethylamine oxide to trimethylamine in lingcod muscle during storage at 0 and 4°C. However, under the conditions of the tests there was not a corresponding reduction in the bacterial population. Similar results were obtained from a test in which small fillets were used rather than comminuted muscle. This lack of parallelism between trimethylamine nitrogen content and viable bacterial count of treated samples indicates that the antibiotics may inhibit the growth of microorganisms responsible for the reduction of trimethylamine oxide and allow the growth of non-reducing bacterial species. It is also possible that the antibiotics evaluated may have interfered with a trimethylamine oxide reducing enzyme system. A reasonable degree of agreement was obtained between viable bacterial count, trimethylamine content and organoleptic score from tests on control samples.

TABLE I - Effect of tetracycline antibiotics on the viable bacterial count, trimethylamine content and organoleptic score of lingcod muscle stored at 0°C.

Test	Days Stored	Bacteria x 10^6/g muscle						TMA-milligrams nitrogen/100 g muscle		
		Control		CTC		OTC		Control	CTC	OTC
1	1	0.013	(9)*	0.007	(9)	0.022	(9)	0.23	0.26	0.35
	2	0.045	(8)	0.005	(9)	0.023	(9)	0.39	0.35	0.34
	4	0.170	(8)	0.013	(9)	0.120	(9)	0.79	0.38	0.46
	5	0.690	(7)	0.110	(8)	1.3	(8)	3.0	0.40	0.36
	6	8.2	(5)	2.7	(7)	10.5	(6)	19.0	0.40	0.42
	7	15.0	(6)	8.0	(8)	110.0	(7)	21.0	0.46	1.3
	8	40.0	(4)	32.0	(8)	370.0	(6)	25.0	0.50	0.51
	9	228.0	(2)	100.0	(7)	510.0	(6)	39.0	0.50	0.70
	10	125.0	(2)	159.0	(6)	370.0	(5)	52.0	0.51	0.55
2	1	0.24	(9)	0.11	(9)	0.09	(9)	0.20	0.25	0.20
	3	2.0	(8)	0.068	(9)	0.170	(9)	1.4	0.20	0.18
	4	22.0	(6)	0.16	(8)	1.2	(8)	10.0	0.36	0.31
	5	100.0	(5)	0.12	(7)	3.2	(7)	35.0	0.34	0.30
	7	172.0	(5)	4.0	(7)	194.0	(6)	72.0	0.47	1.3
	8	950.0	(1)	33.0	(6)	350.0	(6)	73.0	1.9	9.4
	9			10.0	(6)	640.0	(5)		1.9	25.0

* ( ) = Organoleptic scores = 10 to 5.5 = Acceptable
Less than 5.5 = Unacceptable

It may be concluded from these tests that the measurement of trimethylamine content of certain species of fish may not be a suitable index for the determination of freshness when tetracycline antibiotics are used as preservatives.

TABLE II - Effect of tetracycline antibiotics on the viable bacterial count, trimethylamine content and organoleptic score of lingcod muscle stored at 4°C.

Test	Days stored	Bacteria x 10^6/g muscle						TMA-milligrams nitrogen/100 g muscle		
		Control		CTC		OTC		Control	CTC	OTC
3	1	0.023	(9)*	0.006	(9)	0.006	(9)	0.47	0.35	0.41
	2	0.110	(8)	0.010	(9)	0.010	(8)	2.3	0.53	0.45
	4	50.0	(5)	3.0	(7)	34.0	(8)	53.0	0.54	0.54
	5	300.0	(2)	96.0	(7)	400.0	(7)	61.0	0.71	0.80
	6	-	(2)	290.0	(7)	970.0	(6)	56.5	1.2	4.9
	7			850.0	(6)	2000.0	(5)		2.0	4.4
	8			1400.0	(6)	3500.0	(3)		2.3	6.0
	9			4000.0	(4)	5100.0	(2)		14.0	31.0
4	1	0.23	(9)	0.110	(9)	0.08	(9)	0.21	0.21	0.20
	3	88.0	(6)	0.52	(8)	8.3	(8)	28.0	0.44	0.42
	4	88.0	(5)	6.0	(7)	180.0	(7)	57.0	0.78	2.6
	5	540.0	(2)	500.0	(7)	880.0	(6)	74.0	7.6	14.5
	7	-	(2)	-		-		68.0	24.0	72.0

* ( ) = Organoleptic scores = 10 to 5.5 = Acceptable
Less than 5.5 = Unacceptable.

SUMMARY NO. 31

INHIBITION OF THE ANTIBACTERIAL ACTIVITY OF THE TETRACYCLINES

B.A. Southcott
J.W. Boyd

The presence in sea water of factors influencing the sensitivity of certain microorganisms towards the tetracycline antibiotics has been noted; preliminary experiments in which various ions were added to the several agars used for antibiotic sensitivity tests have been described (Summary No. 17 of this Station's Annual Report for 1960-61). This investigation has been continued.

Experiments were carried out in which the test organisms used to inoculate antibiotic disc sensitivity plates were grown in synthetic broth with known additions of Mg^{++}, washed with solutions varying in Mg^{++} concentration and inoculated into synthetic agars with or without Mg^{++}. In such experiments employing synthetic media, growth of the organisms was poor; the limited results obtained did not indicate any significant difference in the tetracycline sensitivities of variously treated organisms.

In order to study further the interaction of the tetracycline antibiotics with various inorganic ions, the gradient plate technique was adopted. In this procedure, a Petri dish is slightly elevated at one side and 15 ml molten agar poured in then allowed to harden; this results in a wedge-shaped layer of agar. The dish is then levelled and a second portion of molten agar poured on top of the hardened layer, giving a second wedge inverted over the lower wedge. Various substances are added to each layer before pouring, the result being that the plate provides an axis of changing concentrations of the two substances. Since the use of synthetic agar resulted in poor, slow growth of the organisms used in these experiments, Eugonagar (BBL) was routinely employed in the preparation of gradient

plates. The ion being tested was added to the lower layer and the antibiotic to the upper layer of agar. The plates were surface inoculated by spreading 0.5 ml of a diluted overnight broth culture evenly over the surface of the hardened agar with a sterilized bent glass rod. The organisms used in this study had been isolated from fish or shrimp and were pigmented and rapidly growing types; the presence of pigment facilitated the interpretation of partly grown plates.

When the antibiotic is fully effective against the test organism, no growth will occur; when the ion in the bottom layer interferes with the action of the antibiotic, growth will take place at the side of the plate where the thick edge of the lower wedge is situated. By the gradient plate method it has been possible to demonstrate that certain ions (Mg^{++}, Mn^{++}, Ca^{++} and Fe^{+++}) interfere with the action of chlortetracycline (CTC) and oxytetracycline (OTC) against the several organisms used and that OTC is invariably more inactivated by these ions than is CTC.

Figure 1 - The effect of Mg^{++} and Ca^{++} on antibiotic activity of CTC and OTC. (Shaded areas indicate presence of bacterial growth.)

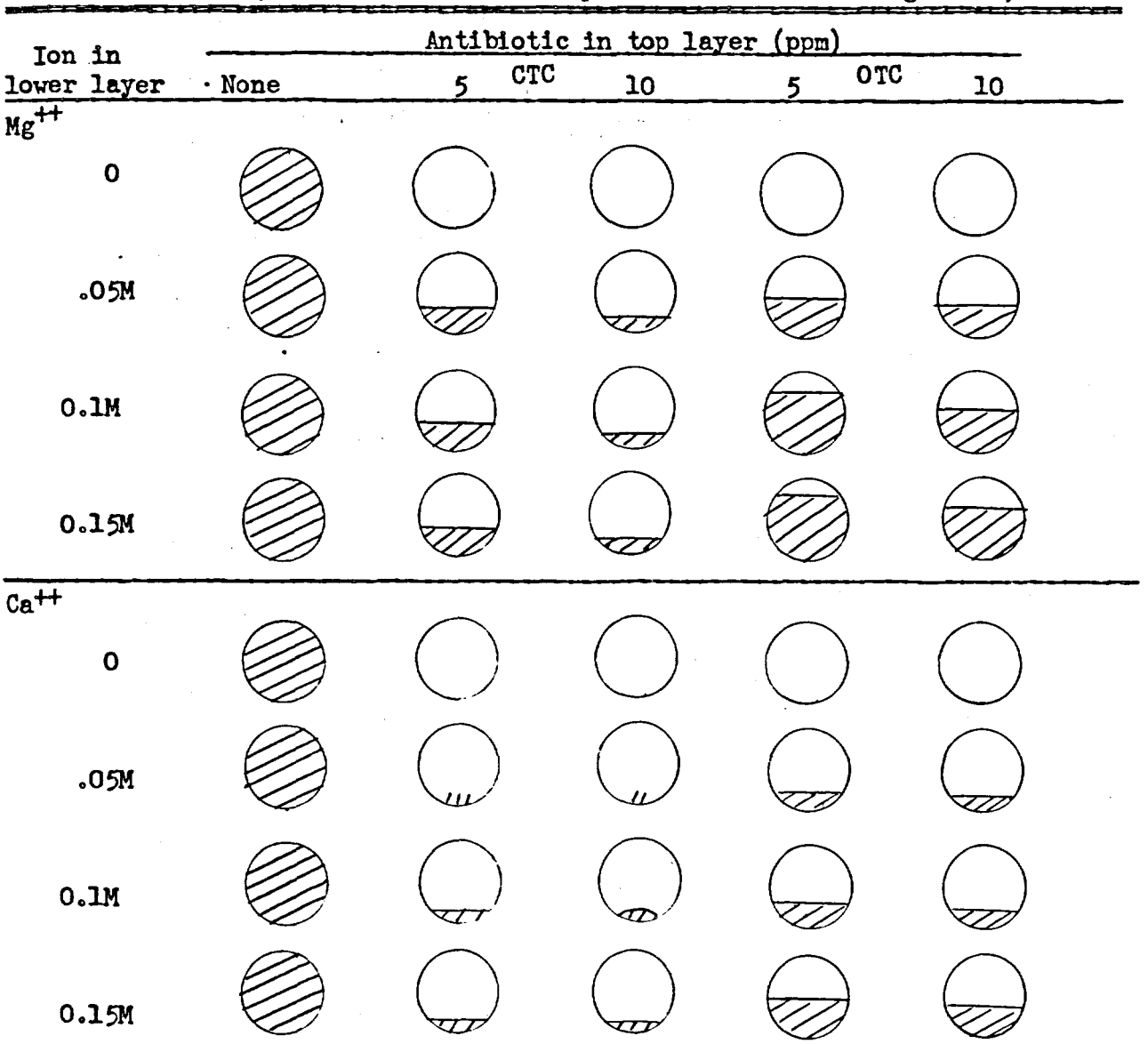
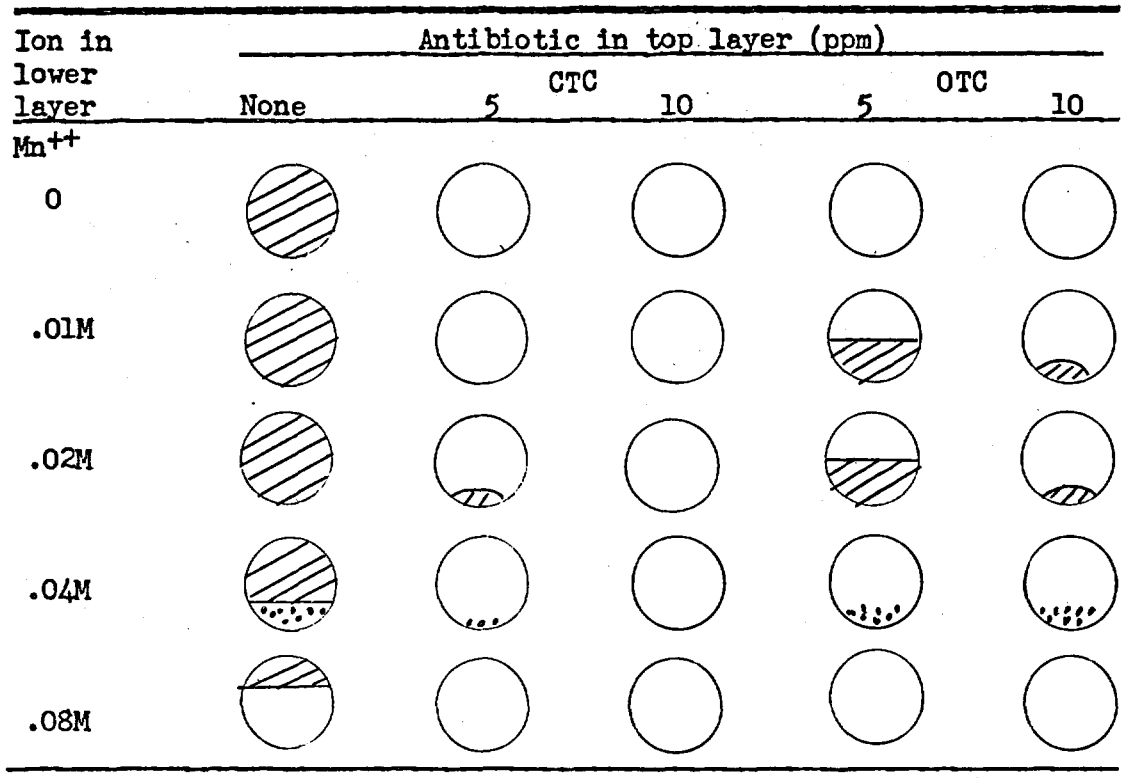


Figure 1 gives the results of a typical test involving the action of 5 and 10 ppm CTC and OTC as affected by Mg^{++} and Ca^{++}. The test organism was a yellow-pigmented

coccus which, in previous work, had been shown to be much less sensitive to tetracyclines on sea-water agar than on nutrient agar.

When Mn^{++} was tested it was found to be itself inhibitory to the organisms employed when used at a concentration of .04M in the gradient. Tested with two different organisms, Mn^{++} at a level of .02M inhibited somewhat the action of 5 and 10 ppm OTC and, to a smaller extent, the action of 5 ppm CTC (Figure 2).

Figure 2 - The effect of Mn^{++} on antibiotic activity of CTC and OTC.



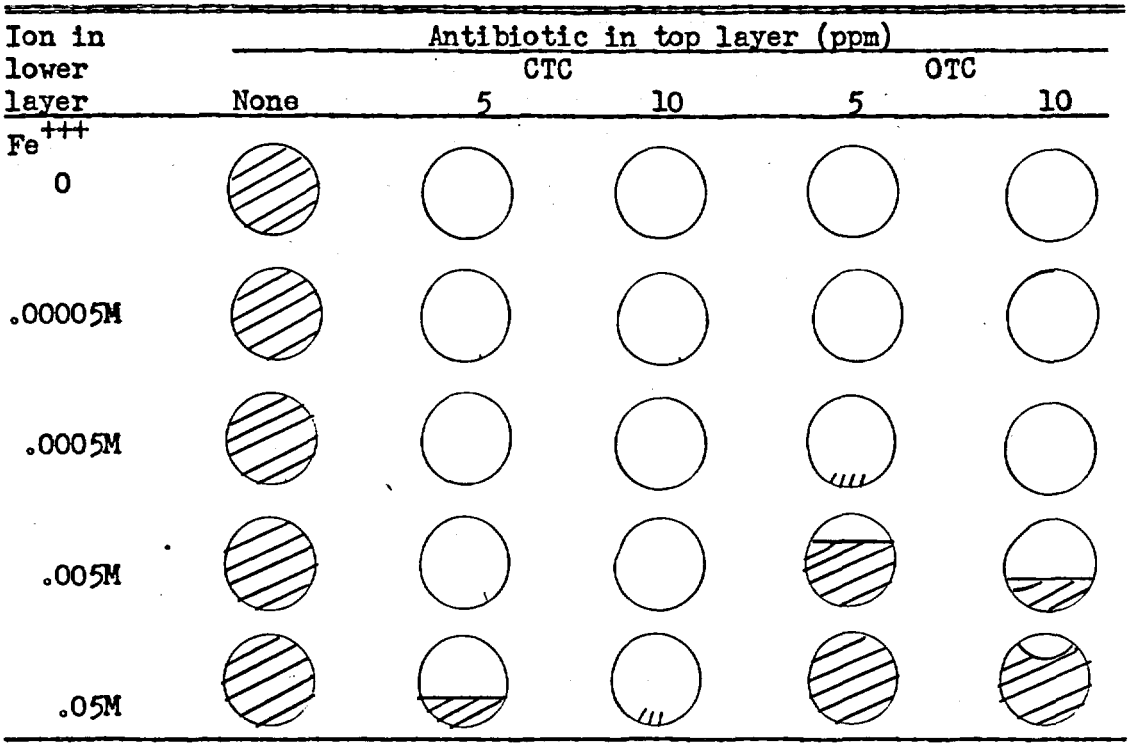
In the tests with Fe^{+++}, it was found that there was a greater difference between the extent of inactivation of OTC and of CTC than with the other ions tested. The organisms used were a Gram + coccus and a Gram + rod, both yellow pigmented. Results of these tests are in Figure 3.

From the results of the above experiments and many similar ones performed with different organisms, it appears that the presence of certain di- or trivalent cations may interfere to a considerable extent with the action of the tetracycline antibiotics. It has been found that higher levels of tetracycline antibiotics are needed for the preservation of shrimp than for the preservation of other fish (e.g. cod). For this reason it was thought desirable to test the effect of shrimp extracts upon tetracycline activity.

Preliminary experiments to determine the effect of shrimp extracts upon the sensitivity of microorganisms to the tetracyclines included: (1) testing of the antibacterial action of CTC solutions prepared in shrimp juice compared to those prepared in water; (2) addition of EDTA at various levels to shrimp juice agar and to Ca^{++} agar; (3) use of crossed filter-paper strips on hardened agar to test antagonism between antibiotic and shrimp solutions; (4) measurement of antibiotic disc zone diameters when the discs had been dipped into water or shrimp juice and then placed on inoculated regular agar or shrimp agar; (5) measurement of antibiotic disc zone diameters when dry discs were placed on inoculated agars of several kinds: nutrient

agar, modified sea-water agar, eugonagar, cod juice agar and shrimp juice agar.

Figure 3 - The effect of Fe^{+++} on antibiotic activity of CTC and OTC.



The results of the experiments indicated that when water extracts of cooked shrimp are used, inhibition of tetracycline activity could sometimes be demonstrated, but could not always be repeated. In one test, the CTC solution prepared in shrimp juice was as active against a shrimp organism as was the CTC solution prepared in water; in a second trial, the shrimp-CTC solution was only about 2/3 as active as the water preparation.

In a further attempt to demonstrate shrimp interference with tetracycline action, agar was prepared containing pieces of shrimp instead of merely the extracted juices. Nine-gram lots of finely chopped pieces of cooked shrimp or cod were weighed into flasks, 15 ml molten agar added and the suspension sterilized. These agars were used on the bottom layers of gradient plates; lumps of flesh made smooth spreading of layers and even inoculation difficult; however, results were obtained as shown in Figure 4.

In order to avoid the inconvenience of having pieces of fish or shrimp in the agar, the next series of tests was carried out using a paste made of equal weights of flesh and water ground in a Waring blendor. The required amounts of flesh homogenate were weighed into flasks, molten agar added, a square of cheesecloth tied over the neck of the flask instead of a cotton plug and the top covered by an inverted beaker. After sterilization had caused some coagulation and expression of juice, the agar, presumably containing substances from the flesh, was poured through the cheesecloth into the Petri dish, resulting in an agar layer substantially free of flesh particles.

When 2.5 and 5.0 gm of shrimp flesh (5 and 10 gm of homogenate) were added to agar, the activity of OTC was reduced, that of CTC was not. Cod agar used as a control did not affect OTC or CTC. A further test employed 5 and 10 gm lots of shrimp or cod in the agars; both levels of shrimp reduced the activity of OTC considerably, the

larger amounts of shrimp reduced the activity of CTC only slightly, and cod plates showed complete inhibition of the test organism by both antibiotics. Tests with other organisms and lower antibiotic levels produced almost the same results. Thus, when shrimp meat is homogenized and added to agar, the resulting inhibition of antibiotic action is not as extensive as when whole pieces of meat are added to the agar.

Figure 4 - The effect of shrimp and cod on antibiotic activity.

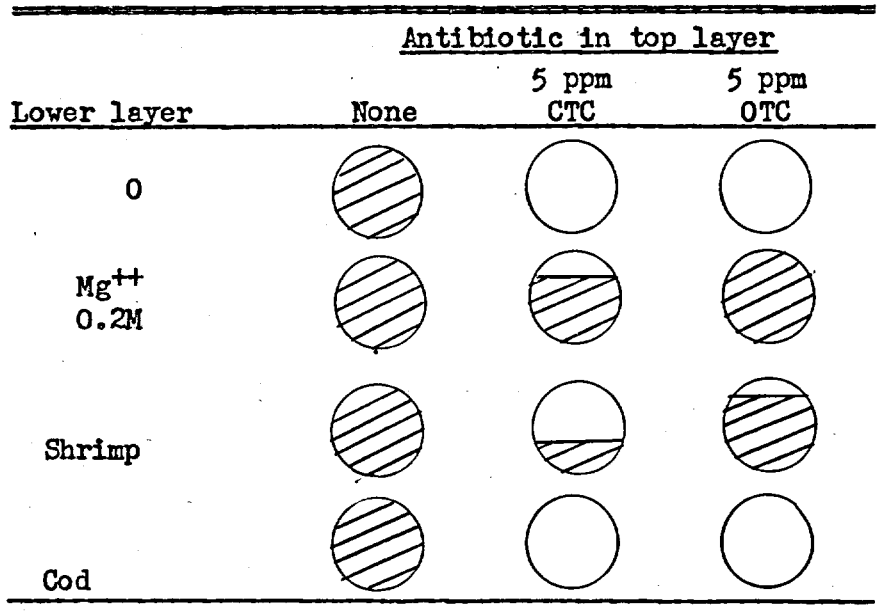
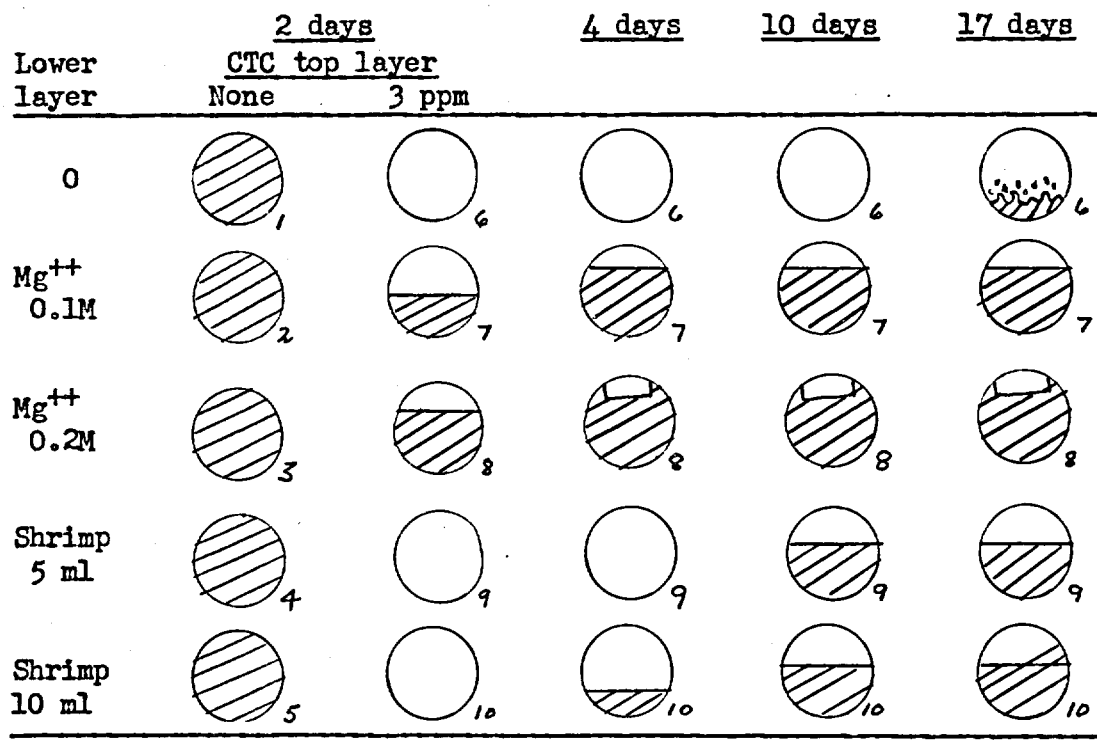


Figure 5 - The effect of shrimp on antibiotic activity.

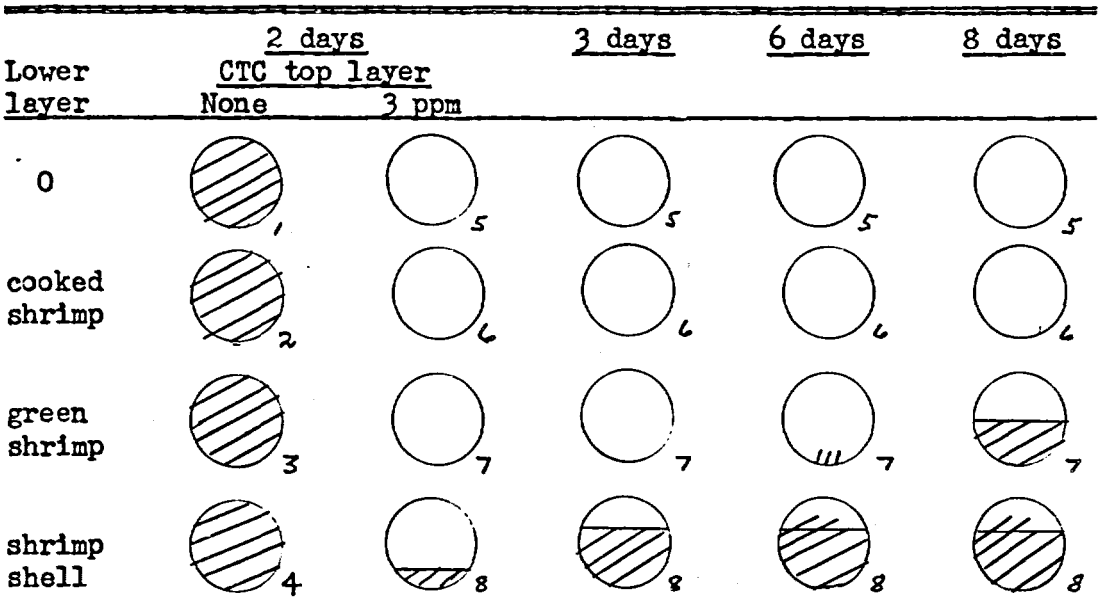


In order to obtain a more concentrated shrimp preparation to be added to the agars, shrimp juice was prepared by subjecting cooked shrimp meat to 15,000 lb pressure per square inch in a press. Double strength agar was prepared so that larger amounts

of juice could be added. When portions of juice representing 10 or 25 gm of shrimp meat were added to agars, there was inhibition of the antibiotic action of 2 ppm CTC but not of 5 ppm CTC. As had been noted in previous tests with various organisms, full growth on control plates and partial growth on plates containing antibiotic and a known ion was thick and pigmented at 24 or 48 hours; however, growth on plates where the antibiotic activity was being reversed by shrimp preparations developed more slowly. This can be seen in the results in Figure 5 in which long incubation was used. After 17 days the control CTC plate showed growth but it differed in appearance from the growth on the other plates.

When shrimp are treated with antibiotic under commercial conditions, the procedure is carried out on beheaded uncooked shrimp; for this reason a series of tests was planned using uncooked ("green") shrimp. It was found that shrimp with shells on could not be pressed successfully. The green shrimp were shelled by hand, the meat blended with water, autoclaved and filtered through glass wool; the shells were soaked in water, autoclaved then filtered similarly. In this way, substances usually extracted during the commercial cook and discarded were retained in the extracts. Figure 6 shows the results of a test using these preparations and a pressed cooked-shrimp sample.

Figure 6 - The effect of shrimp on antibiotic activity.

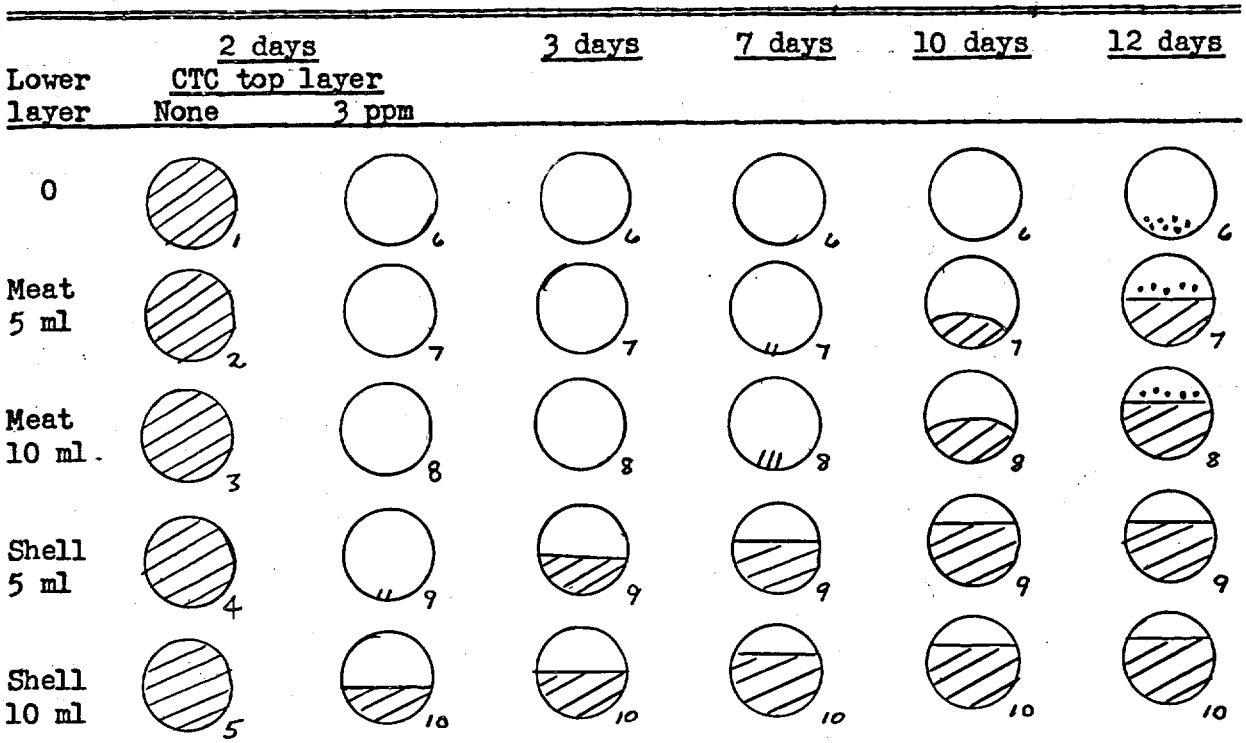


A similar extraction was made of the meat and shells of prawns; the resultant liquids were concentrated to half volume using a pyro-magnestir at 95-99°C. The pH's of these preparations were: meat 7.0, shells 9.0. Plates were prepared using extracts with unadjusted pH's; the results of the test are given in Figure 7.

The test was repeated using both samples at pH 7.0. After incubation for two days, heavy growth occurred on control plates; after 5 days there was considerable growth on plates containing shell extract, while only a slight sign of growth was seen on plates containing meat extract. At 7 days there was almost as much growth on the meat plates as on the shell plates.

The results of the above tests indicate that certain ions antagonize to a considerable extent the antibiotic action of CTC and, to a greater degree, that of OTC against certain organisms isolated from fishery products.

Figure 7 - The effect of shrimp on antibiotic activity.



When extracts of shrimp are used as a source of antagonistic substances, the inactivation of the antibiotic seems to extend over a longer period of time than when induced by a known cation. The extract of shrimp shell appears to be more active in antagonizing tetracycline action than does the extract of shrimp meat.

SUMMARY NO. 32

EFFECT OF ULTRAVIOLET LIGHT ON FISH SPOILAGE MICROORGANISMS

J.W. Boyd
B.A. Southcott

The purpose of this trial was to develop a method which could be used to study the germicidal effect of ultraviolet light (UVL) on microorganisms associated with the spoilage of different species of fish during storage in refrigerated brine.

For the initial tests gray cod fillets were stored at 0°C in a 3% sodium chloride solution contained in a polypropylene container. At intervals samples of the brine were removed for viable bacterial counts and for treatment with UVL.

In the first test, samples of brine were dispensed into sterile petri plates to a depth of 1 mm. The plates were placed approximately 7.5 inches beneath an 8-watt UVL germicidal lamp which was fitted with a reflector. After 5, 10 and 20 minutes of exposure to the radiation, samples were taken for viable bacterial counts.

In test 2 the brine samples were treated similarly to those in test 1 with the exceptions that the time of exposure to UVL was 10 and 15 minutes and the depth of brine solutions was 1 and 2 mm.

The results of the two initial tests are presented in the accompanying table.

The effects of time of exposure and depth of solution on the germicidal activity of ultraviolet light for the sterilization of chilled fish storage brines.

Test	Time of Exposure - min	Solution Depth - mm	Bacteria x 10^6/ml	% Kill
1	None - control		6.0	
	5	1	3.0	50
	10	1	0.20	97
	20	1	0.10	98
2	None - control		73.0	
	10	1	10.0	86
	15	1	7.7	90
	10	2	70.0	4
	15	2	9.9	86

SUMMARY NO. 33

MICROBIOLOGICAL STUDY OF DISEASED SALMON FROM HORSEFLY RIVER

J.W. Boyd

In collaboration with the Salmon Commission an attempt was made to isolate a microorganism(s) from infected gills of migrating sockeye salmon which were obtained from the Horsefly River spawning area during a period of high mortality.

Although it is desirable to make a primary isolation from diseased fish which are still alive or have just succumbed, it was not possible in this study to obtain fish alive or with a short post-mortem age. For the primary isolation, heads of infected salmon were collected at the spawning area and transported in ice to the laboratory. The post-mortem age at the time of examination was approximately 24 hours. Gross examination of the gills revealed that a type of proteolysis had taken place for small areas of gills appeared to have dissolved.

For the primary bacteriological isolation, swabs were obtained from the gills and inoculated into Eugon and Leetown Broths; inoculated tubes were incubated at 25°C. Also for the purpose of obtaining initial cultures, portions of infected gills were aseptically cut away with sterile scalpels and forceps and then placed in sterile petri plates. Eugonagar was poured over the gill portions in the plates and then the poured plates were incubated at 25°C. After subsequent transfers into broth and streaking onto agar, five pure cultures were obtained from the initial broth and agar mixed cultures. All cultures appeared to be Gram positive rods, either large or small, occurring singly, in pairs, and at times in short chains.

To test the proteolytic activity of the isolates fresh sockeye salmon gills were used for the protein material. A sockeye salmon was sacrificed, the gills were aseptically removed with sterile scalpels and forceps and then portions of the gills were placed into sterile petri plates. Five ml of Eugonagar (9°C) which had been inoculated with 0.1 ml of 18-hour broth cultures of organisms isolated from the gills of infected fish were poured into the plates containing the gill portions.

For control plates, uninoculated tubes of agar were poured similarly over gill portions contained in petri plates. Plates were incubated at 25°C. After 48-hr incubation the plates were examined for growth and proteolytic activity. From observations made on the controls it was quite evident that the gills which were obtained for this test were quite sterile because of the fact that colonies had not developed on any control plates. There was also no evidence of proteolysis taking place on the control plates. Three of the isolates were found to be proteolytic, with one culture being more proteolytic than the other two cultures. The gill portions appeared to inhibit the growth of the remaining two cultures since there were zones of inhibition around the gill portions in the agar.

To determine the pathogenicity of the proteolytic isolates toward migrating sockeye salmon, 18-hr broth cultures of each isolate were prepared in physiological saline, and either injected into the heart or body cavity or sprayed onto the gills of migrating salmon at Cultus Lake. Subsequent to treatment the salmon were placed in an isolated area and observed daily.

Since there was not any method available at the time of the initial trial to control the temperature of the water, which had dropped considerably, the fish were sacrificed after one month. At this time swabs of the gills and pieces of gill arches were taken for bacteriological examination. There was evidence of a physiological change in the gill tissues. Isolates obtained from swabs of the gills and from the gill arches were found to be morphologically similar to the cells which were initially injected into the salmon.

This collaborative problem will be continued when facilities for controlling water temperature are available at Cultus Lake.

SUMMARY NO. 34

A STUDY OF CERTAIN ORGANISMS ISOLATED FROM DISEASED FISH — B.A. Southcott

Among fish held in aquaria at this Station there appeared some whose external appearance resembled that described for the disease "fish furunculosis". Reports in the literature indicated that Aeromonas salmonicida had been established as the causative organism of fish furunculosis, and attempts were made to isolate this organism from the diseased fish in question.

In one test, the external surfaces of typical lesions in five diseased salmon were swabbed; the swabs were used to inoculate tubes of Brain Heart Infusion broth. In 24 hours dilutions were made of the well-grown broth cultures and streak plates on Eugonagar (BBL) were prepared. After a 24-hour incubation at 25°C, the several plates had white, shiny, abundant growth of identical appearance. Gram stains from each plate showed short, wide, Gram-rods, occurring mostly singly but with a few pairs and short chains. There was deeper staining at the ends of each cell. Later stains showed a more variable morphology, especially from broth cultures. Single colonies were picked into broth.

Plates of Eugonagar were surface-spread from a pure broth culture and antibiotic discs were applied. The organism was found to be sensitive to CTC, OTC, TC, DCTC; it was slightly sensitive to streptomycin and not sensitive to penicillin.

Three-day old plates on Eugonagar showed a slight brown coloration of colonies; at 6 days the pigment had become darker brown and had diffused slightly

into the medium. When 1.0% aqueous p-phenylenediamine was added to such a plate and then poured off, the streak of growth immediately turned black-purple in colour. This positive reaction is typical of Aeromonas salmonicida; however, it could not be repeated with later transfers of the isolate on Eugonagar or on a special "Leetown Standard Furunculosis Medium".

The organism was found to grow well at temperatures from 5°C to 37°C (the highest tested) but did not grow at 0°C. These temperature relationships do not correspond to those described for Aeromonas salmonicida. On NaCl-containing agars the organism grew luxuriantly with 5%, slightly with 10% and not at all with 15% of the salt. Nutrient gelatin first showed infundibuliform and later complete liquefaction; litmus milk was slightly acidified and then peptonized; the organism was motile. Further tests of the antibiotic sensitivity of the organism were made using antibiotic discs (Table I, Organism 1). Acid and gas production from carbohydrate broths containing brom cresol purple as the indicator was studied (Table II, Organism 1). The carbohydrate reactions were not at all similar to those reported for Aeromonas.

TABLE I - Antibiotic sensitivities of isolates from diseased fish.

		Zone diameter (mm) Organism					
Antibiotic	Conc	1	2	3	4	5	6
Chlortetracycline	10 mcg	20	19	27	25	24	25
Oxytetracycline	10 "	21	21	24	25	25	21
Demethylchlortetracycline	10 "	19	19	26	26	26	23
Tetracycline	10 "	18	17	25	26	25	22
Penicillin	5 units	0	0	0	0	0	0
Streptomycin	5 mcg	9	8	15	+	0	0
Triple Sulfa	1 mgm	23	23	23	46	46	29
Chloromycetin	30 mcg	23	23	13	15	18	16
Furoxone	100 "	10	10	11	0	0	0
Furadantin	100 "	8	8	0	0	0	0
Neomycin	5 "	+	+	10	12	12	0
Kantrex	30 "	10	13	18	16	23	13

TABLE II - Carbohydrate reactions of isolates from diseased fish.

	Organism					
Carbohydrate	1	2	3	4	5	6
Dextrose	ag	ag	a		a	
Maltose	"	"		ag		
Arabinose	"	"	a	"		
Galactose	"	"	a			
Fructose	"	"				
Starch	"	"	ag	ag		
Lactose	"	"				
Xylose	"	"	ag		a	
Raffinose	"	"				
Sucrose	"	"				
Glycerol	"	"		a		

a = acid produced
g = gas produced

In a second test, swabs were taken into Leetown broth from an open scrape on the side of a diseased Rainbow trout. The preliminary stains showed Gram-rods of various lengths, with rounded ends and deeper staining at each end of the cells. Other stains and tests indicated that this organism was identical with that isolated in the former test. The results of antibiotic and carbohydrate tests are in Tables I and II (Organism 2).

Further isolations were made from various sites on both living and dead diseased fish: the outside of swollen areas, open lesions, blood and muscle in lesion area, kidney and fin. In no case did the reactions of the isolates indicate that they might be Aeromonas salmonicida (Organisms 3, 4, 5, 6 in Tables I and II are examples). Classification of the organisms was not attempted as the occurrence among the aquarium fish of diseased specimens was not widespread.

SUMMARY NO. 35

BACTERIAL CLASSIFICATION STUDIES

B.A. Southcott

In a continuation of the classification studies described in Summary No. 19 of this Station's Annual Report for 1960-61, further tests were carried out. A study was made of the extent of growth and degree of pigment production of 34 Gram + cocci and 30 Gram + rods on agars of varying NaCl concentration (0, 5, 10 and 15%). Indole, Methyl Red and Voges-Proskauer tests were also done on the cocci.

Further intensive work on the classification of large groups of microorganisms isolated from fishery products has, for the present, been suspended, as it is felt that no useful purpose is served by this procedure unless the organisms concerned are connected with some specific problem under investigation in this laboratory. Surveys of the bacterial population of various kinds of fish are being made at many institutions using the newer methods involving computer analysis of very large numbers of reactions; this large-scale procedure appears to offer the most promise for future fundamental classification studies.

BIOCHEMISTRY OF FISH MUSCLES

SUMMARY NO. 36

POST-MORTEM BIOCHEMICAL CHANGES IN FISH MUSCLE
A - INTERRELATIONSHIPS BETWEEN CHANGES IN PROTEIN SOLUBILITY, PROTEIN-BOUND NUCLEOTIDES, THE COURSE OF RIGOR MORTIS AND FREEZING AND FROZEN STORAGE OF FISH MUSCLE

N. Tomlinson
Shirley E. Geiger

Last year the course of rigor-mortis in certain species of fish and some of the biochemical changes in the muscle that accompany the process were investigated (Summary No. 26 of this Station's Annual Report for 1960-61). This year a study is being made of the influence of the stage of rigor mortis in which a fish is frozen on the solubility of the muscle proteins, both during freezing and subsequent frozen storage. The possible role of protein-bound nucleotides in changes in protein solubility is also being investigated.

During the frozen storage of fish denaturation of protein occurs and it has been shown by Dyer (1950) and others that this denaturation is confined almost exclusively to the globulin (high ionic strength-soluble) fraction of the muscle proteins and particularly to actomyosin.

It has been reported by the Torry Research Station (Annual Report, 1959) that a greater amount of protein is extractable in 5% salt solution from cod frozen pre rigor than from those frozen after rigor mortis at all stages of frozen storage. On the other hand Nikkila and Linko (1954, 1956) found that in Baltic herring less protein was extractable with 5% NaCl solution from fish frozen pre rigor than from those frozen in rigor mortis or post rigor, although with fish in the fresh condition these workers found that protein solubility, was lower during rigor than at other times. Similarly Partman (1960), working with trout and carp, observed that less actomyosin was extractable during frozen storage from fish frozen at the time of killing than from muscle from the same fish held 1 to 4 days at 5° to 10°C before being frozen. The reason for these different findings is not clear. They could have resulted from species differences. A second possibility is that while the Torry group was apparently working with commercially caught fish, Partmann was using essentially unexercised fish. It is interesting that Migita and Otake (1961) found that somewhat more protein was extractable in salt solution from carp allowed to struggle to death before freezing than from fish killed immediately after capture. The condition of Nikkila and Linko's fish at death was not described. Work from several laboratories has shown that, among other differences that can occur, very different quantities of adenine nucleotides are present in fish muscle at death depending on the degree of exhaustion of the fish, and one of these nucleotides (ATP) can very greatly influence the state of both actin and actomyosin in vitro. Also, recent work by Jones (1961) has indicated that different quantities of adenine nucleotides are present in cod muscle post-rigor depending on the degree of exhaustion of the fish at death. Although the quantities of these are very small, it is likely that a large part of them are bound to actin. Finally, a third possibility is that different experimental methods used by the investigators have influenced the results.

The rate of freezing has been shown by Reay and by Dyer and others to have a definite influence on the solubility of actomyosin and that this was related to the time taken for the flesh to cool through the range between -2° and -6°C. In this connection it is interesting that Partmann (1961) has shown that ATP disappears at a very much greater rate from fish muscle at temperatures within this range than it does at somewhat higher or at lower temperatures.

Connell (1960) has investigated the changes that occur in the actin of cod muscle during storage at -14°C and, while his results indicate a very slow loss of extractable actin, this loss occurred more slowly than did the loss of solubility of actomyosin in 5% NaCl. Connell suggested that changes in myosin, rather than in the actin moiety, were chiefly responsible for the observed loss of solubility of actomyosin. He pointed out, however, that the changes he observed in extractable actin might be the result either of slow denaturation of actin or of loss or alteration of the bound nucleotide, but that his results could not definitely distinguish between these possibilities. Since Straub and Feuer (1950) demonstrated the presence of adenosinetriphosphate in G-actin (and that this ATP was split to ADP during the polymerization of G-actin with the formation of F-actin) this protein-bound nucleotide has been the object of numerous investigations and evidence is accumulating that it is either essential for several characteristic properties of the protein or for their protection. Thus Barany, Nagy, Finkelman and Chrambach (1961) have found that if ATP is removed from G-actin the protein loses (a) its ability to polymerize to F-actin, (b) to combine with myosin at high ionic strength, and (c) to activate the ATPase activity of L-myosin at low ionic strength. Certain procedures which remove ATP from

G-actin do not remove ADP from F-actin and do not result in the impairment of certain properties of F-actin [(b) and (c) above] that are common to both forms.

Methods

Lingcod were caught off the mouth of the Fraser River and held live at the Vancouver Public Aquarium until needed. Rainbow trout were obtained from the Sun Valley Trout Farm, Port Coquitlam, B.C. and were held live at the Station. Non-protein nitrogen was measured by microkjeldahl procedure and protein N by the same procedure or by the biuret method (Snow). Extraction of muscle protein was carried out by homogenizing 2.5 g samples of lateral-dorsal muscle in 60 ml of the appropriate extractant with an omni-mixer operated at about one half maximum speed for 70 seconds, the homogenizer being stopped and restarted at 10-second intervals. A plastic baffle plate was fitted on the drive shaft of the homogenizer in such a way that it was submerged in the extractant during homogenization and thus prevented vortex formation and consequent foaming. Following homogenization, the suspension was made up to a volume of 250 ml with extractant and then placed on a reciprocating shaker (132 one and one-half inch strokes/min) in a stainless steel centrifuge bottle for 1 hour. The suspension was then centrifuged 15 min at 14,600 g and the protein in the supernatant solution was measured. Extracts of lingcod muscle in 5% NaCl-0.02 M $NaHCO_3$ or KCl-borate buffer could be shaken for 20 hours under these conditions with only very small losses in protein solubility. If the extract was shaken in a glass Erlenmeyer flask, however, very large losses in soluble protein occurred. All procedures were conducted at 0°C. Determination of total nitrogen in duplicate samples of lingcod and trout muscle yielded values of 29.4 and 32.3 mg N/g muscle and of non-protein nitrogen 4.76 and 3.85 mg N/g muscle, respectively.

Fish were killed by a heavy blow on the head. When desired they were "exhausted" before killing by being made to swim against a current for 30 minutes, then being placed in air for 10-15 minutes.

Results

1. Studies with lingcod muscle

In order that the protein-bound nucleotides of muscle may be examined, it is necessary that they first be separated from the very much greater quantity of "free" nucleotides present in the muscle. One means of accomplishing this is to remove these free nucleotides from the muscle by extracting it with a buffer of low ionic strength. Such a procedure was used by Perry in studying the bound nucleotides of the myofibrils of rabbit muscle and in the present work Perry's procedure has been modified for use with lingcod muscle. The data in Table I show that extraction by the modified procedure (B), using KCl-borate as extractant gives results that are quite comparable with respect to phosphorus remaining in the washed muscle residue to Perry's method (A), and has the advantage of being much less time-consuming. As will be shown below, practically the whole of the acid-labile P in the residue can be accounted for as adenosinediphosphate (ADP). The results for the seven samples extracted with KCl-borate indicate that quantitative agreement between different samples is only fair and that small changes occurring during storage would be difficult to detect. KCl (0.1 M) or KCl plus EDTA as extractants gave results similar to KCl-borate. Under certain circumstances EDTA is known to remove bound nucleotide from G-actin, but it had no apparent effect on bound nucleotide under the present conditions. The use of water alone as an extractant resulted in a lowered recovery of acid-labile P in the washed residue and the addition of mersalyl to the KCl-borate buffer caused nearly complete loss of acid-labile P. This last finding was somewhat unexpected as it has been reported that F-actin containing ADP can be prepared from

mammalian muscle washed with mersalyl solution, although this reagent removes ATP from G-actin. There seems to be a discrepancy between the effects of EDTA and mersalyl that should be examined further. The use of $NaCl-NaHCO_3$ solution as extractant, as expected, resulted in the solution of most of the protein in the KCl-borate washed residue, and this was accompanied by an almost complete loss of bound phosphorus. It was noted that more protein could be extracted by a single extraction with $NaCl-NaHCO_3$ than by two extractions with KCl-borate followed by one with $NaCl-NaHCO_3$ so that some denaturation of protein must be occurring during the KCl-borate extraction. As will be seen later, mersalyl also dissolves much of the muscle proteins not usually soluble in low ionic strength solutions.

TABLE I - Phosphorus in lingcod muscle residues after extraction by different procedures and extracting solutions.[1]

Procedure[2]	Extractant[3]	P in residue[4] µg/gm muscle PML	PAL	PAS	PT
A	KCl-borate	12.4	12.4	14.8	39.6
"	" "	12.0	12.0	14.4	38.4
"	" "	11.5	12.5	14.4	38.4
"	" "	10.4	12.0	16.0	38.4
B	" "	13.8	10.0	12.0	35.8
"	" "	12.6	12.2	12.0	36.8
"	" "	15.8	13.2	14.7	43.7
"	KCl	12.5	10.8	22.3	45.6
"	KCl, EDTA	14.6	13.0	23.8	51.4
"	H_2O	12.0	7.9	18.9	39.0
"	KCl, borate, mersalyl	6.6	1.2	3.0	10.8
C	$NaCl, NaHCO_3$	0.7	0.7	1.2	2.6

[1] All muscle samples extracted had been frozen in liquid N_2 in pre-rigor condition and stored for a few days at -30°C before being used.

[2] Procedure A. Muscle homogenized in 9 volumes of extractant, centrifuged at low speed (600xg), resuspended and rewashed in fresh extractant 9 times. This and the following procedures were all carried out at 0°C.
Procedure B. Muscle homogenized in 100 volumes of extractant, centrifuged at high speed (14,600xg), resuspended and rewashed once in same volume of fresh buffer.
Procedure C. Muscle homogenized and washed as in B with KCl-borate, then once with 100 volumes of extractant.

[3] Extractants: KCl, borate (0.025 M, 0.039 M, pH 7.1)
KCl (0.1 M)
KCl, EDTA (0.1 M, 0.004 M sodium ethylene diamine tetra-acetate)
KCl, borate, mersalyl (0.025 M, 0.039 M, 0.12 mg/ml)
$NaCl, NaHCO_3$ (5%, 0.02 M)

[4] PML = Inorganic P plus molybdate labile P.
PAL = Acid-labile P.
PAS = Acid-stable P.
PT = Total P in washed muscle residue extractable in 10% trichloracetic acid. This is equal to the sum of PML, PAL and PAS (for details see Summary No. 26 of this Station's Annual Report for 1960-61).

A preliminary examination of the bound nucleotides has been made by means of column chromatography. Samples of lingcod muscle frozen prerigor in liquid nitrogen then held at -30°C for a few days or at -10°C for 11 months were extracted by the modified procedure. The washed residue was extracted with 4% perchloric acid, and the extract, freed from protein by centrifugation, was neutralized with 4 M KOH and the precipitated potassium perchlorate removed by centrifugation. The clear extract was then chromatographed on a column of Dowex 1(x8) in the chloride form. Elution of nucleotides was carried out by stepwise procedure using increasing concentrations of hydrochloric acid (Fig. 1). All procedures were carried out at 0°C.

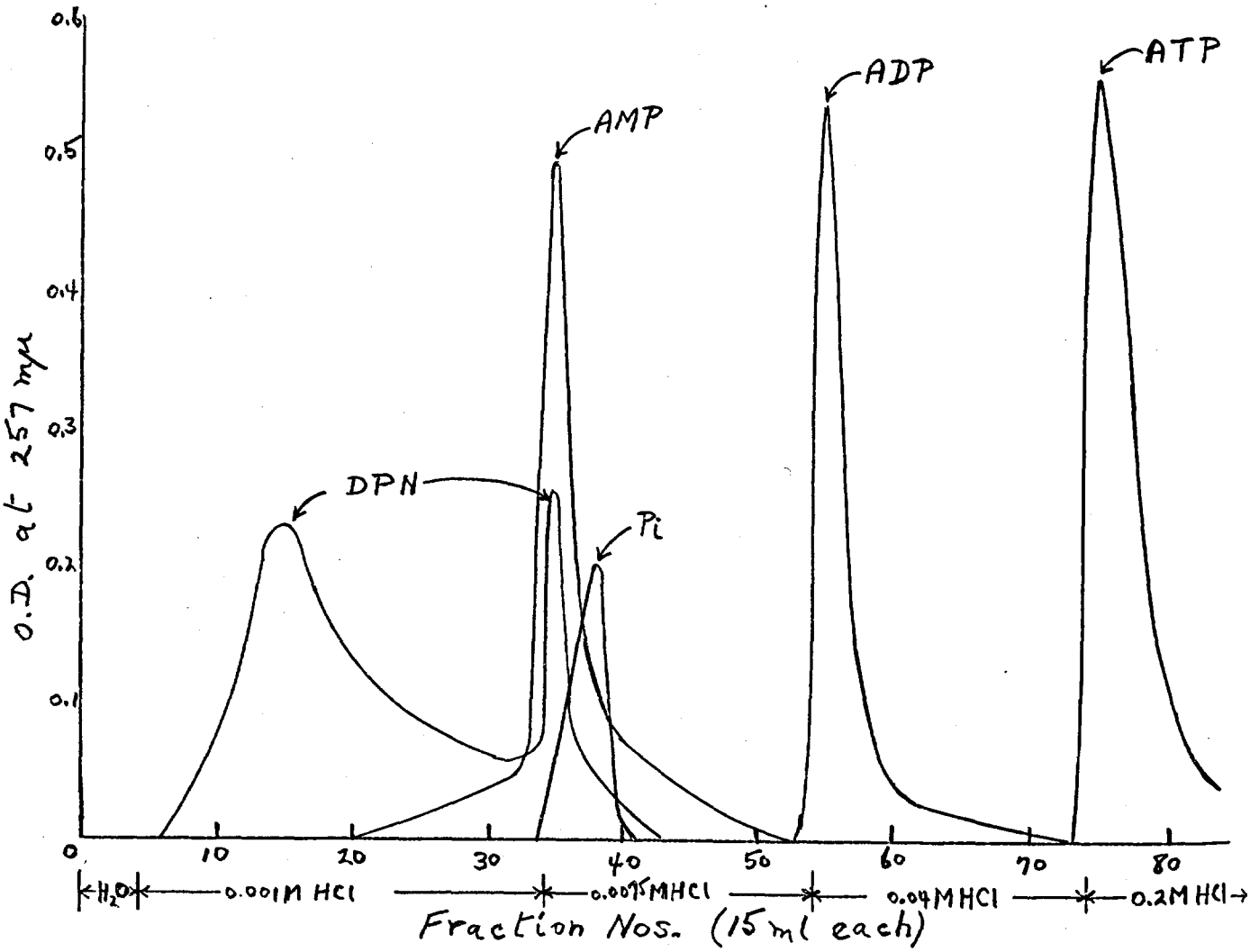


Fig. 1. Chromatography of known compounds on Dowex 1x8, Cl^- (60 mesh). Column 1 cm x 14 cm, refrigerated. Compounds applied to the column in 20 ml of solution containing .023 M KCl and .036 M sodium borate. AMP, ADP and ATP are adenosinemono-, adenosinedi- and adenosinetriphosphate, respectively. DPN is nicotinamide-adenine dinucleotide. The second peak that appears (in 0.0075 M HCl) when DPN is chromatographed is presumably adenosine diphosphoribose present as an impurity. Pi is inorganic phosphate.

The data (Table II) show clearly that adenosinediphosphate accounts for well over 90% of the total protein-bound nucleotide in preparations of this kind from both freshly frozen and severely denatured frozen lingcod muscle. This seems to be in accordance with expectations, for any G-actin present in the muscle would presumably polymerize during the washing procedure. ADP was identified on the basis of its chromatographic behaviour, its ultraviolet absorption spectrum, and its adenine: acid labile phosphorus:total phosphorus ratio (1:0.97:1.91). The quantities of ADP found are similar to those found by Saito et al (1959) in acid extracts of whole post-rigor muscle of rainbow trout killed in an unexercised condition but the amounts of AMP and ATP are much smaller. The quantities of all 3 nucleotides are quite different from the quantities found by Jones and Murray (1961) in post rigor muscle from cod killed in either an unexercised or exhausted condition. They are also very different from quantities reported by Jones and Murray (1961) to be found in "leached" cod muscle homogenates. Differences in technique may account for the latter discrepancies. Too little work has yet been done to be able to attach any significance to the apparent relatively small decrease in bound ADP during storage at -10°C. If the assumption is made that all the ADP was bound to actin and that the actin has a molecular weight of 62,500 similar to that of rabbit actin (Connell has calculated a molecular weight of an apparent dimer of cod actin to be 130,000) then only about 14% of the extractable protein of lingcod muscle is actin.

A second set of comparable muscle samples was examined by a different procedure. In this part of the work actin was extracted from the muscle according to Connell's (1960) slight modification of the procedure of Barany et al (1957). The extract of actin (pH 7.6) was treated with 40 mg of Dowex 1x8 Cl^- per ml for 5 minutes at room temperature to remove free nucleotides and inorganic phosphate. Sufficient ice-cold 50% perchloric acid was then added to the treated chilled extract to bring the perchloric acid concentration to 5%, the precipitated protein was removed by centrifugation, the supernatant solution neutralized with cold 4 M KOH and the clarified extract was chromatographed as above.

The data (Table III) show that ATP is the predominant nucleotide. This is again in accordance with expectations, for G-actin would be the form of actin expected to be extractable under the conditions used. The fact that G-actin can apparently be extracted from severely denatured muscle is in accordance with Connell's previous findings and the small decrease in ATP on long storage also is in agreement with his observations of decrease in "active" extractable actin. The amount of protein in these extracts suggests that they contain other proteins in addition to actin but the ratio of nucleotide to protein is similar to that reported for crude actin extracts prepared from rabbit muscle by Szent-Györgyi (1951).

In Table IV data are presented showing the differences in extractability of lingcod muscle protein in high ionic strength solution and in low ionic strength solution with mersalyl (O-[(3-hydroxymercuri-2-methoxypropyl) carbamyl] phenoxyacetic acid, sodium) added. It appears that the use of mersalyl (a reagent that combines with sulfhydryl groups) reveals changes in protein during frozen storage that do not appear to be detected by the use of NaCl-$NaHCO_3$. The rather large difference between the quantities of protein extractable from the freshly-killed fish by the two extractants has not always been seen, but the small increase in solubility in NaCl-$NaHCO_3$ and definite decrease in solubility in KCl-borate-mersalyl during freezing seems to be consistent for lingcod killed in this condition. In the late stages of loss of protein solubility in NaCl-$NaHCO_3$ it is clear that protein is much more readily extracted by mersalyl than by the high ionic strength salt solution. It appears that mersalyl might be a useful reagent in studies of protein denaturation in fish muscle.

TABLE II - Nucleotides from washed muscle residue of lingcod frozen pre-rigor in liquid N_2.

	Stored 1 month at -30°C µM/gm muscle[1]		Stored 11 months at -10°C µM/gm muscle[2]
Sample No.	(1)	(2)	(3)
DPN	-	-	-
AMP	-	0.013	-
ADP	0.362	0.384	0.313
ATP	-	0.009	0.015
Pi	0.645	0.82	0.67

1 - = none detected
Identity of AMP and ATP not certainly established - quantity shown is maximum possible (U-V absorption spectrum suggests adenine, but not pure.)

2 A very small amount of material with U-V absorption spectrum similar to adenine emerged earlier than expected for DPN. Trace of material possibly ATP - but U-V absorption spectrum showed impure.

Protein	mg prot N/gm muscle soluble in	-30°C storage	-10°C storage
	KCl-borate (0.025 M, 0.039 M pH 8.3)	8.64	7.0
	NaCl 5%, $NaHCO_3$ 0.02 M	27.32	(12.0) 7.74[3]
	KCl-borate + 1 mg/ml mersalyl	22.20	(14.2) 12.58

3 Figures in brackets show prot N extracted in 20 hours, unbracketed figures in 1 hour.

TABLE III - Nucleotides in a crude actin extract from pre-rigor frozen lingcod muscle.

	Stored 1 month at -30°C µM/gm muscle[1]		Stored 11 months at -10°C µM/gm muscle[2]
Sample No.	(1)	(2)	(3)
DPN	-	-	-
AMP	-	-	-
ADP	-	0.020	.016
ATP	.129	0.100	.088
Pi	-	-	0

1 AMP and ADP not identifiable in sample (1), apparently result of use of too little Dowex in pre-treatment.

2 Value for ADP is a maximum as U-V absorption spectrum of eluate shows impurities were present (IMP?).

Protein	Prot N in actin extract (mg/gm muscle)		
	(1)	(2)	(3)
	3.34	5.46	4.19

TABLE IV - Solubility of lingcod muscle proteins during frozen storage.

Storage of muscle	mg prot N extracted/gm muscle[1] Extracting solution: $NaCl-NaHCO_3$ (5%, .02M)	KCl-borate-mersalyl (.025M, .039M/mg/ml pH 8.3)
Freshly killed[2]	15.8	18.4
Frozen in liquid nitrogen	17.96	13.3
do, then stored 1 mo at -30°C	18.7	13.6
do, " " 11 mos " -30°C	14.96	13.1
do, " " 11 " " -20°C	11.2	10.
do, " " 11 " " -10°C[3]	0.74(5.02)	5.6(7.2)

[1] Protein extracted at low ionic strength subtracted (7.0 to 8.6 mg protein N/gm muscle).

[2] Fish killed in a rested condition after being held in captivity about one month.

[3] Values in brackets are maximum quantity extracted after the homogenate was shaken for 20 hrs at 0°C. Unbracketed values were those extracted in 1 hour from the time of homogenization.

2. Studies with rainbow trout muscle

This portion of the work is being directed towards investigating the relationship, if any, that exists between the various stages of rigor mortis and the solubility of muscle protein and also the influence that the stage of rigor in which a fish is frozen has on protein solubility, in the hope that it might be possible to reconcile some of the apparently contradictory findings that have been recorded in the literature.

The trout used were all 10 to 12 inches long and weighed an average of about 250 g. In order to avoid as much as possible differences in protein concentration that are known to exist between muscle samples taken from different sections along the length of a fish, samples (one from each side of the fish) were taken from the lateral-dorsal muscle in a region about $1\frac{1}{4}$ inches in length centered below the leading edge of the dorsal fin. When comparison of soluble protein extractable from a number of fish sampled by this procedure was made (Table V), it was found that larger variations occurred between individual fish than between samples from the same fish. As a result, in order to reduce errors that would be introduced by making comparisons between different fish following different treatments, the practice of taking two samples only from each fish, one at the beginning of the experimental treatment and the second at the end, was adopted. In this way changes resulting from experimental procedure were always measured in the same fish and comparisons could be made between changes occurring in different fish.

In view of the observations made during the work with lingcod (above) regarding the influence of mersalyl on muscle protein solubility, a comparison was made between $NaCl-NaHCO_3$ and mersalyl (0.002 M) solutions as protein extractants. Results are tabulated in Table VI. The mersalyl solution appeared to extract a little more protein from prerigor muscle than did the NaCl solution, although the difference may not be significant.

Changes in protein solubility during the course of rigor mortis were examined (Tables VII and VIII). There does not appear to be any significant change in protein solubility until the fish has begun to soften or has become quite soft again following

rigor. It also appears that this either does not occur in all fish treated in this fashion (held in the round) or else there are considerable differences in the time post mortem when it occurs. The latter possibility is the more likely, as losses have always been observed in such fish that have reached an advanced stage of post rigor softening (see also Table IX). These findings seem to indicate that no important degree of denaturation occurs during rigor mortis but that it may begin as rigor resolves. It is possible that milder methods of homogenization and shorter extraction periods would reveal protein solubility changes during rigor. This will be investigated, but if such changes occur in these fish under our conditions of storage they must be reversible during the extraction procedure.

A limited investigation of the effect of very rapid freezing (in liquid nitrogen) on protein solubility has been made (Table IX). Apart from the possibility that freezing in this manner during post rigor softening may hasten denaturation, it does not appear to have any marked influence on protein solubility.

TABLE V - Soluble protein nitrogen extractable from lateral dorsal muscle from opposite sides of the same fish[1].

Fish No	Condition	1st side mg N/g muscle	2nd side mg N/g muscle	Difference mg N/g muscle
1	Unexercised	24.1	23.9	- 0.2
2	"	25.5	23.9	- 1.6
3	"	25.5	25.3	- 0.2
4	Exhausted	22.8	24.6	+ 1.8
5	"	22.7	23.2	+ 0.5
6	"	21.4	22.1	+ 0.7
1	Unexercised-frozen[2]	23.8	22.9	- 0.9
2	" "	21.4	22.2	+ 0.8
3	" "	24.7	24.8	+ 0.1
4	Exhausted- "	23.4	21.7	- 1.7
5	" "	23.4	23.1	- 0.3
6	" "	22.7	21.9	- 0.8

[1] Extractant, NaCl 5%, $NaHCO_3$ .02 M.
[2] Frozen in air at -30°C and stored for 20 hours.

TABLE VI - Protein nitrogen extractable by different extractants from opposite sides of the same fish.

Fish No	Condition	Extractant[1] $NaCl-NaHCO_3$ mg N/g muscle	Extractant[1] Mersalyl mg N/g muscle	Difference mg N/g muscle
1	Unexercised-freshly killed	23.9	24.8	+ 0.9
2	" "	22.5	23.7	+ 1.2
3	" "	22.2	23.2	+ 1.0
4	Exhausted "	24.4	25.7	+ 1.3
5	" "	25.6	26.2	+ 0.6
6	" "	22.8	24.2	+ 1.4

[1] NaCl 5%, $NaHCO_3$ 0.02 M.
Mersalyl (sodium) 0.002 M.

TABLE VII - Changes in quantity of protein nitrogen extractable from trout muscle during the course of rigor mortis[1].

Fish No	1st sample Taken at time of killing mg N/g muscle	Time hrs	Condition of fish	2nd sample Taken at post mortem time indicated mg N/g muscle	Change mg N/g muscle
By NaCl, $NaHCO_3$ solution					
1	24.5	0	Limp	24.3	- 0.2
2	22.4	2	"	24.1	+ 1.7
3	24.3	4	Slight stiffening	24.6	+ 0.3
4	25.0	6	Stiff	26.0	+ 1.0
5	25.2	8	"	21.9	- 3.3
6	24.9	10	"	23.5	- 1.4
7	25.1	10	"	23.7	- 1.4
8	26.0	12	"	25.7	- 0.3
9	26.0	14	Softening	24.8	- 1.2
10	23.8	18	"	23.4	- 0.4
11	23.2	22	"	17.2	- 6.0
12	21.4	26	"	19.2	- 2.2
13	24.8	36	Soft	21.3	- 3.5
By mersalyl solution					
1	25.9	0	Limp	25.9	0
2	22.3	2	"	24.3	+ 2.0
3	25.5	4	Slight stiffening	24.7	- 0.8
4	25.6	6	Stiff	24.7	- 0.9
5	25.8	8	"	25.8	0
6	27.1	10	"	23.3	- 3.8
7	25.3	10	"	23.4	- 1.9
8	25.0	12	"	24.2	- 0.8
9	27.5	14	Softening	25.4	- 2.1
10	27.6	18	"	19.6	- 8.0
11	25.5	22	"	24.3	- 1.2
12	24.1	26	"	23.0	- 1.1
13	25.5	36	Soft	21.1	- 4.4

[1] Fish unexercised at time of killing. Stored round at 72°F.

TABLE VIII-Changes in quantity of protein nitrogen extractable from trout muscle during the course or rigor mortis[1].

Fish No	1st sample Taken at time of killing mg N/g muscle	Time hrs	2nd sample Taken at post mortem time indicated Condition of fish	mg N/g muscle	Change mg N/g muscle
By NaCl, $NaHCO_3$ solution					
1	26.2	0	Limp	24.8	- 1.4
2	23.4	1½	Stiff	23.7	+ 0.3
3	25.0	3	"	26.0	+ 1.0
4	24.2	5	"	24.0	- 0.2
5	21.2	24	Softening	20.9	- 0.3
By mersalyl solution					
1	26.1	0	Limp	24.9	- 1.2
2	25.8	1½	Stiff	26.1	+ 0.3
3	25.2	3	"	28.2	+ 3.0
4	24.1	5	"	23.6	- 0.5
5	21.4	24	Soft	17.7	- 3.7

[1] Fish exhausted at time of killing. Stored round at 72°F.

TABLE IX - Changes in protein nitrogen extractability caused by freezing trout muscle at different stages of rigor mortis[1].

Fish No	Time post mortem hrs	Condition of fish	Condition of muscle Fresh mg N/g muscle	Frozen mg N/g muscle	Change mg N/g muscle
Unexercised fish					
1	0	Limp	25.3	25.0	- 0.3
2	5½	Slight stiffening	25.4	25.4	0
3	10	Stiff	24.2	22.5	- 1.7
4	23	Softening	20.1	16.2	- 3.9
5	36	Soft	16.2	17.4	+ 1.2
Exhausted fish					
1	0	Limp	26.0	24.2	- 1.8
2	3	Stiff	23.9	23.2	- 0.7
3	18	Soft	14.4	14.9	+ 0.5

[1] Fish stored round at 72°F. Extracting solution NaCl, $NaHCO_3$. Muscle frozen in liquid nitrogen.

SUMMARY NO. 37

POST-MORTEM BIOCHEMICAL CHANGES IN FISH MUSCLE
B - CHANGES AT STORAGE TEMPERATURES BELOW 0°C

R.E.E. Jonas
N. Tomlinson

This investigation is a continuation of that described in Summary No. 26, part B, of this Station's Annual Report for 1960-61. In work described last year fish were frozen in air at the temperature of storage. In the present work, in order to avoid differences introduced by different freezing temperatures, samples were all rapidly frozen in liquid nitrogen before being placed in storage. Changes that occur in the phospholipid content of lingcod muscle have been examined this year, in addition to the components examined previously.

Methods and Materials

The lingcod differed from those used previously in that they did not eat in captivity, possibly because they were in spawning condition. This appears to be reflected in the smaller quantity of lactic acid produced post mortem in the muscle, in comparison with that which occurred in the earlier work. The only change in methods was an increase in sample size from 5 g to 20 g. This was made possible by the larger size of the fish available.

Results

In Table I are recorded the results of analyses of muscle samples (from three lingcod) that had been held in frozen storage for various periods of time up to a maximum of six months. Some trends appear to be clear. Lactic acid was formed at -10 and -20°C but not at -30°C. Phosphorus was lost from the phospholipid fraction at each storage temperature but very much more slowly at -30°C than at -10 and -20°C. Results with acid-labile and acid-stable fractions were more irregular than in the previous work, particularly with the former. While in all three fish (ignoring the very low value in #3 at the time of killing) there was a decrease in the P of the acid-labile fraction at -10°C from the beginning of storage, at -20°C there appeared to be an increase in this fraction in fish #1 during the early stages of storage and a slow decrease in #2 and #3. At -30°C the tendency seemed to be toward an increase in all three, although this was not particularly clear in fish #2. The acid-stable P tended to decrease at -10°C but to be relatively stable at -20 and -30°C. Although in earlier work with feeding lingcod duplicate samples analysed at the time of killing gave results in fairly good agreement with each other, certain of the results in the present work (the value for lactic acid in fish #1, and for PAL in fish #3 at the time of killing) suggested that samples from these fish might not be as nearly comparable with each other as was the case previously. For this reason another large, non-feeding lingcod was killed, the lateral-dorsal muscle was frozen and stored at -30°C. Sets of four samples are to be taken for analysis after the same time intervals as were used in the work described in Table I. This experiment has just been started, but results for samples taken at the time of killing and after two days' storage are presented in Table II. The variations between samples from this fish are such as to indicate that this could be the source of some, if not all, of the seemingly contradictory or irregular findings mentioned above.

TABLE I - Influence of storage at temperatures of -10, -20 and -30°C on the lactic acid content and certain phosphorus-containing fractions of lingcod muscle.[1]

Storage time days	Fish No	Storage temp °C	Lactic acid or P(mg/g muscle) Lactic acid	PML	PAL	PAS	PI	PLIP
0	1		1.54	.26	.39	.43	1.11	.22
0	2		1.16	.12	.35	.40	1.31	.23
0	3		1.10	.10	.13	.38	1.33	.22
2	1	-10	1.64	.07	.36	.48	1.34	.20
		-20	1.06	.21	.46	.47	1.09	.19
		-30	.95	.14	.39	.41	1.15	.22
	2	-10	1.75	.09	.26	.46	1.40	.22
		-20	1.27	.05	.24	.43	1.47	.24
		-30	1.16	.23	.31	.40	1.25	.26
	3	-10	1.26	.04	.27	.39	1.29	.19
		-20	1.04	.11	.30	.51	1.53	.22
		-30	1.06	.12	.34	.35	1.30	.22
30	1	-10	2.33	.08	.09	.39	1.69	.14
		-20	1.38	.17	.55	.37	1.31	.17
		-30	1.14	.07	.41	.42	1.29	.22
	2	-10	2.51	.08	.09	.41	1.63	.16
		-20	1.76	.10	.38	.37	1.51	.18
		-30	1.11	.09	.33	.41	1.24	.27
	3	-10	1.93	.09	.09	.35	1.53	.11
		-20	1.25	.06	.33	.30	1.40	.13
		-30	1.09	.12	.42	.40	1.14	.21
90	1	-10	2.43	.0	.04	.32	2.03	.11
		-20	1.25	.12	.23	.41	1.42	.11
		-30	.98	.15	.59	.38	.96	.19
	2	-10	2.78	.04	.11	.34	1.72	.10
		-20	1.91	.08	.22	.32	1.69	.16
		-30	1.53	.07	.39	.38	1.25	.21
	3	-10	1.78	.04	.06	.35	1.60	.08
		-20	1.50	.12	.17	.33	1.51	.10
		-30	1.02	.04	.52	.41	1.07	.19
184	1	-10	2.53	.0	.02	.30	2.0	.09
		-20	1.69	.0	.15	.45	1.81	.10
		-30	1.09	.15	.52	.32	1.24	.17
	2	-10	3.26	.14	.04	.28	1.95	.09
		-20	2.71	.22	.12	.47	2.05	.11
		-20	1.14	.18	.47	.33	1.57	.21
	3	-10	2.43	.10	.03	.31	1.90	.06
		-20	1.85	.05	.15	.35	1.89	.07
		-30	1.10	.06	.64	.36	1.10	.14

[1] These fish had been in captivity without eating for about 2 months when they were killed.

TABLE II - The lactic acid content and certain phosphorus-containing fractions of lingcod muscle during storage at -30°C. Variations between samples from the same fish.[1]

Storage time days	Sample No	Lactic acid or P(mg/g muscle) Lactic acid	PML	PAL	PAS	PI	PLIP
0	1	1.42	.16	.17	.41	1.30	.22
	2	1.07	.06	.28	.38	1.35	.22
	3	0.98	.10	.36	.38	1.22	.23
	4	1.09	.06	.28	.40	1.39	.23
2	1	1.16	.03	.33	.34	1.31	.24
	2	1.07	.09	.20	.32	1.37	.24
	3	1.29	.05	.29	.35	1.41	.23
	4	1.35	.04	.25	.33	1.42	.24
Average values							
0		1.14	.095	.27	.39	1.31	.225
2		1.22	.05	.27	.33	1.38	.237

[1] This fish had been in captivity without eating for about 1 month when it was killed.

SUMMARY NO. 38

LINGCOD MUSCLE PHOSPHOMONOESTERASES
PHOSPHOSERINE PHOSPHATASE

G.F. Grant
N. Tomlinson

This study was begun last year and preliminary results were described in Summary No. 35 of this Station's Annual Report for 1960-61.

Materials and Methods

Many of these have been described previously (vide supra); new ones used are described as appropriate below.

Results

Further attempts to increase the purity of the enzyme preparation were made using three different methods. These consisted of (a) removal of inactive protein by isoelectric precipitation, (b) acetone fractionation, and (c) column chromatography. Tables I and II summarize the results of investigations of methods (a) and (b). The most satisfactory procedure from the point of view of improvement in purity (as judged by increase in specific activity and reduction in activity toward p-nitrophenyl phosphate as substrate), and least loss of total activity, was that employing heat treatment at pH 8.0 followed by acetone fractionation (after Neuhaus and Byrne, 1959). Several attempts were made to improve the purity of the preparation by means of method (c), using diethylamino-ethyl cellulose in the column, but the results were not satisfactory.

TABLE I - Partial purification of phosphoserine phosphatase from lingcod muscle by isoelectric precipitation of protein.

Treatment[1]	Soluble protein after treatment	Activity	Specific activity	Recovery of enzyme	Activity vs PNP[2]
	mg/ml	units/ml	units/mg protein	%	units/ml
None (initial preparation[3])	97.5	144	1.48		24
pH 4.0 at 0°C	14.4	72	5.0	50	1.12
pH 4.5 " "	31.2	114	3.6	79	2.0
pH 5.0 " "	45.	133	2.96	92	-
pH 6.0 " "	95.	139	1.43	97	-
pH 7.0 " "	92.5	117	1.27	81	9.75
pH 7.5 " "	82.8	139	1.6	97	7.75
pH 8.0 " "	76.3	127	1.7	88	5.8
pH 9.0 " "	59.0	114	1.93	79	5.5
pH 8.0 " 40°C	56.8	136	2.4	95	1.2
pH 8.0 " 50°C	41.8	129	3.1	90	1.16
pH 8.0 " 60°C	25.6	86	3.36	60	0.4
pH 8.0 " 50°C (10 min)	31.5	138	4.4	96	1.0
Followed by pH 4.5 at 0°C	27.0	96	3.43	67	0.6

[1] Treatments at 0°C were of 10 min duration, at higher temperatures of 5 min duration. After treatment pH was adjusted to 6.0 to 6.2 and insoluble protein was removed by centrifugation.

[2] A unit of activity vs PNP (p-nitrophenylphosphate) as substrate is that activity which would release 1 µM of p-nitrophenol under the assay conditions.

[3] Initial preparation was a dialysed 40-60% saturated ammonium sulphate fraction of an aqueous extract of lingcod muscle.

TABLE II - Partial purification of phosphoserine phosphatase from lingcod muscle by fractionation with acetone.

Fraction	Protein in the fraction	Specific activity	Activity vs PNP
	mg/ml	units/mg protein	units/ml
Starting material[1]	41.8	3.1	
0-35% acetone	20.0	0.4	
35-41% "	10.7	1.1	
41-46% "	7.2	1.4	
46-62% " [2]	6.6	21.3	0.2
Soluble in 62% acetone	5.3	0	

[1] Preparation pretreated by heating at pH 8.0 for 5 min. Vol 29 ml.

[2] Recovery of activity equal to 53% of starting material. Volume of dialysed fraction 18 ml.

The influence of several possible activators and inhibitors on the activity of the enzyme were investigated with a preparation partially purified by isoelectric precipitation and acetone fractionation. The results are presented in Table III.

TABLE III - Influence of potential activators and inhibitors on activity of phosphoserine phosphatase from lingcod muscle.

Activator or inhibitor added	% Activation or inhibition	
Salts alone (each .01 M)		
None	0	0
$MgCl_2$	107	
$CoCl_2$	63	
$ZnCl_2$	20	
$FeSO_4$		56
$BaCl_2$		72
$MnSO_4$		68
$CaCl_2$		100
$CuSO_4$		100
NaCl	0	0
$NaSO_4$		10
Salts in addition to 0.01 M $MgCl_2$		
$MgCl_2$	0 (107)	0
" + $MgCl_2$	37	
" + $CoCl_2$		1.5
" + $ZnCl_2$		11
" + $FeSO_4$		70
" + $BaCl_2$		13
" + $MnSO_4$		70
" + $CaCl_2$		99
" + $CuSO_4$		100
" + NaCl		0
" + Na_2SO_4	3	
Compounds in presence of $MgCl_2$ (.02 M)		
Iodoacetamide .005 M	0	0
Mersalyl .005 M		98
NaF .01 M		71
NaF .001 M		36
EDTA .02 M	0	0
Cysteine .01 M		14
L-serine .04 M		100
" .02 M		100
" .01 M		96
" .004 M		85
D-serine .04 M		81
" .02 M		61
" .01 M		42
" .004 M		11

Enzyme (acetone-fraction dialysed vs distilled water for 18 hours at 0°C before use) was incubated in the complete system except for substrate for 30 minutes before reaction started by adding substrate.

It appears that a metal ion is necessary for the enzymatic activity and that while Mg ion was the most effective of those tested, both Co and Zn had a favourable effect in the presence of very low concentrations of Mg. Ca and Cu ions were very strong inhibitors, while Ba, Mn and Fe had a somewhat weaker inhibitory effect. The -SH reagent, mersalyl, was found to be a strong inhibitor at low concentration. Free serine also inhibited the enzyme, the L-isomer being much the more potent of the two forms. EDTA did not inhibit at the concentration tested. The influence of higher concentrations of this compound should be examined.

Figures I and II illustrate the influence of Mg ion concentration and of substrate concentration on the enzymatic activity. It appears that 0.02 M Mg ion is sufficient to provide optimum activity. Figure III shows the time-course of the release of inorganic P from the substrate when different low concentrations of substrate are available and indicates that both the D- and L-forms of phosphoserine are attacked, although the change in rate suggests one may be attacked more readily than the other.

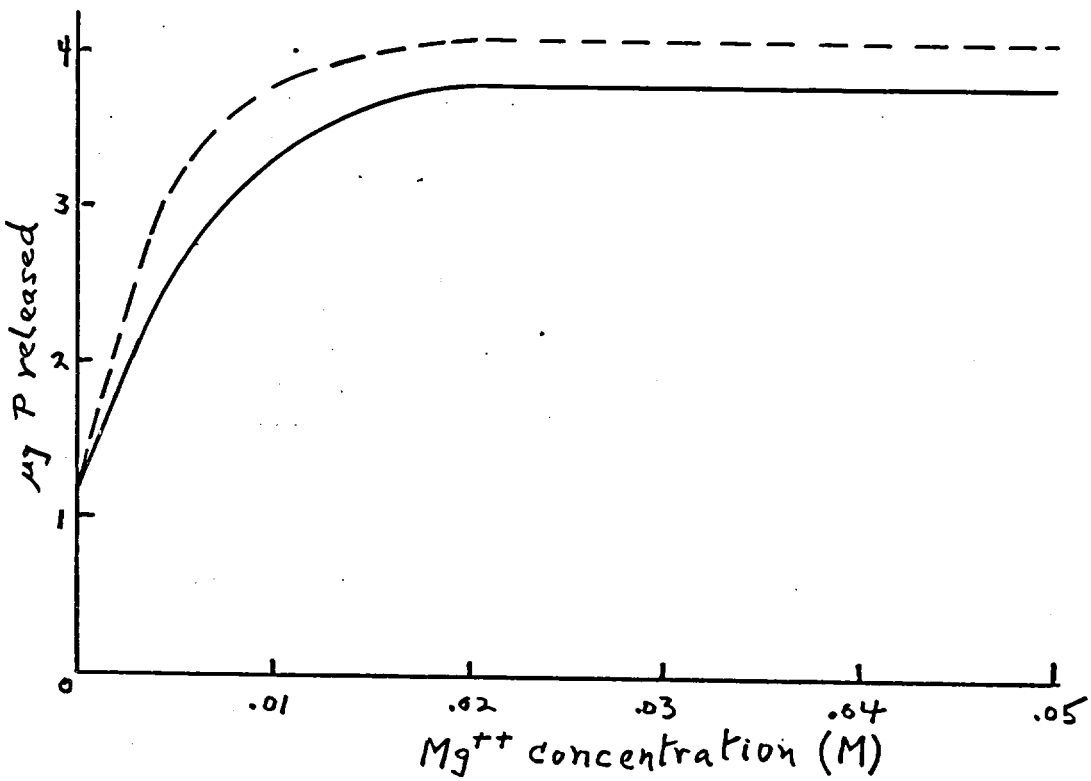


Fig. 1. Influence of Mg ion concentration on the activity of phosphoserine phosphatase from lingcod muscle. Enzyme: - - - - heat treated at pH 8.0, protein 2.8 mg; ——— acetone fraction, protein 0.3 mg.

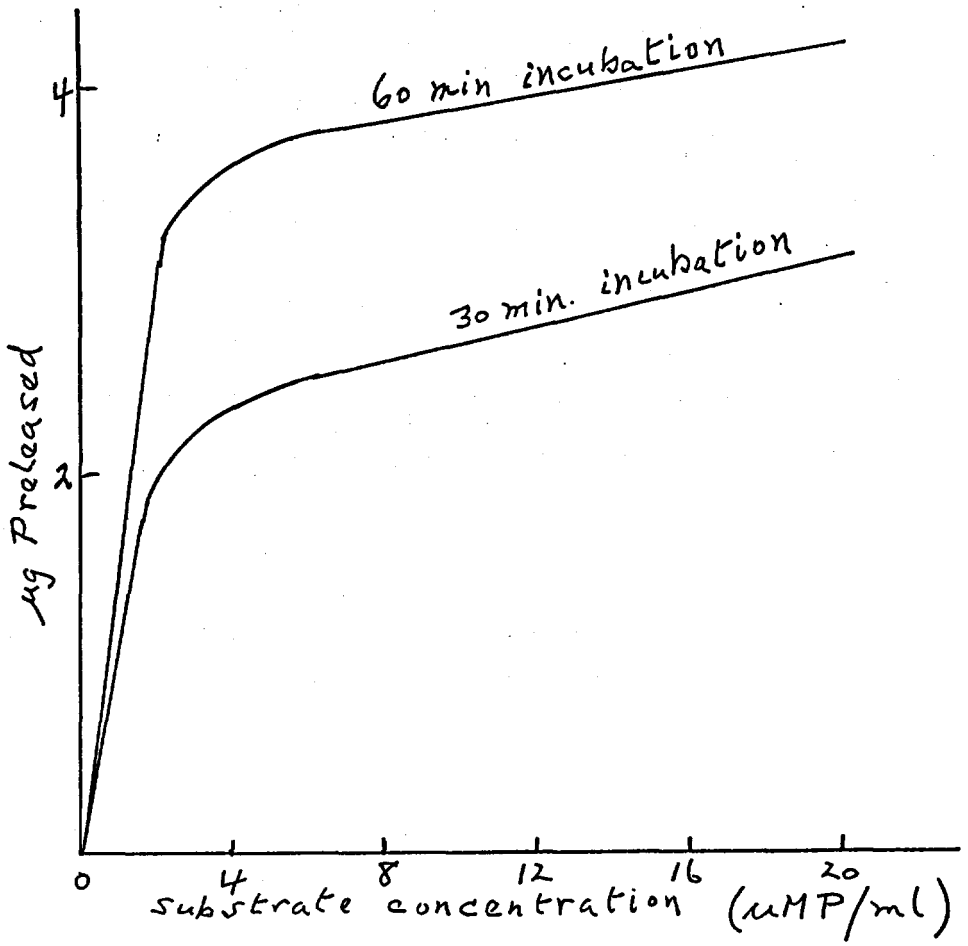


Fig. 2. Influence of substrate concentration on the activity of phosphoserine phosphatase from lingcod muscle. Enzyme, acetone fraction, 1.3 mg protein.

In addition to a very small amount of activity with p-nitrophenyl phosphate as substrate (Table II) the enzyme preparation obtained by acetone fractionation released inorganic P from the following substrates:

	Activity (as % of that with phosphoserine as substrate in 90 min)
Glucose-1-phosphate	14
Adenosinediphosphate	15
Adenosinetriphosphate	25
Inositol monophosphate	36
Phosphothreonine	11
Phosphoethanolamine	2
Casein	10

Adenosinemonophosphate, glucose-6-phosphate, 6-phosphogluconic acid, 3-phosphoglyceric acid and 2-phosphoglyceric acid were not attacked.

This enzyme seems to be fairly stable. Preparations containing Mg ions have been frozen and stored at -30°C with little or no loss in activity and have

been held at 0°C for one week with a loss of only 5-10%. There is some evidence of greater lability when a preparation is dialysed relatively free from Mg ions before storage.

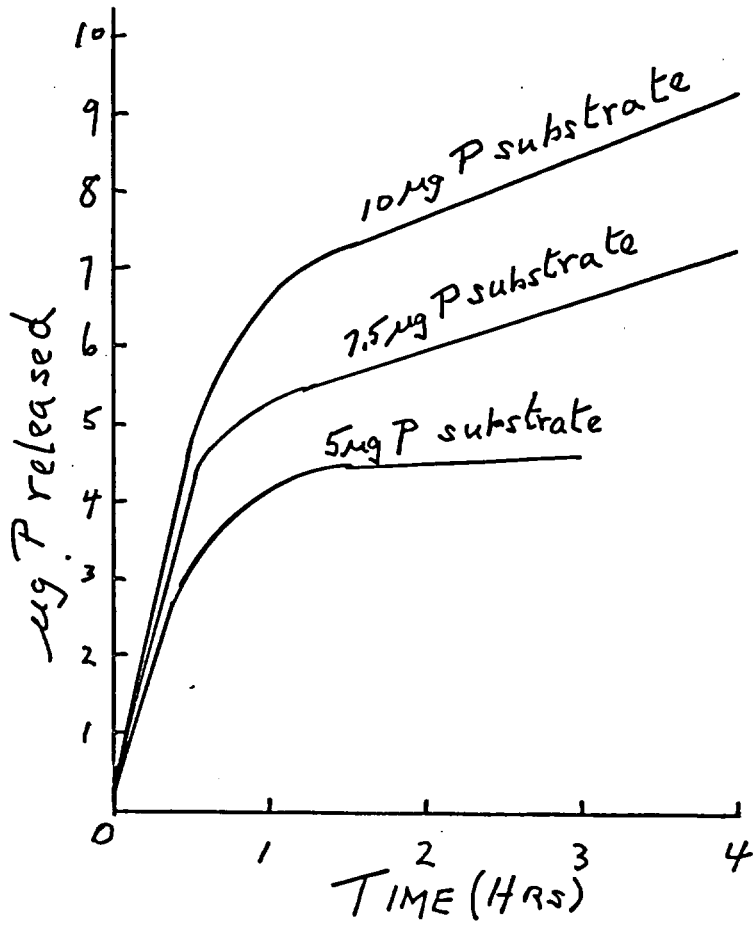


Fig. 3. Time-course of release of inorganic P from DL-phosphoserine by lingcod muscle phosphoserine phosphatase.

SUMMARY NO. 39

BIOSYNTHESIS OF TRIMETHYLAMINE OXIDE — E. Bilinski

This investigation is a continuation of the work initiated at Grande Rivière Technological Station, which has been described in Summaries Nos. 1.4 and 6 of Grande Rivière Station's Annual Reports for 1959-60 and 1960-61, respectively.

In the previous studies of this series, tracer experiments conducted with living lobster have shown that choline is a precursor of betaine and trimethylamine oxide. It was also observed that the oxidation of choline to betaine is not likely to be an intermediate step in formation of trimethylamine oxide. In connection with the previous work with lobster it was thought desirable to conduct with another species of marine crustacea a similar study on formation of trimethylamine oxide and in the present report experiments on utilization of choline-methyl-C^{14} by the crab are described. This material together with previously unpublished experiments on formation of phospholipid-bound choline in lobster has been the object of a

publication dealing with the metabolism of choline in marine crustacea.

Materials and Methods

Edible crabs (Cancer magister) were caught in English Bay, Vancouver, B.C. Choline-methyl-C^{14} chloride was injected with a clinical needle into each side of the body between the first and second legs. After injection the animals were kept in sea water until they were sacrificed at the end of a metabolic period of 24 hours. The whole body including the carapace was employed in the isolation procedure.

The methods used for the isolation of trimethylamine oxide and phospholipid bound choline and for the determination of radioactivity have been described in the previous reports.

TABLE I - Utilization of choline-methyl-C^{14} in crab*.

Isolated compound	Amount present per 100 g body weight	Specific radioactivity	% of total radioactivity
	millimoles	counts/min**	
Trimethylamine oxide	0.45	120	>0.1
Phospholipid choline	0.07	8800	>1.9

* 2.7 μ curies as 3.5 μM choline-methyl-C^{14}-chloride was administered per 100 g body weight. Two crabs (total weight 184 g) were sacrificed after a metabolic period of 24 hours.

** Counts per minute per infinitely thick planchet (3.8 sq cm) of $BaCO_3$.

Results

The results of experiments conducted with crabs are presented in Table I. Choline-methyl-C^{14} was the only compound administered. The metabolic pathways of choline observed in experiments with lobsters were: conversion of choline to betaine and trimethylamine oxide and its incorporation into phospholipids. However, the present data indicate the occurrence in crab of only the last two of these pathways, as insufficient betaine was found to permit measurement of its radioactivity. The values for the percentage conversion of choline-methyl-C^{14} are expressed on the basis of amounts of administered choline and of trimethylamine oxide and phospholipid choline present in crabs at the end of the experiment. These values are minimal ones only, as no allowance was made for the radioactivity eliminated from the body during the experimental period. Another indication of the magnitude of conversion of choline to trimethylamine oxide might be obtained by comparing it to the conversion of choline to phospholipid bound choline. It appears from the figures in Table I that the incorporation of free choline into phospholipids is about 20 times greater than its conversion to trimethylamine oxide.

These experiments confirm the earlier observation made with lobsters that choline may serve as precursor of trimethylamine oxide. Trimethylamine is the most likely intermediate in this process and it is well established that the oxidation of trimethylamine to trimethylamine oxide takes place in animals. Conflicting evidence has been presented regarding the ability of animals to convert choline to trimethylamine. In mammals the conversion of choline to trimethylamine

has been attributed to microbial activity in the digestive tract or to an enzymic system in the animal. In our experiments an attempt was made to eliminate the enterference from microbial flora by avoiding the digestive tract during the administration of labelled compounds and the results suggest the presence in each species of marine crustacea under study of an enzyme system capable of converting choline to trimethylamine oxide.

SUMMARY NO. 40

FAT HYDROLYSIS IN FROZEN FILLETS OF LINGCOD AND PACIFIC GRAY COD

J.D. Wood
S.A. Haqq

It has been known for several years that lipids in fish muscle undergo changes during storage. Dyer found that fat spoilage was involved in the deterioration occurring under frozen storage even in the non-fatty species of fish. The changes taking place in the muscle lipids were studied for various Atlantic species and it was found that the lipids were hydrolyzed with the formation of free fatty acids. Lovern et al. found that it was the phospholipids which were breaking down and recent work by Bligh has shown that hydrolysis of phosphatidylethanolamine and phosphatidylcholine is mainly responsible for the increase in free fatty acids. Apart from some early work by Brocklesby in 1933 on salmon, there are no data available for the information of free fatty acids in Pacific species, and an investigation was therefore commenced to study the free fatty acid formation in lingcod (Ophiodon elongatus) and the Pacific gray cod (Gadus macrocephalus), both species being caught commercially off the coast of British Columbia.

Fillets were obtained from freshly caught fish, wrapped in cellophane and stored in wax cartons at +10°F (-12°C). The lipids were extracted from the muscle tissue as described by Bligh and Dyer. The troublesome cloudy upper layer mentioned by Olley and Lovern was not encountered under the conditions of storage used in the present experiment. The extracted lipids were chromatographed on silicic acid to remove phospholipids and the free fatty acids in ethanol solution were titrated with 0.02 N KOH using phenolphthalein as indicator.

TABLE I - Lipid composition of fresh lingcod and Pacific gray cod fillets.

Species	Total lipid (% of wet muscle)	Free fatty acids (% of total lipid)
Pacific gray cod	0.58 ± 0.03*	5.2 ± 0.3
Lingcod	0.85 ± 0.09	3.4 ± 0.4

* Each value in the table is the mean for 10 fillets ± the standard deviation.

Table I shows the lipid composition of fresh lingcod and Pacific gray cod fillets. The total lipid and free fatty acid composition of the Pacific gray cod resembles closely the values for the Atlantic cod. The lingcod has a slightly higher lipid content but free fatty acid as a percentage of the total lipid is somewhat less than that in the gray cod. These two effects balance out so that the free fatty acid comprises 0.030 and 0.029% of the wet muscle in Pacific gray cod and lingcod, respectively.

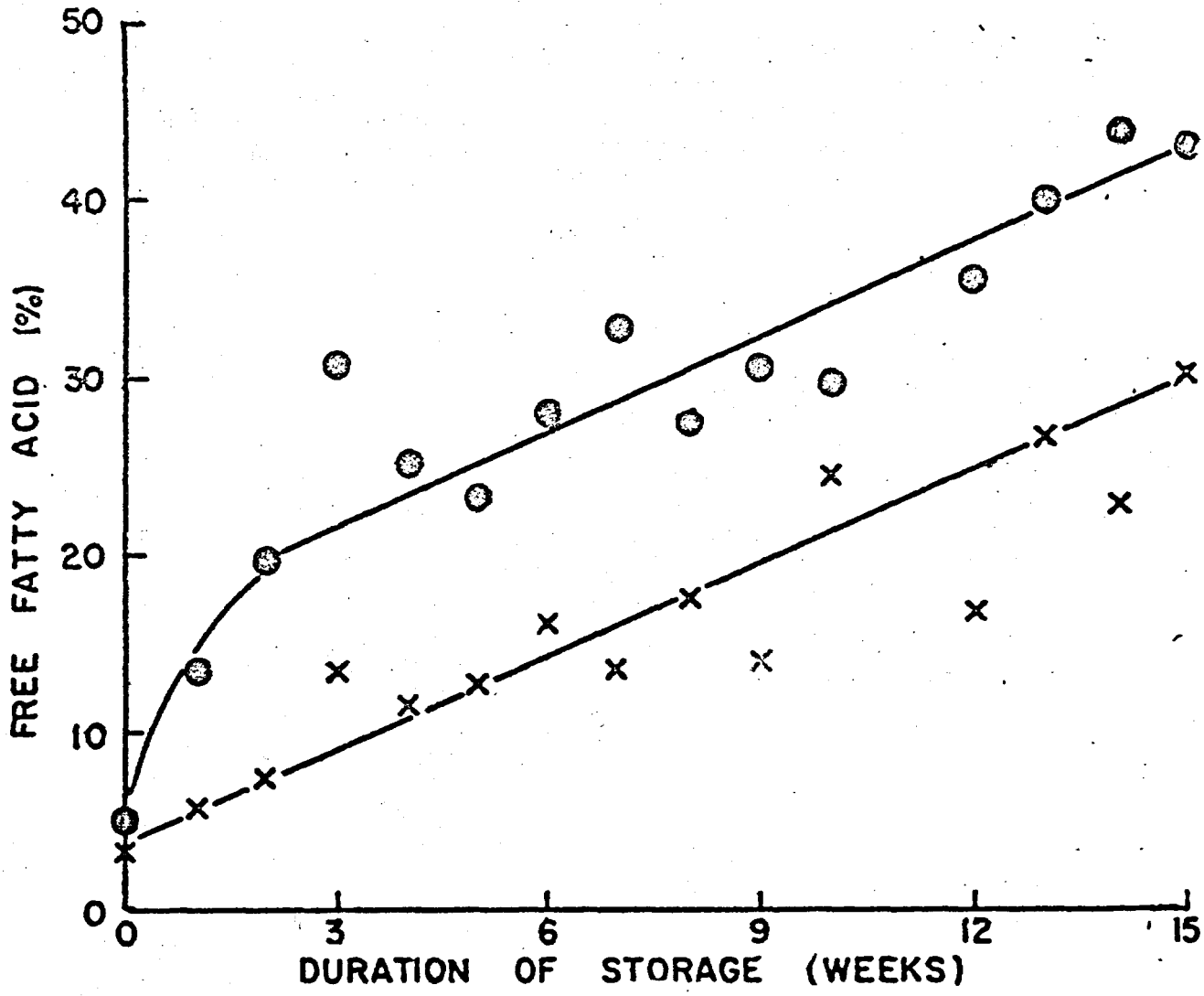


Fig. 1. The formation of free fatty acids in lingcod and Pacific gray cod fillets. ● —— ● Pacific gray cod; x —— x lingcod. Each value in the figure is the mean value for 10 fillets.

The increase in the free fatty acid content of the fillets is shown in Fig. 1. The rates of hydrolysis differ in the two species. The Pacific gray cod lipids hydrolyze rapidly during the initial two or three weeks storage but thereafter a less rapid but continuous process ensues. The lingcod lipids on the other hand exhibit a more uniform rate of hydrolysis during the 15-week experimental period. The results for the Pacific gray cod resemble closely those obtained for the Atlantic cod at similar storage temperatures.

SUMMARY NO. 41

REPORT ON THE STUDY OF DETERIORATION OF PHOSPHOLIPID OF GRAY COD

Wing Wai
J.D. Wood

The deterioration of phospholipid while in storage at -10°C was reported. This study was intended to determine the cause of the hydrolysis of phospholipid

into fatty acid. It had been suggested that it was due to either an enzyme or a catalyst or both.

Experimental (See Methods in Enzymology, Vol. I, page 660 et seq. for general methods used.)

Enzyme Preparation: The gray cod used for preparation of enzyme were freshly killed, beheaded, gutted and frozen immediately. The enzyme was prepared by homogenising the flesh in one and a half times volume of distilled water (w:v) for one minute. The homogenised material was centrifuged at 11,000 rpm for 20 min. The supernatant fluid was filtered through glass wool. The filtrate was dried in the freeze-drier. The dry enzyme preparation was then dissolved again to the desired volume of $CaCl_2$.

Methods

1. Manometric Method:
   Incubation was done in a Warburg apparatus. Egg lecithin was used as substrate. Conditions:
   (a) various pH buffers;
   (b) boiled and unboiled enzyme preparation;
   (c) temperature.

2. Direct reaction of enzyme preparation on phospholipid extract of gray cod in ether medium (modified Hannahan Method).

3. Estimation of rate of hydrolysis for minced and whole fillets at -10°C.

4. Estimation of hydrolysis of phospholipid in water at -10°C.

5. Estimation of hydrolysis of phospholipid in aqueous system.

6. Estimation of hydrolysis of phospholipid with butanol - extracted enzyme preparation.

Remarks

The results of Manometric Method did not indicate any activity due to enzymes. The Modified Hannahan Method showed that the phospholipid was broken down gradually both in the control and enzyme extract samples. As the reaction solution turned from colourless to yellow gradually, it was difficult to judge the end-point during titration. It was suggested that a spectrophotometric method should be used in order to obtain consistent results. The rate of hydrolysis of phospholipid in whole and minced fillets was found to be about the same. The hydrolysis of phospholipid in water at -10°C proceeded gradually. Attempts to estimate the hydrolysis in aqueous system by spectrophotometric method was not successful. It is possible that extraction of phospholipase is not possible with those methods tried. Butanol method of extraction was tried. However, the experiment was not completed due to limitation of time.

From the preliminary study the hydrolysis of phospholipid seemed to be due to a catalytic agent. However, more concrete methods such as spectrophotometric study should be employed in order to obtain the complete elucidation of the cause of hydrolysis.

SUMMARY NO. 42

PHYSICAL AND CHEMICAL CHANGES IN FISH MUSCLE DURING COLD STORAGE

Hans Buttkus
H.L.A. Tarr

Loss of tenderness and development of toughness in fish muscle during frozen storage coincide with myosin denaturation as measured by its decreasing solubility in cold neutral salt solutions of comparatively high ionic strength. Some doubt has been expressed regarding the reliability of measurements of decrease in myosin solubility as an index of toughness development. Although physical devices for measuring the texture of mammalian muscle have been described, these have failed to give satisfactory results in measuring the toughness of fish muscle. Love and Nemitz and Partmann attributed this failure to the more frequent distribution of myocommata between the myotomes of fish muscle as opposed to mammals where long parallel fibres can be separated from certain muscles.

The need for a routine test capable of measuring texture changes in freezer stored fish is therefore evident. Love in 1958 proposed a method for studying denaturation based partly on chemical reactions of formaldehyde with fish proteins and partly on the mechanical disintegration of muscle fibres. The decrease in optical density of a muscle fibre suspension with increasing storage time was stated to correlate with increasing "toughness" of muscle samples. Before adopting this test, however, it seemed desirable to compare its results to those of a different method, preferably less complex in nature. It was found that the Mangold sclerometer was well suited for this purpose. Relative measurements of the hardness (skleros) of fish muscle were possible with this instrument if care was taken in selecting the muscle sample. Explanations of terms used in connection with the sclerometer are given under "Methods".

Studies were also initiated to investigate post mortem drip in fish muscle. It has been found that the main factor on which the onset of rigor mortis in mammalian muscle depends is the disappearance of ATP. In fish muscle a very similar relation was shown to exist by Tomlinson et al in 1961. The pre-rigor ATP level in muscle can be maintained in the frozen state at -30°. It decreases rapidly, however, on thawing. Accompanying this ATP breakdown is a shortening of the muscle fibres and the appearance of drip. If, therefore, an agent could be found which would prevent the breakdown of the ATP, it was felt the shortening of muscle fibres and the formation of drip might be prevented. ATP added to defrosted mammalian muscle is hydrolysed two to five times and in fish muscle 50 to 200 times as fast as its hydrolysis in unfrozen muscle immediately after death. Magnesium ions (Mg^{++}), however, have been known by physiologists to exert, at certain concentrations, a depressing effect on the activity of various types of tissue. It has been discovered that magnesium anaesthesia minimized the breakdown of ATP which ordinarily occurs when animals are killed by decapitation without anaesthesia. It was felt, therefore, that the use of Mg^{++} might delay, or even partly prevent, the storage deterioration of fish as far as toughness and drip after thawing are concerned.

Methods and Material

The fish used in the present experiments were lingcod (Ophiodon elongatus) which, after having been caught at sea, were kept alive at the Vancouver Public Aquarium until the beginning of the experiments. In order to test the effect of magnesium on the storage quality of fish muscle, 1 to 1.5 kg lingcod were injected intraperitoneally with a total of 4.0 ml 50% $MgSO_4 \cdot 7H_2O$, equivalent to 200 mg Mg^{++}

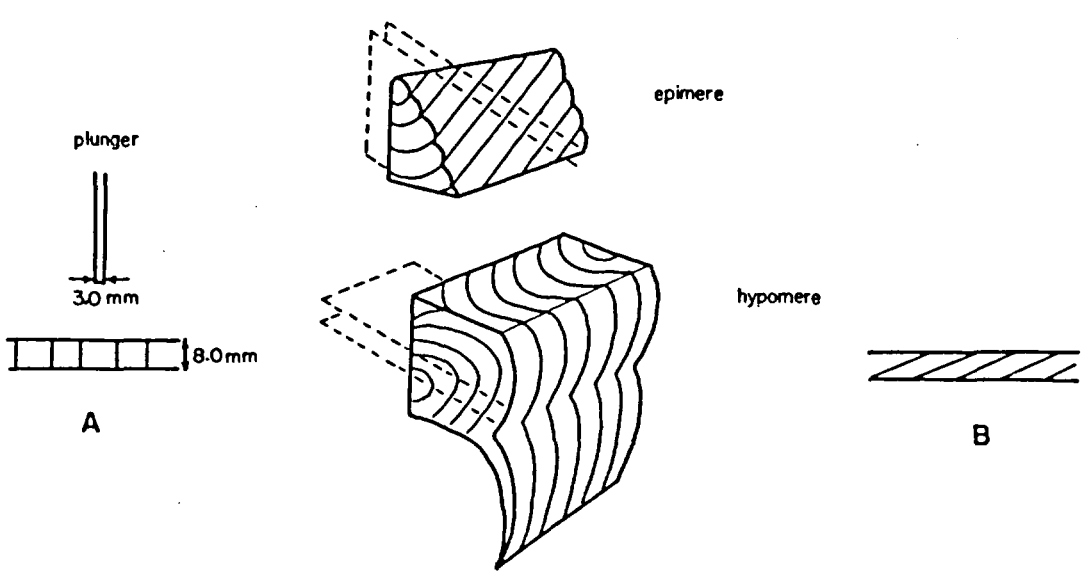
per kg of fish. The injections were administered over a period of 20 minutes, until the muscle remained completely relaxed. After each injection the fish were returned to a tank containing sea water (10°C) saturated with oxygen. One half hour after the first injection the fish were killed, dissected and the fillets were stored over ice in air-tight jars at -5 and -30°C. Two lingcod, each about 1.5 kg, were injected with a total of 5 ml 0.5 M sodium hexametaphosphate, a calcium and magnesium complexing compound and were prepared in the same manner as those injected with $MgSO_4$. Fishes used as controls during the experiments were stunned and decapitated without previous injections.

Storage

A problem arising during cold storage of fishery products is the loss of moisture (desiccation) and a consequent toughening of the product. Even when the product is wrapped in polyethylene bags a certain amount of desiccation of the surface layers will take place. This is accompanied by an accumulation of free ice crystals in the bag with increasing storage time. To reduce moisture losses during the present experiments, the samples were wrapped in polyethylene. They were then frozen on metal plates in direct contact with the evaporator coils of the refrigeration system and stored in jars containing a layer of ice pieces. The jars were sealed so that the atmosphere in them would remain saturated with water vapour.

Sclerometer

A sclerometer similar to that described by Mangold in 1922 was constructed and used in the present work. With this instrument the hardness of a muscle, i.e. its resistance to a deforming force, is measured by determining the distance through which a plunger, resting on the surface of the muscle, will fall at constant force during a constant time interval. The muscle blocks to be tested were about 8 mm thick and approximately 6 cm^2 in top area. The sections were cut from the frozen tissue with a fine-toothed saw in such a way that the myocommata were arranged in a vertical direction, i.e., parallel to the penetrating plunger (see diagrams).



Diagrams- Selection of muscle samples for the sclerometer test as indicated by the broken lines. In A the myocommata are arranged in a vertical direction. If the myocommata proceed diagonally through the sample, as in B, lower penetration readings will be obtained.

The cut samples were defrosted for 30 minutes at room temperature before making measurements. During the defrosting they were covered with a smooth piece of aluminum foil, making intimate contact with the surface of the sample to prevent drying. The defrosted sample was then placed on the platform of a "Lab Jack" and was raised by means of the fine screw adjustment. It was adjusted so that when the plunger was in zero position it would just touch the surface of the tissue between two myocommata. At zero time a weight of 2.0 g was attached to the lever 14 cm from the fulcrum and 10 cm from the plunger. The plunger moved downward, indenting the myotome section. After 3 minutes the distance which the plunger had moved into the tissue was recorded. The total distance through the tissue (thickness or height) was determined by lowering the plunger to the platform of the "Lab Jack". The readings were recorded as percent penetration. The tenderness of the tissue is directly proportional to the percent penetration values. For convenience percent penetration and tenderness are therefore used interchangeably. The relation between tenderness and toughness would accordingly be given by: 100% - % tenderness = % toughness. Since the sclerometer measurements have not been formally correlated with taste panel evaluations, tenderness measured during these experiments is referred to in quotation marks. The sensitivity of the presently employed sclerometer is 0.02 cm per scale division.

Drip

To simplify the procedure for collecting and measuring press drip, a sieve plate with holes of 1-mm diameter (24 holes per cm^2) was made from stainless steel which could be positioned 5 cm from the bottom of a Servall 316 stainless steel centrifuging tube. Whatman No. 1 filter paper was placed on top of the sieve plate and the frozen muscle sample (2.5 g) was weighed into the centrifuging tube, defrosted for 10 minutes at 30°C and centrifuged at 0°C for 10 minutes at 2500 x g. The tissue remaining on the filter paper was reweighed and the weight loss expressed as percent drip. Larger samples were used when collecting drip for magnesium analysis.

Chemical Methods

Magnesium analysis was carried out on drip samples deproteinized with trichloracetic acid (TCA). 4.5 ml of 10% TCA were added to 0.5 ml of drip, stirred well and centrifuged at 17,300 x g. The magnesium determination was carried out on 0.5 ml of the supernatant according to the colorimetric method of Orange and Rhein in 1951. Calcium was precipitated as the oxalate from deproteinized drip samples. The calcium concentration of the precipitate was determined with a Beckman DU spectrophotometer (flame attachment). Protein determinations were made using the colorimetric biuret reaction according to Snow in 1950. Acid labile $\Delta^{7'}$ phosphorus was determined as described by Tomlinson *et al* in 1961. All dissections were made at 0°C and samples weighed for analysis were immediately homogenized in ice-cold 11.4% (w/v) trichloroacetic acid.

Results and Discussion

Samples were removed from storage at weekly intervals and tested for toughness with the Mangold sclerometer. It was found that previously reported interference in physical measurements by the myocommata could be avoided by placing a sufficiently small plunger of the modified Mangold sclerometer directly on the myotome section of a carefully-selected muscle block. With larger fish the space between the myocommata is greater, thus simplifying measurements. Triplicate penetration recordings of the sclerometer generally showed less than $\pm 4\%$ deviation. Penetration measurements of fresh tissue with the sclerometer were considerably more difficult to make than were those of the defrosted muscle. The penetration values of fresh tissue varied also to

a much greater degree between different regions of the muscle than was the case for defrosted samples.

It has been stated previously that the toughness of frozen fish is always greater than than that of unfrozen fish. On this basis our penetration measurements appeared to be reasonably valid. At no time after frozen storage, regardless of the storage temperature and treatment of the fish, did they attain values for "tenderness" as high as those of the freshly-killed and unfrozen fish tissue. Also, samples stored at -30°C were always more "tender" than those stored at -5°C. This was, however, not the case when using the method proposed by Love. It was found that lingcod muscle, especially when the fish were killed during Mg^{++} anaesthesia, would actually increase in tenderness during the first two weeks of storage at both -5° and -30°C (Fig. 1A). In general, Love's method seems to be more useful in differentiating "toughness" of fish in long-term experiments, i.e., after about 60 days, when a clearer separation of the optical density curves for the -5° and -30°C stored fish becomes evident (Fig. 1B).

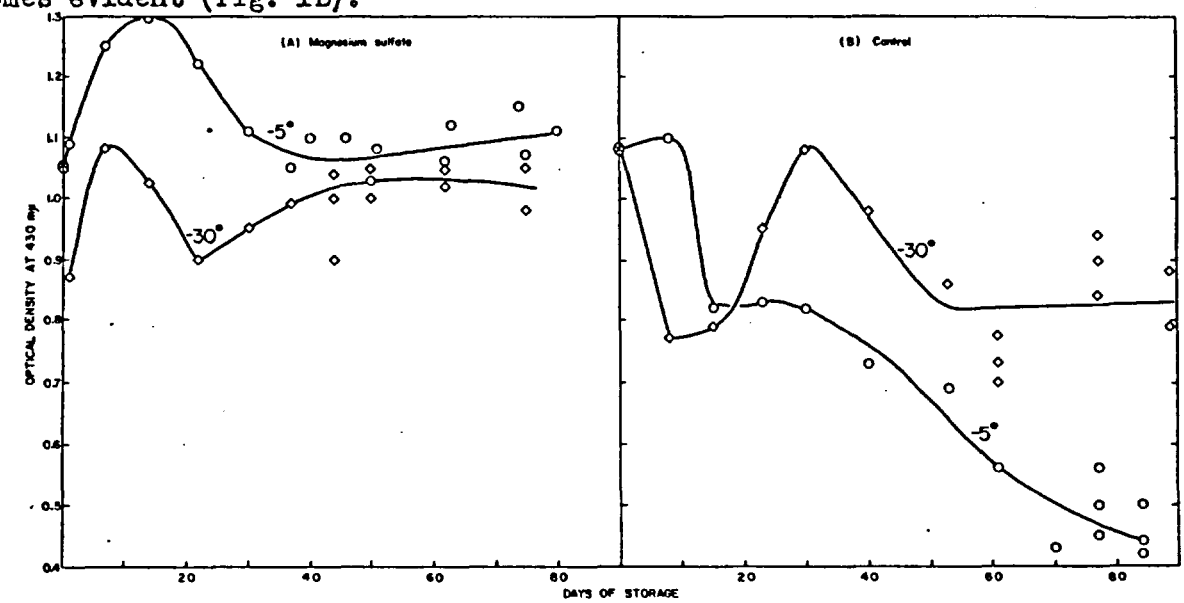


Fig. 1. Development of toughness in lingcod muscle during cold storage as measured by Love's method (A) on a fish which was killed while in magnesium anaesthesia and (B) on a control fish.

Evaluation of the sclerometer measurements of the Mg^{++}-treated fish muscle as compared with untreated fish showed that the Mg^{++} injection effected the preservation of a more "tender" texture at the higher storage temperature of -5°C (Fig. 2A and B). In some cases the muscle of the Mg^{++}-injected fish was between 12-20% more "tender" than the controls. Others showed as little as 9% difference after 20 days of storage. This "tenderizing" effect of the magnesium was also confirmed by a small taste panel on cooked samples. Although both treated and untreated fillets developed an oxidized flavour after 20 days at -5°C, penetration tests were continued in order to follow further changes in texture. When stored at -30°C, the difference in texture between the muscle of the Mg^{++}-treated and the untreated fish was not always detectable. Any agent having a protective action against protein denaturation would be expected to show its greatest effect at the storage temperature producing the highest degree of denaturation, in this case -5°C. At -30°C, where denaturation is less pronounced, the protective action of Mg^{++} would then be expected to be less noticeable. Generally it was found that samples stored at -30°C would attain, after 70 to 100 days, an almost absolute value in the region of 35-40% penetration, while those stored at -5°C would approach a value of 20%. The penetration values of freshly-killed fishes varied between 48 to 62%.

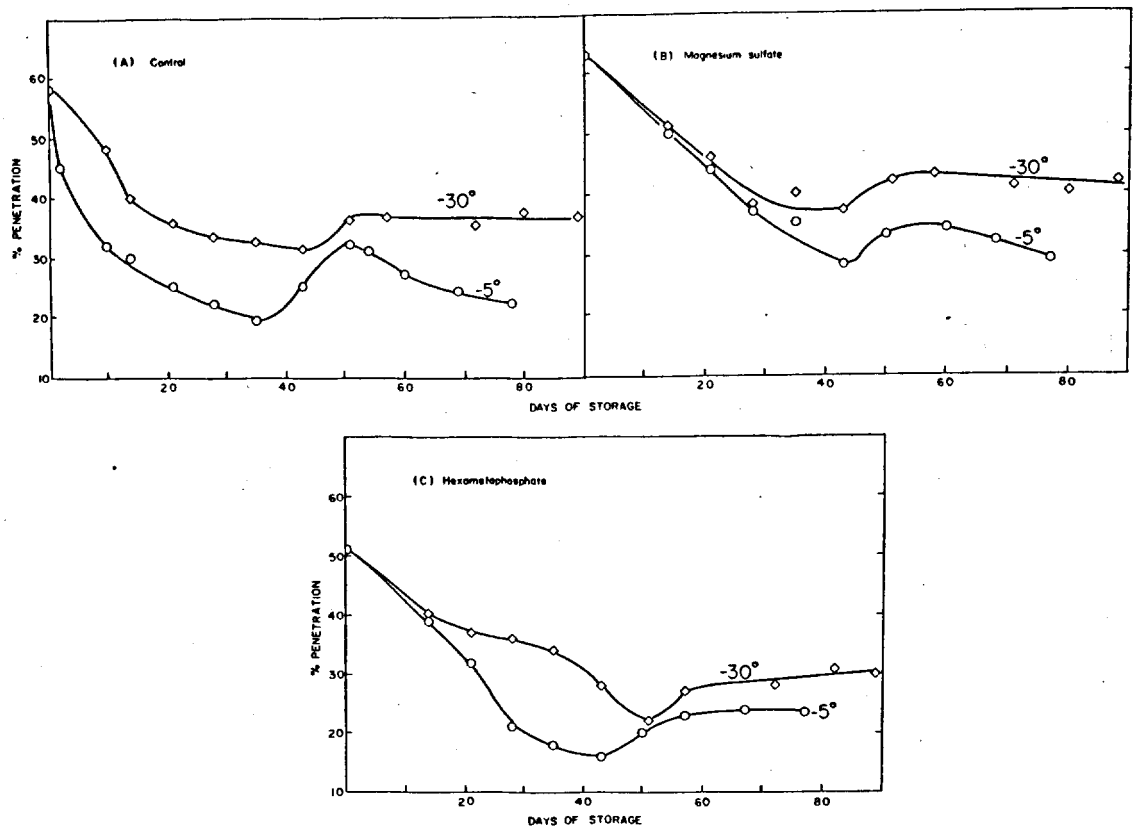


Fig. 2. Effect of pre-slaughter, intraperitoneal magnesium sulphate and sodium hexametaphosphate injections on textural changes of ling-cod muscle during cold storage. Penetration measurements were made with a modified Mangold sclerometer.

As can be seen from Fig. 2A, 2B, 2C, the curves representing texture changes at different storage temperatures show very characteristic minima. These low points on the tenderness scale varied in location and intensity from fish to fish and were usually found between 10 to 50 days. As all fish were frozen pre-rigor, a possible explanation for this phenomenon might be that the "tenderness" during the first week is obscured by the intensity of thaw rigor. During this time a combination of factors would be measured, namely, reduction in "tenderness" caused by protein denaturation as well as a persisting rigor hardness. After a storage period of 50-60 days, including the thaw period of 30-40 minutes, texture changes in the white muscle due to rigor could have been partly resolved. Following this period only texture changes in firmness and elasticity due to protein denaturation are measured with the sclerometer and an almost absolute value in the region of 35 to 40% penetration is obtained for fish stored at -30°C. In order to eliminate the supposed rigor interference it might be advantageous to make further penetration measurements on cooked samples rather than those on raw, defrosted muscle.

Chemical analyses showed that the Mg^{++} content of the magnesium sulphate injected fishes had risen by 2-3 mg per 100 g of tissue, while the calcium level usually responded to such an injection by an increase of 3 mg%. In attempting to correlate the ATP concentration with the observed texture changes, it was found that the $\Delta^{7'}$ phosphorus level in the magnesium-injected fishes had dropped within the first 24 hours of freezer storage by 75-85% of the level found immediately post mortem in unfrozen fish (Table I). This rapid hydrolysis of the ATP took place even at -30°C. Extraction of this muscle with perchloric acid at 0°C and separation of the nucleotide components on a Dowex-1 (Cl^-) column confirmed that the ATP

concentration had decreased and inosinic acid had correspondingly increased (Tarr and Leroux, submitted for publication). Such a rapid decrease in the $\Delta^{7'}$ phosphorus level was, however, not observed with muscle from rabbits which were treated, killed and stored under the same conditions.

TABLE I - Representative $\Delta^{7'}$ phosphorus levels (mg P/g tissue) of lingcod and rabbit muscle.

Post-mortem time	0	1	3	4	6	32	39	Days
Lingcod (control)	.42	.41	.42	.41	.41	.40	.39	mg P/g
Lingcod (Mg)*	.56	.14	-	.20	.21	.24	.24	"
Lingcod (Mg)*	.36	.06	.07	-	.05	-	-	"
Rabbit (Mg)*	.57	.54	.58	-	.58	.63	.64	"

* The samples were obtained from animals which had been killed while in magnesium anaesthesia. They were frozen at -30°C immediately following dissection.

In post-mortem fish muscle the in situ activation of an ATPase by magnesium is difficult to explain in comparison with rabbit muscle. Dubois et al. in 1943 postulated that an increased yield of ATP from rabbit and rat muscle after magnesium injection was possible because myosin ATPase is inhibited by magnesium and the muscles are thus prevented from contracting. That magnesium has also an anaesthetic effect on fishes was shown in the present experiments. However, the higher post-mortem activity of the ATPase in the magnesium-treated fish muscle as compared with that of the rabbit points toward some interesting biochemical difference between the muscle of a fish and a mammal. Dingle and Hines in their study with isolated muscle proteins made the following interesting observation: "It is usually stated that actomyosin ATPase is characteristically "strongly" activated by Mg^{++}. Though this does seem to occur with cod actomyosin, it appears to be much less marked with mammalian actomyosin." Also, the presence of a sarcoplasmic ATPase has been demonstrated in carp muscle by Reuter. He showed that the activity (Q_p) of the magnesium-activated sarcoplasmic ATPase is higher in the carp than in the rabbit. These observations point to a difference in activity rather than one of fundamental nature. On the other hand a direct extraction of myosin, as carried out on mammalian tissue, has so far not been successfully applied to fishes. Extraction of fish muscle (carp and cod) yielded only actomyosin but both myosin and actomyosin were obtained when extracting rabbit muscle under the same conditions. It may be concluded from these results that in fish muscle a much more rapid post-mortem formation of actomyosin takes place than in rabbit muscle. In the Mg^{++}-injected fish this change from myosin plus actin (Mg^{++} suppressed ATPase) to actomyosin (Mg^{++} activated ATPase) would then lead to an accelerated ATP breakdown.

As the muscle from the Mg^{++}-treated fishes had lower ATP concentrations and a more "tender" texture than that from untreated fish, it seems that high ATP concentrations are not involved in preserving good texture quality of frozen stored fish muscle. As to the function of the Mg^{++} in "tenderizing" fish meat, it may be interesting to note that some ions (Ca, Mn) have been shown to protect albumin from heat denaturation. It could be possible that Mg^{++} ions during cold storage perform a similar protective action on the fish proteins. If this is so, precooling and holding fish in refrigerated sea water may have beneficial effects on the texture of fish. As the Mg^{++} concentration in sea water (127 mg Mg%) is higher than in fish muscle (lingcod, 26.8 mg Mg%), these ions would migrate into the fish muscle after the selective permeability of the membranes has ceased to be active.

Evaluation of drip measurements showed that hexametaphosphate was the best agent to prevent the formation of drip (Fig. 3). Unfortunately it had a toughening effect on texture of the fish as measured by sclerometer (Fig. 2C). This fact was brought out after about forty days of storage when the "tenderness" of the hexametaphosphate-treated fish decreased below that of the control fish.

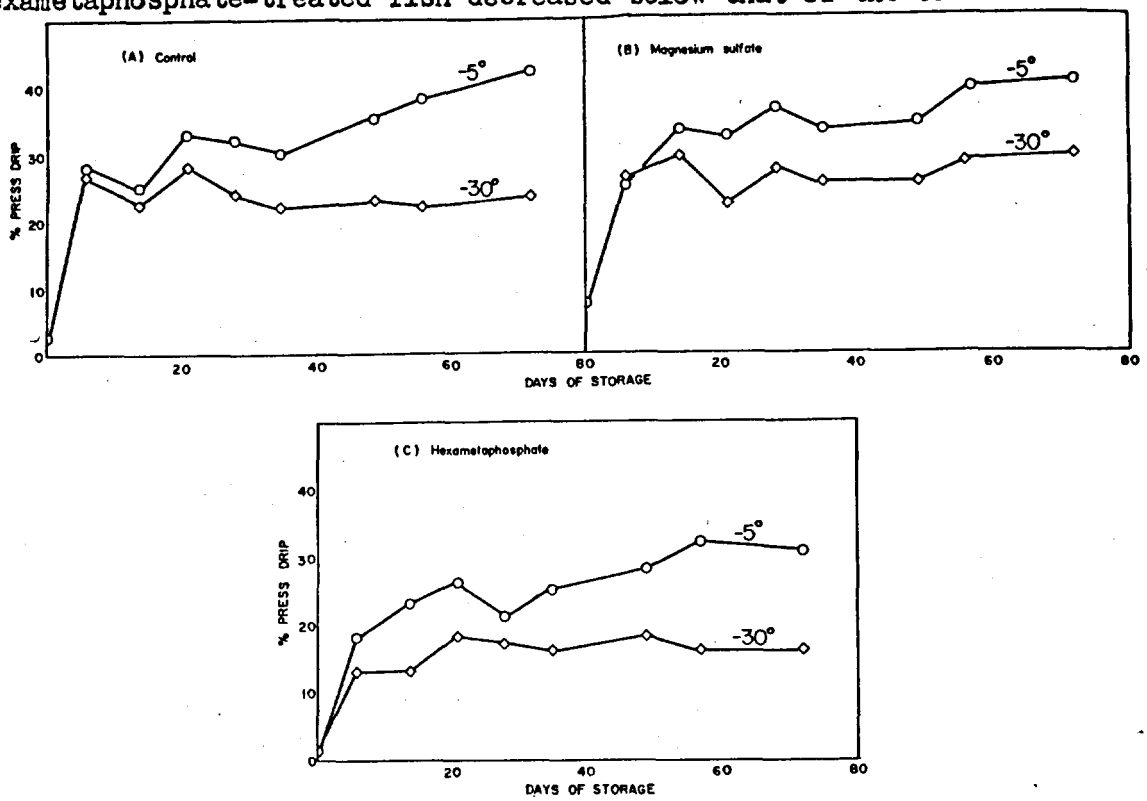


Fig. 3. Effect of pre-slaughter, intraperitoneal magnesium sulphate and sodium hexametaphosphate injections on the water retention of lingcod muscle during cold storage.

Analysis of the drip, taken at weekly intervals, was carried out to determine possible changes in its composition. The protein content of the drip collected from a Mg^{++}-injected fish remained constant at 5.1 ± .4%, that of a control fish at 7.0 ± .6%, and that of a hexametaphosphate-injected fish at 6.5 ± .4% over a time period of three months.

Drip analyses, carried out on untreated lingcod fillets and whole fish after storage at -5°C and -30°C, showed differences in the Mg^{++} concentration. The drip collected from samples stored at -30°C showed no significant variation in the Mg^{++} content. However, if samples were defrosted and refrozen the Mg^{++} content decreased from 19 to 21 mg to 13 to 14 mg per 100 ml of drip. An uneviscerated 50-cm lingcod, just going into rigor, was frozen at -30°C (Fig. 4). The Mg^{++} level in the drip collected from it the following week remained at a constant high level. Upon thawing the fish overnight at room temperature, the magnesium contained in the exuding fluid had dropped from 19.8 to 16.5 mg Mg^{++}%. The fish was again frozen at -30°C and the drip collected from it then had dropped further to 14 mg Mg^{++}%. It remained at this level when stored at -30°C. Some samples were first frozen at -30°C (12 hours) and then stored at -5°C. The Mg^{++} level in the drip collected from these samples dropped more gradually from 20 to 21 mg after the second day to 15 to 16 mg after three months storage. The described changes in Mg^{++} concentration seemed to be more pronounced in drip collected from fillets than from whole fish, when both were held under the same storage conditions. For instance, one fillet stored at

-5°C gave a value of 15 mg% after six weeks, while the control gave 21 mg%.

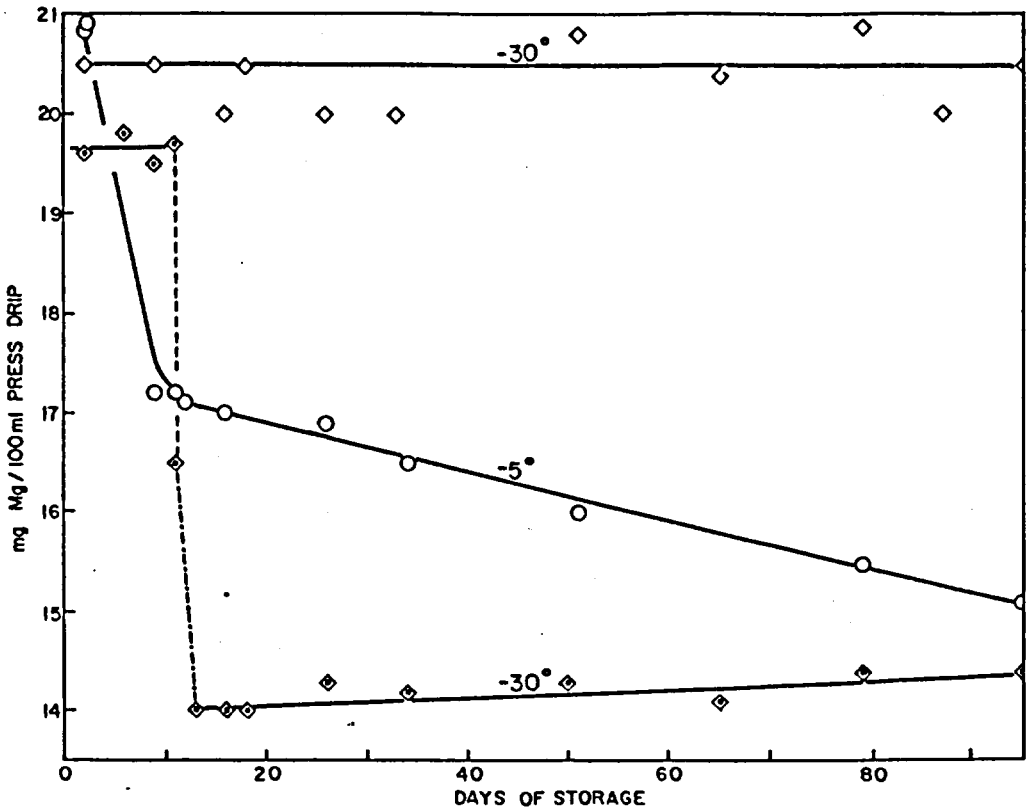


Fig. 4. Varying magnesium concentrations in press drip collected from lingcod, held under different storage conditions.

The change in the Mg^{++} concentration of the drip may be due to an increased affinity of denatured muscle proteins for small molecules. If this is so, the changing magnesium concentration may provide a useful measure of protein denaturation without altering the protein structurally as during extraction procedures. Kauzmann refers to the ability of a protein to adsorb small molecules as dyes, ions, lipids, etc. When a protein changes its configuration, some bonding sites may be destroyed and new sites may become available.

Conclusion

It is felt that the sclerometer used in the present experiments does not provide a complete solution to the difficulty experienced in measuring texture in fish products but the results seem to warrant a search for more refined mechanical measuring methods which may make it possible to express texture in the form of numerical values. Although the cause of toughening of fish muscle during frozen storage is very likely to be of a chemical nature, the effect is a change in the mechanical properties of the muscle and must be measurable in terms of such properties before an intelligent search and correlation can be carried out to determine the causative agent and the effect of different conditions on it. Taste panel evaluations of mechanical methods are very necessary but organoleptic evaluations are cumbersome and time-consuming and cannot always be taken as absolute since even trained judges may differ in their opinions under different conditions. In view of this and because of the need for a reliable method which can be used for rapid routine evaluations of texture in cold stored fish, it is felt that there are definite advantages to mechanical measurements.

Previous attempts to measure texture of fish muscle objectively have been

carried out on raw samples. However, since the final organoleptic evaluation for texture is always made on cooked samples, it seems to be desirable to make mechanical or chemical texture evaluations on cooked samples also, or at least establish a relationship between the texture of raw and cooked muscle.

SUMMARY NO. 43

RED MUSCLE AND RIGOR MORTIS IN LINGCOD

Hans Buttkus

The first major post-mortem changes in the appearance and structure of fish muscle became noticeable with the development of rigor mortis. The importance of rigor mortis as an influence on the keeping properties of fresh fish and on the quality of frozen fish has been realized and some work on this subject has been reviewed by Amlacher (1961). Generally the progress of rigor mortis was evaluated by touch on the basis of stiffness of the post-mortem fish. More refined mechanical devices were later developed by Messtorff (1954) and Nemitz and Partmann (1960) which measured changing mechanical properties of the fish muscle. Noguchi and Yamamoto (1955) utilized muscle contraction, caused by perfusing water, to determine rigor.

In this laboratory it was found that the lateral red muscle seems to have a major influence on the development of the stiffness associated with rigor mortis in lingcod. The unique post-mortem contraction of the superficial red muscle could therefore be used to provide information as to the rate of rigor development. Measurements of mechanical properties of the red muscle provided also evidence that some resolution of rigor mortis does take place in fish. The greater contraction of the red in comparison to the white muscle was also shown to lead to greater drip formation during thaw rigor in fish fillets.

Experimental

The lingcod (Ophiodon elongatus), 1-2 kg, used in the present experiments were caught at sea by trawl net and kept at the Vancouver Public Aquarium. When needed for an experiment they were transported to this laboratory under constant oxygen supply and with care to prevent excitement of the fish. They were then killed by stunning. Fillets to be used in thaw rigor experiments were stored immediately in plastic bags at -30°C. After a few days of storage they were cut into blocks about 4 cm^2 top area. The red muscle on the surface of the fillet was cut off some blocks and left in position on others.

Red muscle was obtained by filletting only one half of a fish. After removing the skin with a sharp knife, a portion of predetermined length of the red muscle was marked off with fine wood splinters and was then quickly dissected from the fillet. Marking of the original length of the muscle in the body of the fish was done to be able to express the contraction of the muscle relative to its original length. This was especially necessary in exhausted or exercised fish, where a very notable contraction takes place during and after the dissection. The white muscle used in these experiments was cut from the epimere. For the contraction experiments with a kymograph and lever arrangement, 1.5 to 2.0 cm wide and 10.0 cm long strips of red and white muscle were used. One end of the muscle was clamped into a plastic vice and suspended in a humidity chamber. A silk thread was tied to the lower end of the muscle and attached to one end of an aluminum lever (magnification 3.3). The changing length of the muscle was thus recorded with an ink pen on a kymograph drum turning at 2.2 revolutions per day. Penetration

measurements were made simultaneously with a Mangold sclerometer (as described in Summary No. 27 of this Station's Annual Report for 1960-61) on a section of white muscle only, a portion of white muscle with red muscle attached to it and a section of red muscle only.

To measure the elastic modulus and the work potential, the red muscle of trout was cut into strips 1.0 cm wide and 5.0 cm long. They were loaded within their elastic limit and unloaded every 15 minutes for 15 seconds. The muscles were kept throughout the experiments in a humidity chamber. The remaining portion of the fish from which the muscle had been cut was wrapped in polyethylene and held as a control at the same temperature at which the experiment was conducted. Comparisons with the amount of contraction, the elastic modulus and the stiffness of the fish could be carried out in this manner.

Results and Discussion

It was found during cold storage studies of fish fillets that the contraction of red muscle during thaw rigor is more pronounced than that of the white muscle. Preliminary studies of the different contraction characteristics showed that red muscle, although present only as a thin layer, contributes very much more to the shrinkage of fillets and leads to greater drip exudation during thaw rigor than white muscle. When pieces of frozen fillets from which the red muscle had been cut off were thawed at room temperature, the total contraction in length amounted to 13%. It rose to as much as 33% on pieces which contained white and red muscle. Such a large decrease in the dimension of length, on a section of white and red muscle, resulted, however, at the same time in an increase of height, so that the actual volume shrinkage remained comparatively small. The large alteration of dimensions which such a muscle block underwent during thaw rigor, resulted in an increased exudation of drip.

It seemed therefore also interesting to determine the contraction characteristics of red and white muscle in the course of normal rigor development, i.e. post-mortem conditions leading to rigor at room temperature. Of particular interest hereby was the question whether the maximum contraction of an isolated muscle would coincide with the beginning of the maximum rigor hardness of the fish from which the muscle had been excised. Contraction and simultaneous hardness measurements carried out at room temperature (19-21°C), gave some information as to the site of the major physical changes in the fish during rigor mortis. Although white muscle constitutes the bulk of the fish body, it did not increase appreciably in hardness with increasing post-mortem time. Even when cut at full rigor from the right half of the body, which had been left as a control, only a small change in hardness was noticed. The white and red muscle combined and the red muscle alone showed a marked increase in hardness with increasing rigor development. On the control fish the tail section and head portion rises slowly under the increasing tension, associated with increasing rigor development. This tension seems to originate mainly under the skin where the main portion of the superficial red muscle is located. On a beheaded fish, the skin portion, measured to the tail fin, decreased from its original length of 42 cm, immediately post mortem, to 36 cm in rigor. During this contraction it would pull the underlying white muscle with it towards the back, thus making it appear harder. After 24 hours post mortem at room temperature, the "resolution" of rigor, the length of the skin had returned to 39.5 cm.

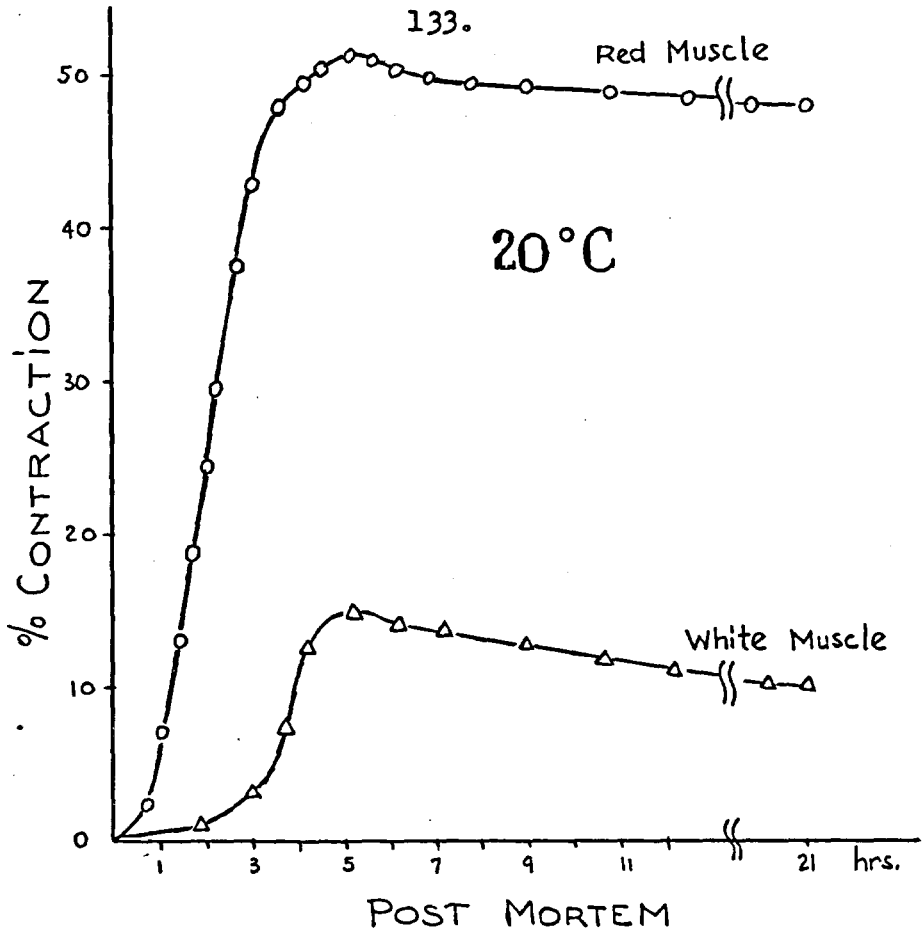
During these studies it was found that the point of maximum contraction of the red muscle, as measured with a lever and kymograph arrangement, usually coincided with the maximum hardness development of the control half of the fish, i.e. the beginning of full rigor. Although Bate-Smith and Bendall in their researches

on rabbit muscle state that considerable shortening sometimes occurs during the rapid phase of rigor, this shortening is not a necessary concomitant of stiffening and rarely occurs under the normal conditions of rigor at room temperature (10-25°C). For the red muscle of lingcod, however, the post-mortem contraction at 20°C is a consistent phenomenon and the rate of contraction can even provide information as to the condition of the fish before death. In the case of exhaustion of a fish before killing, the muscle seems to become "activated" and contracts at a higher rate, thus leading to the point of maximum contraction and rigor quicker than in a rested fish.

Factors which will alter the muscle contraction so that it does not coincide with the rigor development of the control half of the fish body result when the excised muscle is surrounded by a nitrogen atmosphere, when it is loaded beyond its elastic limit or when it is held at a different temperature.

Bate-Smith, Bendall and Marsh have described and used a method to determine certain mechanical characteristics for mammalian muscle. Since the short fibers of white fish muscle were not considered suitable for such measurements, comparative studies on this basis have never been carried out on fish. Red muscle fibers, however, are arranged more uniformly in one direction. This made it possible to compare the elastic modulus and work potential of the red muscle of a fish to mammals. Measurements were carried out from the time immediately post mortem to rigor mortis and its "resolution". At 6.52% initial extension of the red muscle of a 250-g trout (Salmo gairdnerii), the elastic modulus was found to be 1070 g/cm^2 (1.05 x 10^6 dynes/cm^2) and increased in rigor to 6000 g/cm^2 (5.9 x 10^6 dynes/cm^2). With the onset of rigor, the modulus of elasticity increases. For rabbit muscle (psoas) it rises from 500 to about 10,000 (Bate-Smith and Bendall); for beef (longissimus dorsi) from 1110 ± 270 to 34900 ± 17700 (Marsh); for chicken (pectoralis major) from 1000 to 9000 at 14°C or 2000 to 8000 g/cm^2 at 43°C (de Fremery). The work potential of the red lateral muscle from the same trout was found to be 2.3 g cm/g at 20°; that of rabbit is reported to be less than 1.0 and of beef 3.6 g cm/g at 17°C.

The contraction studies of the red and white muscle with the kymograph and lever arrangement showed that the red muscle contracts to 52% of its original length when going into rigor, white muscle only 13%. As can be seen from the graph, after the point of maximum contraction has been reached, a small but definite extension of the red as well as white muscle proceeds with time. A small decrease in the elastic modulus of the red fish muscle, following the initial point of maximum rigor, was also noted with the experimental arrangement according to Bate-Smith and Bendall. This fact may account for the slight but definite resolution of rigor mortis in fish. Beef muscle, on the other hand, when held after the development of rigor at 7°C for 7 days in nitrogen showed no increase in extensibility (Marsh 1954). Bendall concluded from such experiments that rigor does not resolve under aseptic conditions. The slight extension of the excised fish muscle occurs, however, almost immediately after the point of maximum contraction has been reached. This could be because the muscle has been dissected out of its original body site. A fish, on the other hand, going into full rigor after 5 hours, does not begin to soften again before about 12 or more hours post mortem. A definite decrease of rigor tension (softening) of a whole fish was generally noticeable after 24 hours.



The unusual properties of contraction shown by the myoglobin rich, red muscle, suggest that it is a very active tissue which might involve some major biochemical differences from white muscle. Measurements of the pH of a muscle homogenate, prepared from muscle just going into full rigor, gave values as low as 6.5 in some cases as compared to 6.70 to 6.65 for white muscle. Studies on the ATP content of red muscle have already been carried out by Saito (1961) who stated it to be less than in white muscle. He found that IMP formed in the red muscle is decomposed more rapidly, leading to an increase in inosine and hypoxanthine. The lipid content of red muscle of cod was found to be 2% as compared to 0.7% in adjacent white meat (Dyer and Hligh). In mackerel, herring and halibut, the fat content is reported to be as high as 27 to 29% (Braekkan). In his work on the B-vitamin content of red muscle he suggested that the red muscle acts as a supplementary liver rather than as a contractile organ. Braekkan felt that muscles with such high fat content are scarcely suited for strenuous and continued activities. The unusual contractive properties of the superficial, red muscle of lingcod and trout do not seem to warrant, however, their exclusion from the muscular tissues responsible for locomotion. The location of the main portion of the red muscle along the lateral line, its penetration to the spinal cord as well as the aligned direction from head to tail, seem to make it an active component of a fish's muscular equipment which is designed for rapid acceleration.

Electron micrographs of the red muscle of lingcod show the characteristic arrangement of myofibrils. The difference in the fine structure of this muscle, as compared to the adjacent white muscle and skeletal muscle from rabbits, lies in the position which is normally described as the A-band. The H-zone in the red muscle of lingcod is completely missing. The fine structure of the white muscle of lingcod shows all the characteristic features of skeletal muscle of rabbit as obtained by H.E. Huxley. The proportions of the bands are, however, different. The I-band is

very much shorter, while the A-band is proportionately longer. The mitochondria in the two different muscles show also a different structure. Those in the red muscle seem to be of the characteristic cristae type found in mammals. The mitochondria in the white muscle seem to be more of the tubular nature.

SUMMARY NO. 44

FATTY ACID OXIDATION BY THE MUSCULAR TISSUE OF RAINBOW TROUT

E. Bilinski

This investigation was initiated in order to gain information on the enzymic processes involved in utilization of lipids by living fish with special reference to the migrating salmon. An initial study is, however, being conducted with rainbow trout, as live fish of this species are continuously available. The immediate goal is to establish if the muscular tissue of fish has the ability to oxidize fatty acids.

The enzymic mechanism of oxidation of fatty acids is now well established and it is known that in animals such organs as liver and kidney are the important sites of this process. In fish, fatty acid oxidation has been demonstrated to occur in liver and the work of Brown and Tappel (1959) on carp liver mitochondria indicates that the nature of the fatty acid oxidation system is similar to that in mammals. It was long thought that in animals fats are not utilized in the skeletal muscle and that the aerobic metabolism of the muscle is primarily derived from carbohydrate degradation. More recently, however, evidence has been presented which indicates that in mammals fatty acids can be utilized by the muscle and it has been postulated that the lipids may be one of the major substrates of the muscle. Because of the development of this concept it appeared to be of interest to establish if the muscular tissue of fish has the ability to oxidize fatty acids.

In the present study a distinction was drawn between the dark and the white muscle as the two tissues have a very different chemical composition. Especially as far as lipids are concerned it is known that the dark muscle contains more total lipid, fatty acids and phospholipids than does the white muscle. Consequently it was felt that a comparative study of the lipid metabolism in these two tissues might provide some insight into the process of utilization of lipids by the living fish.

The present report presents results of experiments on oxidation of octanoic acid by the dorsal white muscle and the lateral dark muscle of rainbow trout.

Material and Methods

Rainbow trout (Salmo gairdnerii) about 2 years old were obtained from a commercial hatchery and they were held alive until required in the Station's fresh water aquarium.

The conventional Warburg manometric technique was employed in earlier stages of this investigation to measure the rate of oxidation of potassium octanoate. In the exploratory work the procedure was found to be fully satisfactory when applied to fish liver preparations but it was not possible to demonstrate the oxidation of octanoate by muscle homogenates and subcellular fractions with this technique. The various preparation media tried included isotonic, hypertonic sucrose and different phosphate and tris-buffers, with the addition of ATP, EDTA, albumin and carnitine in various combinations. The muscle preparations oxidized dicarboxylic acids but octonoate oxidation could not be demonstrated by them. In view of this, the incubation of tissue slices in presence of C^{14}-labelled substrate was investigated and

found to be a suitable technique and the procedure adopted will be described below.

The fish were stunned, killed by decapitation and skinned. The tissue slices of dark muscle were prepared from about 1 sq cm samples taken along the lateral line. The white muscle samples were taken from the dorsal section of the muscle in the middle of the fish. Tissue slices were cut by means of a Stadie-Riggs tissue slicer.

Incubation was conducted with Warburg apparatus at 25°C in an atmosphere of O_2. The incubation medium was similar to the one described by Ahmed and Scholefield (1961). Calcium was omitted from the Krebs-Ringer medium, the final concentration of various salts being: NaCl, 0.145 M; KCl, 0.0058 M; KH_2PO_4, 0.0015 M; $MgSO_4$, 0.0015 M. Sodium phosphate buffer, pH 7.4, was added to give a final concentration of 0.01 M and final pH 7.3. Octanoic acid employed as substrate, was labelled in the carboxyl group with C^{14} and it was used as the potassium salt adjusted to pH 7.3. The specific radioactivity of potassium octanoate was 0.2 μ curie per μmole. The C^{14}-labelled substrate was placed in the main compartment of the Warburg flask along with the other constituents of the incubation medium. A filter paper soaked with KOH was present in the centre well to collect the $C^{14}O_2$ produced and the side arm contained sulfuric acid.

Freshly prepared tissue slices were placed in chilled Warburg vessels containing the experimental medium and the C^{14}-labelled substrate. The incubation period was started after an initial equilibrium period of 10 minutes. At the end of the incubation, lasting 30 to 120 minutes, the acid was tipped from the side arm to stop the reaction and to liberate any CO_2 dissolved in the medium. After a further 30 min in the bath, the filter papers soaked with alkali were removed with washings from the centre well and they were extracted with water. 0.1 mM of carrier Na_2CO_3 was added to the solution, the carbonate was precipitated as $BaCO_3$ and mounted by filtration on discs of filter paper. The specific radioactivity of $BaCO_3$ was measured using a gas-flow counter provided with a micromil window. The contents of the main compartment of the Warburg vessels were transferred quantitatively to a Kjeldahl flask for the determination of total nitrogen.

An aliquot of the radioactive substrate was converted to CO_2 by wet combustion and it was diluted with carrier Na_2CO_3 and precipitated as $BaCO_3$ by following the procedure used for the determination of the specific radioactivity of the respiratory CO_2 collected in Warburg vessels. The percentage of conversion of the substrate was estimated from the ratio: specific radioactivity of $BaCO_3$ from respiratory CO_2/specific radioactivity of $BaCO_3$ from the total combustion of the substrate. The final results are expressed in terms of mμ moles of substrate converted to $C^{14}O_2$ per 1 mg tissue nitrogen.

Results

The results of experiments conducted on oxidation of potassium octanoate-1-C^{14} by tissue slices from the rainbow trout dark and white muscle are presented in Table I. A very pronounced difference in the rate of oxidation of octanoate was found for the dark and the white muscle. A comparison was made for an incubation period of 60 min at two different concentrations and it was found that at both concentrations the dark muscle oxidized octanoate considerably faster than did the white muscle. In 0.001 M solution (Exp. No. 1 to 6) the rate of octanoate oxidation by the dark muscle was approximately 50 times that of the white muscle. When octanoate concentration was reduced 10 times to 0.0001 M (Exp. No. 7 to 11) its oxidation was similarly about 50 times as great in the dark muscle as in the white one.

TABLE I - Oxidation of potassium octanoate-1-C^{14} by tissue slices from rainbow trout dark and white muscle at 25°C.

Expt No	Tissue	Incubation time minutes	Substrate concentration	mμM substrate converted to $C^{14}O_2$ per 1 mg tissue N
1	Lateral dark muscle	60	0.001 M	10.70
2	"	"	"	10.16
3	"	"	"	10.30
Av 1-2-3	"	"	"	10.39
4	Dorsal white muscle	60	0.001 M	0.25
5	"	"	"	0.16
6	"	"	"	0.14
Av 4-5-6	"	"	"	0.18
7	Lateral dark muscle	60	0.0001 M	2.52
8	"	"	"	4.24
Av 7-8	"	"	"	3.38
9	Dorsal white muscle	60	0.0001 M	0.04
10	"	"	"	0.06
11	"	"	"	0.08
Av 9-10-11	"	"	"	0.06
12	Lateral dark muscle	30	0.001 M	5.96
13	"	"	"	5.44
14	"	"	"	9.20
Av 12-13-14	"	"	"	6.87
15	Lateral dark muscle	90	0.001 M	14.40
16	"	"	"	10.70
17	"	"	"	10.20
Av 15-16-17	"	"	"	11.77
18	Lateral dark muscle	120	0.001 M	15.40
19	"	"	"	14.40
20	"	"	"	19.20
Av 18-19-20	"	"	"	16.33

Regarding the optimum concentration for the oxidation of octanoate it appears from the available data that the amount of octanoate metabolized per 1 mg tissue nitrogen is about 3 times greater in 0.001 M than in 0.0001 M solution for the dark and white muscle.

The incubation of tissue slices of dark muscle was carried out for various lengths of time between 30 and 120 minutes. It appears from the data in Table I (Expt No 12 to 20) that the octanoate oxidizing system maintained activity during the 2 hours experimental period, although the oxidation rate decreased with time.

To make certain that octanoate-1-C^{14} was not spontaneously evolving $C^{14}O_2$ non-enzymatically during the course of the experiments, boiled preparations of dark and white muscle were incubated in presence of labelled octanoate in a fashion otherwise identical with the usual procedure. The absence of any appreciable

release of $C^{14}O_2$ under these conditions is an indication of the enzymic nature of the observed oxidation of octanoate.

It may be concluded from the present investigation that fish muscle contains an enzyme system that will oxidize octanoic acid. This transformation can be demonstrated in both the white and dark muscle; however, quantitatively a very pronounced difference exists between the two tissues, the dark muscle being considerably more active. The oxidation by the dark and white fish muscle of other fatty acids of various chain lengths is at present under investigation.

SUMMARY NO. 45

BLOOD pH AND MORTALITY IN RAINBOW TROUT (SALMO GAIRDNERII) AND SOCKEYE SALMON (ONCORHYNCHUS NERKA)

R.E.E. Jonas
Harcharan S. Sehdev
N. Tomlinson

This was a new project, undertaken and completed during the present year. A number of studies of the effect of exercise on the pH and lactic acid content of fish blood have been made. These have been reviewed in detail by Black (1958). He has pointed out that while in some species of fish death may result from severe exercise, the primary cause of death has not been determined, although it has been suggested that two of the factors that might be concerned are (1) a decrease in blood pH and (2) an increase in lactate concentration in the blood. It appears that the tolerance of fish toward lowering of blood pH has not been established (see Hunn, 1959). The present report describes an investigation of the ability of rainbow trout (Salmo Gairdnerii) and sockeye salmon (Oncorhynchus nerka) to survive a lowering of blood pH brought about by injections of dilute acids.

Materials and Methods

Rainbow trout were supplied by the British Columbia Provincial Hatchery at Cultus Lake, B.C. and sockeye salmon fingerlings by the Biological Station of the Fisheries Research Board of Canada, Nanaimo, B.C. The sockeye were held at the Vancouver Public Aquarium and were converted to living in sea water soon after being received. The trout were about two years old and the sockeye salmon about two and one-half years old when the experimental work was carried out. The fish were maintained on a diet of pelleted fish food (J.R. Clark Co., Salt Lake City, Utah) supplemented once a week by ground horse heart with added riboflavin.

When fish were to be injected with acid or salt solutions, or when blood was to be drawn from them, they were anaesthetized by immersion in a 1 part in 12,000 solution of MS222 (tricaine methanesulfonate, Sandoz Ltd.). The period of immersion was such that the fish were not completely anaesthetized but were quiet and easy to handle. Injections or blood withdrawals were made via the Cuvierian duct by means of a hypodermic syringe fitted with a No. 26 needle according to the method of Sano (1960) while the fish were held in air. When blood was to be withdrawn, the syringe was first rinsed with a solution of heparin (0.4 mg/ml) and then drained. The quantity of blood drawn varied between 0.3 and 0.6 ml per 100 g body weight of fish. Measurement of blood pH was made with a model G Beckman pH meter, the blood being ejected directly onto the electrodes from the syringe. Acid or salt solutions were injected at a rate of about 0.1 ml/30 seconds. Rapid injection of even a small volume of acid solution usually resulted in death of injected fish. Following the injection the fish were placed in water for one minute, after which blood was withdrawn from the duct on the opposite side of the fish from that into which the

injection was made, then the fish were returned to water to recover from the anaesthetic. For measurement of blood lactate, blood was mixed with an equal volume of 10% (w/v) trichloroacetic acid and lactic acid was determined in the clarified supernatant solution by the method of Barker and Summerson (1941). Solutions injected were 0.135 and 0.54 M lactic acid, 0.135 M hydrochloric acid, 0.54 M sodium lactate and 0.54 M sodium chloride. All solutions were sterilized by autoclaving before use. Water temperatures in which the fish were held varied between 11° and 14°C during the course of this work. Fish were exercised by chasing them in a small tank by means of a dip net.

Results and Discussion

The pH values of the blood of a group of 12 unexercised trout (average weight 105 g, range 63 to 170 g) fell between 7.05 and 7.5 with a mean of 7.3, while those of a group of 13 exercised trout (average weight 114 g, range 67 to 220 g) fell between 7.1 and 7.4 with a mean of 7.25. The time required to anaesthetize the fish and measure their blood pH was about 10 to 15 minutes. Periods of exercise varied between 15 and 100 minutes but no correlation between duration of exercise and blood pH was observed. Determinations of lactic acid were made on the blood of 4 fish from each group. In unexercised fish the mean value was 18 mg per 100 ml blood (18 mg %) with a range of 5.5 to 28.2 mg %, while in the exercised (30 minutes) fish the mean was 36.3 mg % with a range of 15.4 to 60.4 mg %. These results are comparable to those found by various workers and summarized by Black *et al* (1961).

Twenty-two trout (average weight 160 g, range 81 to 255 g) were injected with sodium lactate in amounts varying between 43 and 150 µM/100 g body weight, without any fatalities occurring. In 15 of these fish measurement of blood pH was made and the mean value was found to be 7.29, with a range of 7.1 to 7.5. Blood lactate in nine fish injected with 100 to 150 µM of sodium lactate/100 gm body weight averaged 198 mg %, range 110 to 309 mg %. Six trout were injected with sodium chloride in amounts varying between 108 and 324 µM/100 g body weight without fatalities.

The results of injections of trout with lactic acid solutions and with hydrochloric acid solutions are tabulated in Tables I and II, respectively. The data indicate that under the conditions employed, a critical range in blood pH is encountered between about 6.9 and 6.8. Above this range the fish usually survived (a single fish died with a blood pH of 7.22), while below it the fish usually died (a single fish lived with a blood pH of 6.6). It should be noted that while a number of fish were injected with the same quantity of each acid, death occurred in only those in which the blood pH was lowered into or below the critical range. This indicates that death was not caused directly by the injection of the acids themselves but from the changes in pH that it brought about. It is possible that the blood of the fish which survived was in a more alkaline condition at the time of commencement of injection than was that of those that died - a possibility that is indicated by the range of pH values found in unexercised fish (*vide supra*). Fish that died did so within a few minutes, usually without recovering from the effects of the anaesthetic. Examination of the data of the Tables, in conjunction with that for injection of sodium lactate and chloride solutions given above, will show that death of the trout cannot be attributed to increases in either the lactate or chloride ion concentrations of the blood *per se* in the present work. However, this observation should probably not be regarded as evidence against the possibility that a high lactate concentration, if maintained for a protracted period of time, could cause death.

Results of a limited study with sockeye salmon are summarized in Table III.

A relationship between blood pH and mortality in these fish similar to that observed in trout is indicated. Blood lactate concentrations in exercised and unexercised fish were comparable to those previously found in two-year-old sockeye by Black (1957).

TABLE I - Survival of two-year-old rainbow trout injected intravenously with lactic acid.

Weight of fish g	Lactic acid injected µM/100 g body weight	Blood pH after injection	Blood lactate after injection mg %	Alive (A) or dead (D)
135	25	7.3	61.2	A
175	37	7.3	69.	A
170	25	7.22	108.	D
140	37	7.2	58.5	A
155	20	7.2	34.2	A
145	25	7.2	46.2	A
135	36	7.16	63.9	A
145	25	7.16	43.5	A
240	25	7.16	63.	A
76	54	7.15	-	A
103	54	7.15	-	A
93	54	7.15	-	A
145	13	7.12	19.5	A
340	12	7.1	51.6	A
180	25	7.08	30.3	A
230	19	7.05	45.	A
140	25	7.03	58.5	A
160	25	7.00	49.1	A
270	19	6.9	51.6	A
240	22	6.9	60.6	A
175	25	6.82	87	D
220	81	6.8	-	A
170	25	6.8	60.3	D
150	25	6.62	149.	D
200	19	6.6	129.	A
140	25	6.5	83.2	D

Ten additional injected fish with a blood pH of 6.4 or lower all died. There is reason to believe that in the fish with a blood pH of 7.3 and above in this and the following Tables that not all of the acid injected entered the duct.

TABLE II - Survival of two-year-old rainbow trout injected intravenously with hydrochloric acid.

Weight of fish g	Hydrochloric acid injected µM/100 g body weight	Blood pH after injection	Blood lactate after injection mg %	Alive (A) or dead (D)
265	20	7.4	24.8	A
130	20	7.3	22.8	A
155	25	7.2	21.	A
240	25	7.2	24.9	A
150	20	7.2	21.0	A
185	25	7.2	19.1	A
150	12	7.2	40.2	A
160	25	7.1	15.5	A
175	25	7.1	13.1	A
145	12	7.1	45.3	A
200	25	7.05	38.7	A
140	20	7.0	13.2	A
175	14	7.0	25.5	A
135	25	6.9	11.4	D
120	25	6.9	12.0	D
170	20	6.9	21.9	A
170	25	6.85	15.0	A
175	25	6.85	22.5	D
330	25	6.8	54.9	D
155	25	6.7	23.7	D

The range of blood pH in which mortality began to occur in either trout or sockeye (6.9 to 6.8) is only slightly below that observed immediately after exercise or during recovery in rainbow trout (e.g. Black *et al*, 1959), but it should be remarked that the results in the present work may have been influenced by the experimental conditions, particularly through the use of an anaesthetic, in such a way that death occurred at a higher pH than might have been the case under other circumstances. However, this is not certain, and in our experience it would be very difficult, if not impossible, to carry out the necessary injections without the use of an anaesthetic. In this connection Leivestad *et al* (1957) have shown that removing an unanaesthetized fish from water for four minutes causes a pronounced bradycardia accompanied by increased lactic acid formation in the muscles. The lactic acid subsequently passes into the blood. These workers also showed that bradycardia can be induced in fish through fear while they are submerged. It is interesting that it has been shown (Crowell and Houston, 1961) that in the dog a reduction in pH to a little below 7.0 results in a remarkable reduction in the clotting time of heparinized blood, and also that lowering the pH of blood initiates coagulation.

TABLE III - Survival of two and one-half-year-old sockeye salmon injected intravenously with lactic acid.

Weight of fish g	Compound injected μM/100 g body weight	Blood pH after treatment	Blood lactate after treatment mg %	Alive (A) or dead (D)
110	None - fish unexercised	7.2	46.7	A
170	do	7.04	25.1	A
185	do	7.03	25.9	A
201	do	7.0	178.	A
180	None - fish exercised 50 min	7.04	76.5	A
165	do	7.3	74.5	A
200	do	7.3	55.8	A
110	do	7.25	63.	A
150	Sodium lactate, 150[1]	7.28	154.	A
145	do , 150	7.27	414.	A
160	Lactic acid, 25	7.3	41.1	A
155	do	7.18	54.	A
140	do	6.88	67.5	A
105	do	6.87	71.5	A
160	do	6.72	85.5	D
120	do	6.72	78.7	D
155	do	5.85	150.	D

[1] This injection apparently did not all enter the duct.

Acknowledgments

The authors' thanks are due to Mr. J.G. Terpenning, Superintendent of Hatcheries, British Columbia Department of Recreation and Conservation, Fish and Game Branch, Vancouver, B.C. and to Dr. J.R. Brett, Fisheries Research Board of Canada, Biological Station, Nanaimo, B.C., for the rainbow trout and sockeye salmon, respectively, used in this work. We also wish to thank Dr. E.C. Black, Department of Physiology, University of British Columbia for his interest and valuable suggestions during this work and Dr. M.A. Newman, Curator, Vancouver Public Aquarium for use of certain facilities.

MAILLARD BROWNING REACTIONS IN MUSCLE

SUMMARY NO. 46

INTRODUCTORY REMARKS — H.L.A. Tarr

The practical significance of a basic research study of Maillard browning reactions in fish muscles, first initiated at this Station in 1947 (Summary No. 1 of this Station's Annual Report for 1947 and Summary No. 25 of this Station's Annual Report for 1948), is gradually emerging. Thus this general reaction is now

being studied in Japan in its relation to various fish-canning problems and in Great Britain in connection with dehydration research. In British Columbia knowledge gained as a result of this early research has found application in a novel canned herring process. Since the carbohydrate compounds of fish muscles play a very prominent role, though not the sole one, in promoting Maillard reactions, a further study of these has been launched. This study has been greatly facilitated by the live fish-holding facilities recently provided at this Station. The problem is being attacked from two angles, namely: (1) origin of the carbohydrate compounds involved, and (2) the means of removal, or control of development, of these compounds and the effect of this removal on browning.

SUMMARY NO. 47

ACID-SOLUBLE PHOSPHORUS COMPOUNDS AND FREE SUGARS IN FISH MUSCLE AND THEIR ORIGIN

H.L.A. Tarr
M. Leroux

The results of the first investigation of the acid-soluble phosphorus compounds of fish skeletal muscles were published over ten years ago. In this study the fractionation methods used were crude by present standards but it was established that fish muscles contained sugar phosphates and nucleotides in concentrations rather similar to those occurring in rat muscle. Later, the occurrence of ribose and glucose in fish muscles post mortem was demonstrated and the comparative significance of these in occasioning Maillard reactions in heated and dried fish flesh demonstrated.

During the past few years there has been increasing activity in this field, research being greatly facilitated by improved chemical techniques. Thus, post-mortem changes in fish muscle nucleotides, sugar phosphates, and free sugars have been studied by Japanese, Canadian and British investigators. The occurrence of these compounds in ox muscle has also been recorded and their possible effect on flavour of flesh foods suggested.

Though it is now well recognized that ribose, and possibly glucose also, occur only post mortem in fish muscles, the precursors of these sugars have not been clearly established. Possible mechanisms for glucose formation include direct amylolytic hydrolysis of glycogen or its formation from glucose phosphates (see below). Those for ribose formation include hydrolysis of ribonucleic acid or nucleotides, or its formation through hydrolysis of R5P which could be formed from G6P via the pentose cycle. The possible role of these mechanisms was suggested by the fact that there is at present abundant evidence for the occurrence of enzymes of both the Embden-Myerhof glycolytic pathway and the hexose monophosphate shunt system in fish tissues. Also, phosphomonoesterases capable of releasing Pi from both hexose and pentose phosphates are present in fish muscle. The present paper is concerned with the origin and quantitative distribution of sugar phosphates and of the predominant nucleotides in fish muscles and with the relation of these and of glycogen to formation of free sugars post mortem.

Materials and Methods

Reference Compounds

The following reference compounds were obtained from the sources indicated: dicyclohexylammonium F1P (Boehringer; through California Corporation for Biochemical Research, Los Angeles); barium F6P (Schwarz Laboratories, Mount Vernon, N.Y.); all

nucleotides (Pabst Laboratories, Milwaukee, Wisconsin); dipotassium G6P and G1P (Sigma Chemical Co., St. Louis, Mo.); shellfish glycogen, (Mann Research Laboratories, New York); dicyclohexylammonium R1P, DR1P and barium RU5P (previously prepared in this laboratory and stored at -30°C; tricyclohexylammonium hydrogen FDP (the product was prepared in this laboratory. It was chromatographically homogeneous and the total P content was 9.30%; theory 9.46%; it melted with decomposition at 262-263°C); anthrone (Eastman Kodak Co.) was recrystallized before use. The ion exchange resins used were purchased from the California Corporation and were analytical grades of Dowex 1 x 4 Cl^- (screened to 60-150 wet mesh) and Dowex 50 x 8 H^+ (200-400 mesh). Barium G6P, C_1C^{14}, and glucose C^{14} uniformly labelled (U.L.) were purchased from New England Nuclear Corporation, Boston, Mass., and ATP C^{14} U.L. from Sigma Chemical Co. BaG6P C^{14} U.L. was prepared by action of hexokinase on a mixture of inactive glucose plus glucose C^{14} U.L. (ratio 135:1) by the method used in preparation of mannose 6-phosphate. The product was 89% pure (calculated assuming a MW of 396), had a P:glucose ratio of 1:1, was chromatographically homogeneous and had a specific activity of 68 x 10^3.

Preparation of C^{14} Glycogen

C^{14} glycogen was prepared from fish muscle employing the method used in making rat liver glycogen. Two Salmo gairdnerii (200-300 g) were made to swim actively for 10 minutes in order to deplete their muscle glycogen. They were anaesthetized with MS222 and each fish was injected intraperitoneally with 1.0 ml of a solution containing 0.33 mC of $K_2C^{14}O_3$. After 18 hours rest to permit resynthesis of muscle glycogen, during which time the fish were fed, they were stunned and the excised muscle (175 g) frozen in liquid nitrogen. Glycogen was isolated by hot KOH treatment of the chopped frozen muscle and alcohol precipitation. It was precipitated three times with 0.8 vol of ethanol and dried in vacuo over P_2O_5 (yield 150 mg). The product was 97% pure when analyzed by the anthrone reaction with Mann shellfish glycogen as standard. The radioactivity did not change appreciably during the last two precipitations and was 700 cpm per mg. When a small portion was hydrolysed with HCl and the glucose separated by paper chromatography (solvent E; see below), it was found to have a specific activity of 200.

Paper Chromatography

Unless otherwise stated paper chromatography was carried out on Whatman No. 40 acid-washed paper employing descending development for 24 hours at 20°C with the following solvent systems: A. 72% phenol (72 g phenol plus 28 ml H_2O); B. tertiary butanol:H_2O:picric acid, 80 ml:20 ml:4 g; C. tertiary butanol:0.1 N HCl, 8.0:20 v/v; D. isobutyric acid:H_2O: 28% NH_4OH, 66:33:1; E. n-butanol:95% ethanol:H_2O, 52:32:16. Sugars , and in some instances sugar phosphates, were developed by aniline hydrogen phthalate spray. The molybdic acid spray method of Hanes and Isherwood was also employed for sugar phosphates, while in certain instances, particularly where the picric acid solvent was used, the method of Loring et al proved both useful and very sensitive. Nucleotides were detected with a Mineralite ultraviolet lamp.

Analytical Methods

Glycogen was determined by the hot KOH method with a 2-g muscle sample. Free sugars were determined as follows: ground muscle (4 g) was extracted at 0° three times with 4 ml of 0.6 M perchloric acid, the mixture being filtered with suction and pressed between each extraction. The clear filtrate was neutralized with 10 N KOH, and after 1 hour at 0°C the potassium perchlorate was removed by filtration, the filter being washed with cold H_2O. The filtrate was run through a 7 x 1.8 (diam) cm Dowex 1 x 4 Cl^- column which was washed with 50 ml of water, and then through a similar Dowex 50 x 8 H^+ column which was also washed. The solution was evaporated to

dryness. Where necessary glycogen was removed by addition of an equal volume of ethanol and centrifuging at 0°C. After drying the residue was made to a known volume (usually 2.0 ml) and the glucose and ribose determined by the anthrone and orcinol reactions respectively (see below). In certain instances the solutions were concentrated to a very small volume in vacuo and chromatographed using solvent system E. In this solvent system ribose (Rf .34), glucose (Rf .21) and maltose (Rf .15) (and a slower moving sugar present in some fish muscles) separated well and could be eluted for radioactivity measurements. The aqueous solutions were filtered carefully since filter paper particles react with the anthrone reagent. Ribose was determined by the method of Mejbaum with ribose as standard. Hexose sugars (glucose and fructose) and their phosphate esters were determined by the anthrone method using the appropriate conditions for each compound and allowing for salt interference. In some cases fructose was determined by the Roe method. Phosphorus was determined by the Gomori technique, using hydrolysis where necessary. Deoxyribose was determined by Racker's modification of the Dische diphenylamine reaction.

Ion Exchange Chromatography

Sugar phosphates and nucleotides in fish muscle samples were separated on ion exchange columns using a system which appears to give a much better resolution than that suggested by Jones and Burt. Dowex 1 x 4 Cl^- was wet sieved, that passing a 60-but not a 150-mesh screen being retained. Initial experiments indicated that there was no significant advantage to be gained by constructing columns from resin of different mesh sizes as was found advantageous for nucleotide separations. Jacketed columns either 1.0 or 1.4 cm in diameter and 100 cm long were used and maintained at 3-6°C.

The fish muscle sample (30 - 100 g) was blended with 2 volumes of 0.6 M PCA at 0°C. The blended material was filtered immediately with suction, the filter cake washed with 0.5 volume of 0.6 M PCA, and the filtrate promptly neutralized (to about pH 7.5) with 10 N KOH. Even though this procedure was carried out as rapidly as possible at 0°C, tests (see experimental part) showed that RIP was rapidly hydrolysed. This ester is completely hydrolysed by 0.1 N PCA in 30 min at 20°C. After an hour at 0°C, the extract was filtered by gravity through Whatman No. 1 paper and the potassium perchlorate precipitate was washed with a little cold water. The filtrate was applied to the resin column under 5 - 7.5-lb pressure and the column washed with 50 to 100 ml of water. The column washings contained the free sugars. The sugar phosphates and nucleotides were eluted as follows: the gradient system used was based on that described for nucleotides by Pontis and Blumson. Two straight-walled glass jars were connected by an inverted U tube passing through rubber stoppers. The mixing vessel (A_1) (18 cm diam) contained 5 litres of a solution containing 0.05 M KCl, 0.04 M CH_3COO Na and 0.005 M $Na_2B_4O_7$, and the reservoir (A_2) (14 cm diam) 3000 ml of 0.02 M HCl containing 0.15 M KCl. Under these conditions the solutions in the two vessels were at the same height and the ratio of areas $\frac{A2}{A1}$ was 0.6. After ensuring that the U tube was filled, 7.5 lb sq in pressure was applied which gave an elution rate of about 1.2 litre/day with a 1 cm diam column and 2.5 litre/day with a 1.4 cm diam column. Twenty-ml fractions were collected at 3-6°C. This elution system gave a slightly concave upward gradient of Cl^- which was calculated using the equation of Pontis and Blumson (Fig. 1). The borate caused complexing of the cis-glycols and especially delayed elution of G6P and R5P. The acetate caused a buffering effect which ensured a fairly good separation of FDP and the nucleotides.

Fig. 1 illustrates the separation which was attained when a solution containing 9 sugar phosphates and 11 nucleotides was applied to a 1-cm diam column. The recoveries of the compounds are given in Table I. In general, separations and

recoveries were good. However, F1P and F6P, and G1P and DR1P were eluted together and FDP was not well separated from AMP. The system proved unsatisfactory for separation of "trace" nucleotides, (e.g. CMP, GMP, UTP, CTP and GTP) which occur in certain fish muscles. It was found that separations were not usually as sharp with fish muscle extracts. Thus R5P and F6P usually emerged together, and in muscle samples stored at 0°C inosine occurred in this general fraction. However, it is difficult to ensure ideal separations when there are considerable variations in the composition of the extract applied.

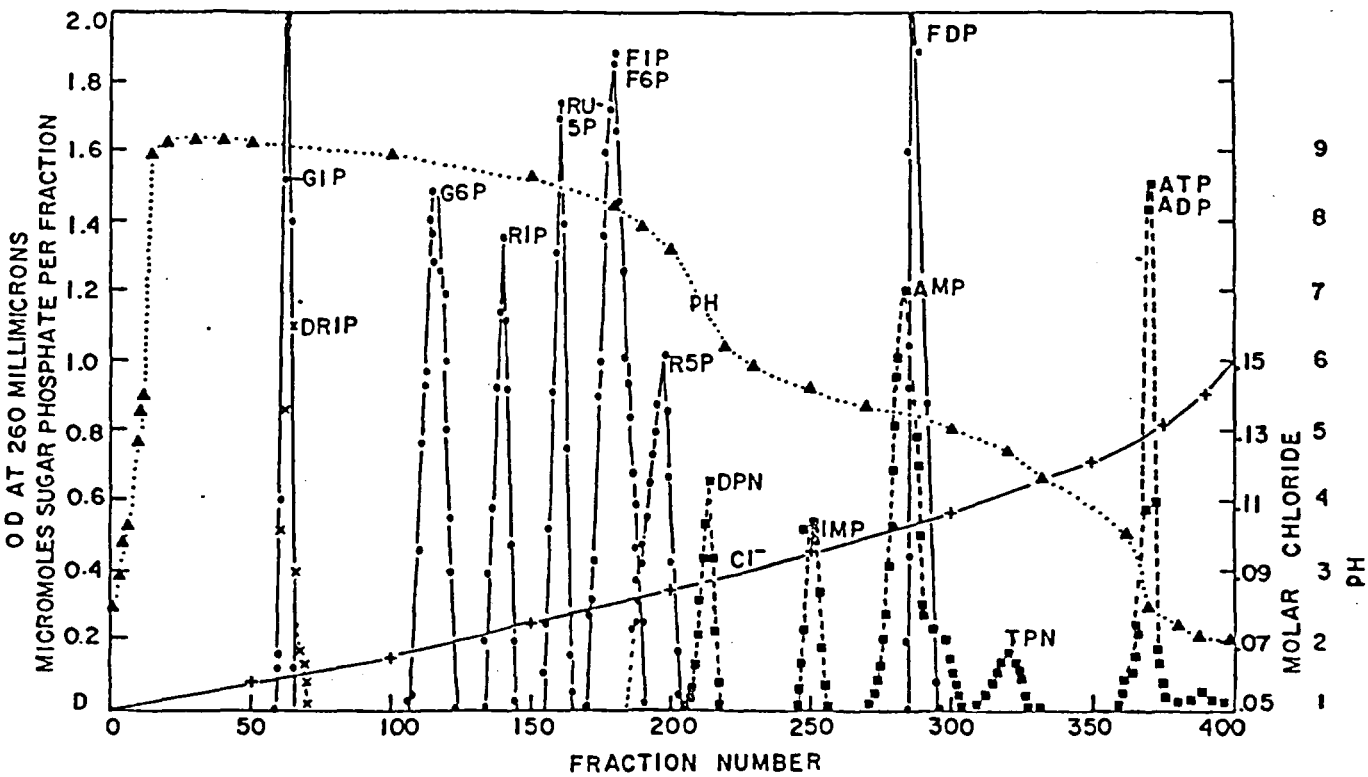


Fig. 1. Separation of a known mixture of sugar phosphates and nucleotides using a Dowex 1 x 4 Cl^- column. Sugar phosphates, o _____ o; nucleotides, ▫ - - - ▫.

Preparation of Lithium Salts

For measurement of radioactivity of certain samples, and for their positive identification in some instances, it was occasionally necessary to isolate the various compounds from pooled ion exchange column eluates. Though in some instances, especially where there were fairly high concentrations of sugar phosphates, it proved feasible to prepare crude barium salts using the classical alcohol precipitation method, it was often found that the comparatively high concentrations of inorganic salts and of borate ions seriously interfered with and often prevented successful isolation. For this reason lithium salts were usually prepared, the following method being used.

TABLE I - Recovery of sugar phosphates and nucleotides from a Dowex-1 Cl^- column.

Compound	μmoles chromatographed	μmoles eluted	% Recovered	Ratios:* P	Sugar	Base	OD ratios** 250/260	280/260
DR1P	10	10.5	105	1	0.96			
R1P	8	7.3	92	1	1.04			
R5P	10	9.4	94	1	1.15			
RU5P	8.7	8.9	101	1	0.92			
G1P	10	9.0	90	1	0.96			
G6P	10	9.7	97	1	0.96			
F1P	10	19.5	97.5	1	0.93			
F6P	10							
FDP	10	10	100	***				
AMP	10			1: 0.206				
CMP	2.5			(0.214)				
GMP	5.0							
ADP	5	8.3	83	2.46	1.0	0.87	0.82(0.85)	0.22(0.22)
ATP	5							
IMP	10	9.35	93.5	0.97	0.95	1.0	1.70(1.68)	0.24(0.25)
DPN	5	5	100	1.8		2.0	0.82(0.83)	0.21(0.22)
TPN	2.5	2.5	100	2.88		1.0	0.85(0.83)	0.19(0.22)
GTP	2.5	These were not recovered. Probably occurred after fraction 400.						
UTP	2.5							
CTP	2.5							

* The values for G1P and DR1P were determined by dividing the total P in the fraction by 2.

** Determined at pH 2 using "peak" fractions; values in brackets are those given in the literature for pure compounds.

*** These fractions were not well separated. FDP was distinguished by the anthrone reaction. Total base was determined at 260 mμ using the factor 1.50×10^4 to convert OD readings to μmoles per ml. Ribose determined with pooled fractions 271-283 was 8.25 μmoles, accounting for most of the 10 μmoles of added AMP. The "shoulder" in Fig. 2 (fractions 295-301) probably consisted of CMP + GMP. The ratio of P contributed by FDP + AMP + CMP + GMP to total base was close to the approximate theoretical value.

The pooled fractions (usually 300 to 1000 ml) were passed through a 15 x 2.8 cm diam column of Dowex 50 x 8 H^+ resin which was washed with 100-200 ml of water. Smaller columns were used where the volumes were less. The clear eluate was evaporated to dryness (in this and subsequent work rotary evaporators with 40°C water bath temperature were used). The dry residue was transferred to a small spherical flask with methanol (25-50 ml) and the methanol distilled as above to remove borate ions as methyl borate. The process was repeated. This procedure removes cations and also borate and acetate ions. The dry residue was dissolved in a small volume of water (usually 0.5-2 ml) and the pH was adjusted to about 7.5 with 1 M LiOH. Acetone was added with shaking (3-5 v) maintaining the pH above 7.5 with LiOH solution. The crude lithium salt was obtained by centrifuging at 0°C. It was dried over P_2O_5 in vacuo. In some cases the salt was reprecipitated to improve the purity of the product. By this method it was possible to isolate a very few milligrams of the crude lithium salts of G1P, G6P, F6P, R5P or FDP from several hundred ml portions of Dowex-1 Cl^- column eluates. In cases where proof of presence or absence of F1P in the fructose monophosphate eluate was sought, the method was

varied by carrying out the ion exchange treatment at 0°C and adjusting the eluate to pH 6 immediately before carrying out the rest of the procedure. The products thus obtained were less pure but F1P did not hydrolyse. It is not known whether F1P will hydrolyse during the normal procedure, but its comparative acid lability suggests that it might. The lithium salts were used directly for paper chromatographic separation of the phosphate esters in suitable solvent systems where it was necessary to determine radioactivity or to separate two compounds such as F6P and R5P or F6P and F1P. The compounds were eluted from the paper with water, the solution filtered to remove filter paper particles, and concentrated to a small known volume for analyses.

Measurement of Radioactivity

Radioactivity was determined with "Nuclear" gas flow equipment employing a micromil window and an automatic sample changer. In some cases a "low background" counter was used. Due to the comparatively small quantities of compounds available in many cases the samples were counted by the "infinitely thin" method. Aluminum planchets (3.2 cm diam) were carefully cleaned with 90% cold formic acid followed by brief immersion in hot "Heikol" alkaline clearing compound, and were then rinsed thoroughly with distilled water and dried. Every holder and planchet was checked for radioactivity before use. The compounds were applied as aqueous solutions, usually in from about 0.02 to 2.0 µmole quantity, care being taken to spread the solution during drying to give a very even film. In this way an approximately linear relationship between quantity and count resulted.

Results

Separation of Sugar Phosphates and Nucleotides in Fish Muscle

The fish employed, unless otherwise stated, were maintained in aquaria at this Station or at the Vancouver Aquarium and were normally rested and well fed. They were obtained with a minimum of struggling and were promptly stunned by a blow on the head. The muscle was quickly excised and frozen in liquid nitrogen or in crushed dry ice and was usually used within a few hours or, if not, stored at -30°C until required.

Fig. 2 shows a typical separation of sugar phosphates and nucleotides obtained using the extract from 38 g of muscle from 2 small rested sockeye salmon (Oncorhynchus nerka) (Sample No. 1, Table II). Table II records the results of similar analyses carried out with a number of samples of muscle excised from freshly-killed fish of widely different species (rainbow trout, Salmo gairdnerii; halibut, Hippoglossus stenolepis; Atlantic cod, Gadus callarias; and lingcod, Ophiodon elongatus). It is obvious that the results indicate extreme variability. This is to be expected since exact duplication of conditions for killing fish and treating samples is extremely difficult to attain and a very large number of enzyme systems are involved in establishing equilibria. A case in point is the result of two separate "runs" with a single Atlantic cod muscle sample which was obtained from a freshly killed and rested fish by courtesy of Dr. P. Odense of this Board's Halifax Station (Nos. 8 and 9, Table II). It is difficult to explain the very large differences encountered, especially since with two opposite fillets from a sockeye salmon rather similar concentration of sugar phosphates and nucleotides were found. Perhaps the concentrations differ greatly in different regions of the muscle. The ratios of G1P to G6P were very variable as might be expected since the concentration of these may be affected by a number of enzymes such as phosphoglucomutase, phosphoglucoisomerase, phosphorylase, G6P dehydrogenase and glucokinase. The ratios of F6P to FDP were also very variable. In the halibut muscle which came from an obviously exhausted and slowly frozen fish all sugar phosphates except a little G6P had disappeared.

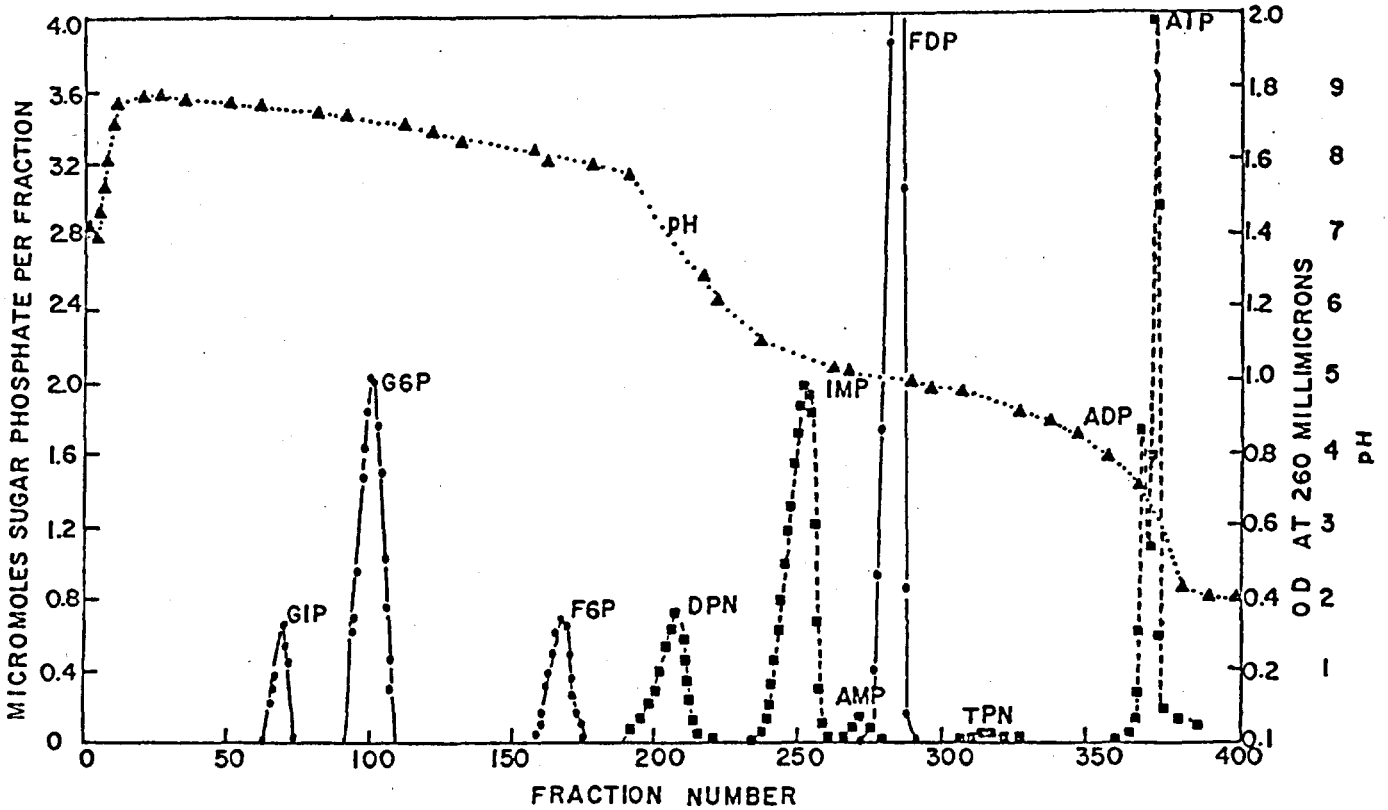


Fig. 2. Separation of sugar phosphates and nucleotides in salmon muscle using a Dowex 1 x 4 Cl^- column. Legends as in Fig. 1.

No R5P was found in muscle from freshly-killed fish and only small concentrations in muscle of stored fish (lingcod). R1P was not found and the probable reason for this is indicated in "recovery" experiments to be reported later. ATP and ADP, which either separated poorly or occurred together, were present in variable amounts in muscles of freshly-killed fish. The concentration of AMP was usually considerably less. The concentration of IMP was, except in the case of stored muscle samples, comparatively high. In the samples which were kept several days at 0°C, several ultraviolet-absorbing fractions were eluted prior to IMP and these, though in most instances they were not identified with certainty, appeared to be inosine, hypoxanthine, adenosine and adenine.

Precursors of Sugar Phosphates and Sugars as Determined with Radioactive Compounds

Live fish. In several instances the fish studied were the same as those used for obtaining the data recorded in Table II, and in such cases this has been referred to. The fish were anaesthetized with MS222 prior to injection.

(a) A 160-g sockeye salmon (Oncorhynchus nerka) was injected in the brachial vein with 66 μC of UL glucose C^{14} in 0.375 ml H_2O. After 15 minutes, during which time the fish recovered and swam freely, it was stunned and frozen in liquid nitrogen. After a few hours the fish was thawed sufficiently to prepare two skinned fillets, one of which was refrozen and stored at -30°C (see below). The other (34 g) was used immediately (No. 4, Table II). The pooled G6P fraction (372 μmoles) yielded 50 mg of BaG6P (purity 81% by the anthrone reaction based on a MW of 396; the ratio of total P to glucose was 0.95:1.0). The specific activity was 17.5. The pooled F6P fraction (64 μmoles) yielded 8.0 mg of BaF6P (purity 70% by the anthrone reaction based on a

TABLE II - Quantitative distribution of the sugar phosphates and principal nucleotides in fish muscles. (μmoles per 100 g)

No	g of muscle used	Diam cm Dowex 1 Cl^- column	Sample description	G1P	G6P	F6P	FDP	R5P	DPN*	TPN	IMP	AMP	ADP	ATP
1	38	1.0	Mixed muscle from 2, 75-g sockeye salmon	7.5	50	16.5	99.5	0	23.5	Trace	81	2.2	38.5	
2	33.5	1.0	1, 85-g sockeye	21	107	27	55	0	11.5	1.9	220	10.2	15	40
3	34	1.0	1, 100-g sockeye	13	475	226	23	0	0	0	710	26	27	27
4	34	1.0	)Fillet 1	32	1,100	188	23	0	Trace	0	775	7	75	
5	31	1.0	160-g sockeye))Fillet 2	**	840	196	29	0	Trace	0	735	14	44	13
6	42	1.0	140-g rainbow trout	16	236	65	117	0	Trace	Trace	575	9	32	
7	33	1.0	11-kg halibut***	0	21	0	0	0	1.3	0	425	0	6	17
8	42	1.0	Atlantic cod	7	38	0	77	0	4.3	0	147	16	74	
9	90	1.4	" "	0	71	14	0	0	4.2	0	670	9	3.8	32
10	81	1.4	1.5-kg lingcod	7	244	70†	2.6	0	28	0	17	4.9	6.5	53
11	100	1.4	As 10 but muscle held 6 days at 0°C	Trace	38	19	0	7.5	Trace	0	27	14	30	
12	87	1.4	Excised from a lingcod and held 3 days at 0°C	Trace	33	21	0	3.7	Nucleotides not studied					
13	100	1.4	Excised from 3.5 kg mature sockeye	35	102	18†	322	0	63		255	7.8	115	

* DPN occurs in large amounts in some fish muscles and may be necessary for optimum activation of some muscle enzymes at the rather low temperatures at which fish often live.

** Several fractions were lost, but the amount recovered in the remainder indicated that the concentration of G1P was about the same as in the first fillet.

*** This fish was thrown into a -20°C storage room at sea and allowed to freeze slowly.

† A very large amount of an ultraviolet-absorbing substance which was not positively identified occurred in this fraction. This obscured the small amount of DPN which might have been present.

MW of 396; glucose to total P ratio = 1:0.98). This compound was used for paper chromatography experiments (see later).

(b) The second fillet (31 g) (No. 5 Table II) from the above fish was extracted after two months at -30°C, 10 µmoles each of R5P and R1P being added to check the recovery of these. The following compounds were prepared and their radioactivity determined.

Fractions 226-253 (227 µmoles IMP) yielded 67 mg (103 µmoles) of barium IMP (87% pure on the basis of the anhydrous compound (MW 484) using absorption at 248 mµ as criterion. The O.D. ratios at pH 2 were: $\frac{250}{260}$ = 1.68 (1.68), $\frac{280}{260}$ = 0.24 (0.25), and the P:ribose:base ratios were 1.0:0.93:1.03. A further 65 mg (55% purity) (41 µmoles) was obtained from the mother liquor from the first precipitation. The total yield was 77% of theory. The specific activity of the IMP was 5.0 as determined with the purest fraction.

Fractions 70-120 (260 µmoles G6P) yielded 55 mg (202 µmoles) of Li G6P of 77% purity based on the glucose content and a MW of 272. The ratio of P to glucose was 1:1.07. The specific activity was 16.7, which compared closely with that obtained with BaG6P isolated from the opposite fillet from the same fish (see (a) above).

Fractions 141-174 contained 61 µmoles of F6P (and also 8.7 µmoles of R5P added to test efficiency of recovery of this ester). The crude mixed lithium salt (9 mg) was 77% pure calculated from its fructose content, and assuming a MW of 272, the recovery was 42% of theory. The ratio of P:fructose was 1:1.06. The mixture contained 5 µmoles of ribose (R5P), which was destroyed by 5 minutes heating at 100°C in 0.1 N NaOH, and was therefore not of nucleotide origin. In order to remove R5P the lithium salt was dissolved in 0.2 ml H_2O, streaked along two 17-cm-wide papers, and developed in solvent A. The F6P zones were eluted and the specific activity of this compound was found to be 41.

The results of (a) and (b) indicate that injected radioactive glucose soon entered the muscle glycolytic system since both G6P and F6P were radioactive. IMP was also slightly radioactive. None of the added 10 µmoles of R1P was recovered, but 87% of the added R5P was recovered.

(c) A 140-g rainbow trout was injected intravenously with 0.5 µc of KG6P C_1 C^{14} in 0.25 ml of H_2O. The fish was kept in a tank of running fresh water (8-10°C) for 5½ hours, then stunned and the muscle (42 g) promptly extracted for ion exchange chromatography (No. 6, Table II). The perchloric acid extract was radioactive (275 x 10^3 cpm). The ion-exchange column "washings" had 137 x 10^3 cpm and fractions 5-17, which contained lactate (see (a) below under "fish muscle post mortem") 96 x 10^3 cpm. Thus, 42 x 10^3 cpm were accounted for as compounds bound more strongly than lactate (sugar phosphates, etc.).

Fractions 77-117 (99 µmoles of G6P) yielded 40 mg of crude Li G6P. The purity was 40% and the yield (59 µmoles) 59.5% of theory. The ratio of P:glucose was 1:0.98. The specific activity was 42.

Fractions 162-183 (27 µmoles of F6P) yielded 10 mg of Li F6P. The purity was 32% by the Roe method and the yield (11.7 µmoles) or 44% of theory. The specific activity was 36.

Fractions 276-288 (49 µmoles FDP) yielded 21 mg of tetralithium FDP. The purity (assuming MW 364) was 91% (by total P content) and the ratio of P to fructose 2:0.96. The yield (52 µmoles) was 88% of theory. The specific activity was 36.

Fractions 355-369 (13.4 μmoles ATP + ADP on the basis of OD measurement at 258 mμ) yielded 7.5 μmoles of ATP + ADP (Li salts), the recovery therefore being 56% of theory. The purity of the crude lithium salt mixture was 75% (by optical density) and the P content 65% of theoretical amount calculated as ATP. The OD ratios at pH 2.0 were $\frac{250}{260} = 0.84$ and $\frac{280}{200} = 0.23$. The specific activity was 9.7.

These results show that, after 5½ hours, injected G6P C_1 C^{14} had been incorporated into, and remained, in both fructose phosphates, and that the specific activity of G6P and these was similar. The ATP-ADP fraction was also radioactive.

(d) The liver (2.3 g) from the salmon used in (c) was extracted with 20 ml of cold 0.6 M PCA and the extract (26 ml; 210 x 10^3 cpm) was subjected to column chromatography on a Dowex 1 x 4 Cl^- 1-cm diam column. The following fractions were pooled, passed through Dowex 50 x 8+ resin columns, evaporated and adjusted to 10-ml volumes. The solutions were then analysed for glucose (anthrone method) and fructose (Roe method), for total P and for radioactivity with the following results:

Fractions	108-117,	5.6 μmoles	G6P.	Ratio P:glucose = 1:1.08.	Specific activity	2000.
"	173-185,	4.3 "	F6P.	" P:fructose = 1:1.1.	" "	11,000.
"	267-276,	0.95 "	FDP.	" P:fructose = 2:0.93.	" "	10,000.

These results show that the hexose phosphates in the liver are much more radioactive than those in the muscle of the same fish. The reason for the higher specific activity of the fructose phosphates is not known.

Fish Muscle Post Mortem

(a) One hundred g of muscle freshly excised from a stunned rested lingcod received a number of sharp incisions across the muscle fibres, and into these 1 ml of K G6P C_1 C^{14} (2.5 μC) was distributed as evenly as possible. After 6 days at 0°C the muscle was extracted with PCA (No.11, Table II). The extract had only 33% (1.1 x 10^6 spm) of the activity (2.5 x 10^6 cpm) of the radioactive G6P originally incorporated. This loss might have been due to liberation of $C^{14}O_2$. Since the column washings had only 6.0% of this activity, presumably due to slight hydrolysis of glucose phosphates to yield free glucose (see below), the remainder of the radioactivity was evidently absorbed on the column. The following substances were eluted, identified and their specific activities determined.

Fractions 4-26 were pooled, and on analysis were found to contain 3,890 μmoles of lactate. The specific activity (assuming that lactate was solely responsible for the observed radioactivity) was 232. This fraction therefore accounted for 87% of the counts which were absorbed on the resin.

Fractions 222-234 (19.4 μmoles F6P + 7.5 μmoles R5P) gave 38 mg of a mixture of the dry barium salts of F6P and R5P. This material was dissolved in 1.5 ml of H_2O, the solution placed on a 7 x 1-cm Dowex 50 x 8 H+ column and the column washed with H_2O. The eluate was evaporated to a small volume and chromatographed using solvent A. The zones corresponding to F6P and R5P were eluted and concentrated, and the radioactivity of the solutions determined. The specific activity of the F6P was 1100 cpm and that of the R5P only 5 cpm. It is very doubtful whether any significance can be attributed to slight radioactivity of the R5P since it is difficult to ensure absolutely clear separation of F6P and R5P in this solvent system, and only a trace of F6P contamination would cause activity in the R5P fraction.

No appreciable radioactivity was found in the AMP- or IMP-containing fractions.

These results show that G6P C_1 C^{14} is incorporated post mortem into lactate and F6P, but not into the nucleotides and probably not into R5P.

(b) An 87-g piece of muscle from the lingcod used in the foregoing experiment which had been promptly frozen and stored at -30°C was defrosted and treated as in (a) above with 1 ml containing 14 μC of UL glucose C^{14}. After 3 days at 0°C the muscle sample was extracted and chromatographed on a 1.4-cm diam Dowex 1 x 4 Cl^- column as usual (No. 12, Table II).

Fractions 131-160 (38 μmoles G6P) yielded 11.6 mg of BaG6P of 20% purity based on a MW of 396 (5.85 μmoles; 15.5% recovery). The specific activity was 570.

Fractions 221-236 (18.5 μmoles F6P) gave 7 mg BaF6P of 20% purity based on a MW of 396 (3.5 μmoles; 19% recovery). The specific activity was 1100. This shows that glucose is phosphorylated in fish muscle post mortem and enters glycolytic intermediates.

(c) Ninety-three g of muscle freshly excised from a stunned lingcod, and which had been frozen and stored 1 day of -30° was treated with 30 μmoles of UL G6P C^{14} (2×10^6 cpm). After 5 days at 0°C the muscle was extracted and the extract (1.2×10^6 cpm) chromatographed on a 1.4-cm diam Dowex 1 x 4 Cl^- column, which was washed with water. The column washings (400 ml) were passed through a 12 x 2.8 (diam) cm Dowex 50 x 8 H^+ column, the column washed with 100 ml of water and the eluate assayed for glucose, ribose and radioactivity with the following results: 324 μMoles of glucose and 410 μMoles of ribose were recovered; the total radioactivity was 92×10^3 cpm, or 7.6% of that in the perchloric acid extract.

A portion of the aqueous extract was concentrated to a small volume and chromatographed in solvent system E. The zones corresponding to glucose and ribose were eluted. Neither sugar was found to be radioactive when amounts of between 0.2 and 4.0 μmoles were subjected to prolonged "counting". Evidently the comparatively small amounts of radioactive G6P used was insufficient to cause radioactivity when diluted by the comparatively large amount of glucose in the muscle. Further experiments showed that only a very small amount of G6P is degraded to yield glucose in fish muscle post mortem (see F below).

The column was eluted as usual. Fractions 151-170 were pooled and treated as usual except that the concentrated aqueous solution obtained after evaporation was used for analyses and no lithium salt was prepared. There were 11.5 μmoles of G6P of specific activity 35.

Fractions 220-233 contained 6.3 umoles of F6P and 1.4 umoles of R5P. The pooled fractions yielded 3 mg of mixed lithium salts (4 umoles F6P and 0.7 μmoles R5P). This material was dissolved in a little H_2O (total radioactivity of the solution was 780 cpm) and chromatographed in solvent system E. The appropriate zones corresponding to F6P and R5P were eluted with water and radioactivity was determined. The results showed that the F6P was radioactive (specific activity 160) while the R5P was inactive.

(d) One μC of C^{14} ATP UL was incorporated into 95 g of thawed lingcod muscle which had been excised from a freshly stunned fish as usual and stored 8 days at -30°C before use. After 2 days at 0°C the muscle was extracted and the extract absorbed on a 1.4-cm (diam) Dowex 1 x 4 Cl^- column. During extraction 20 μmoles of R1P were added. The column washings (265 ml) were passed through a 15 x 2.8-cm (diam) Dowex 50 x 8^+ column. The eluate and washings (410 ml) contained 290 μmoles of ribose and 460 μmoles of glucose. The solution was concentrated to dryness in vacuo and made to 1.0 ml. Half of it was chromatographed on

four 17-cm-wide papers in solvent E. The ribose and glucose were eluted and the solutions concentrated to 2 ml. The ribose fraction was radioactive, the specific activity being 208. The glucose showed slight radioactivity (specific activity 9.8). On rechromatography under the same conditions the isolated glucose was found to be completely free from radioactivity when 2 µmoles were counted. This indicates that ATP is probably largely the precursor of ribose in fish muscles post mortem.

The column was eluted as usual. Fractions 184-191 contained 4.5 µmoles of ribose and 4.38 µmoles of acid labile P (ratio P:ribose, 1:1.03). This represented 22% recovery of the 20 µmoles of added R1P. The solution did not absorb appreciably in the ultraviolet region and was therefore free from nucleoside.

Fractions 113-131 (28 µmoles D1P) were treated with Dowex 50 x 8 H^+ resin and, after the usual methanol treatment, one quarter of the solution was chromatographed in solvent E. G1P, which ran only about 2.5 cm from origin in this solvent, was eluted and 6.2 µmoles (89%) were recovered. This had slight radioactivity (specific activity 5). The solution was rechromatographed in solvent A and the G1P eluted. No radioactivity was found on counting 0.31 µmoles.

Fractions 171-183 (10.5 µmoles G6P) yielded 2.8 mg of 38% pure LiG6P (3.9 µmoles; 38% recovery; P:glucose ratio = 1:0.96). No radioactivity was found on counting 1 µmole.

Fractions 227-261 (430 µmoles; largely IMP) were treated as usual and a crude lithium salt prepared. The salt was dissolved in 2 ml of H_2O and 0.5 ml was chromatographed on 2, 17-cm-wide Whatman 3 mm papers using solvent system D. Two ultraviolet-absorbing bands appeared. The fast-moving band which contained by far the largest amount of ultraviolet-absorbing material was eluted and concentrated to 5.0 ml. This solution contained 38.5 µmoles IMP (OD ratios 1.69 and 0.23; P:hypoxanthine ratio, 1.0:0.95). The specific activity was 610.

(e) Two Salmo gairdnerii weighing about 170 g were stunned and the muscle promptly excised and blended in the cold. The glycogen and glucose content was determined immediately and the remaining muscle was stored at 0°C and used in the following experiments.

Radioactive salmon muscle glycogen (40 mg; 49,000 cpm, based on the radioactivity of the glucose liberated on acid hydrolysis which had a specific activity of 200) was dissolved in 2 ml of warm water and mixed thoroughly into 40 g of blended muscle which was stored, together with a similar sample of untreated muscle, at 0°C. The glycogen content of the sample with added glycogen was determined for the first 3 days, and glucose with both samples at intervals up to 7 days. The results (Table III) showed that there was a very rapid breakdown of glycogen with an equally rapid appearance of glucose, there being no important change after the first day. Paper chromatography of the extracts which had been prepared for determination of glucose revealed that much of the sugar which was being determined was probably maltose or another sugar with an even lower Rf value than maltose. Thus, after one day, the ratio of glucose:maltose (based on the anthrone reaction) eluted from the paper chromatogram was roughly 1:2, and after 2 and 7 days it was about 1:1. Evidently hydrolysis of maltose is quite slow with salmon muscle. The radioactivity was similar for both glucose and maltose. From Table III it will be seen that the difference in glucose content between the muscle containing radioactive glycogen and the untreated muscle was about that expected from the amount of glycogen added.

TABLE III - Glycogen, glucose and radioactivity in stored salmon muscle with and without added C^{14} glycogen.

Days at 0°C	Glycogen* (mg/100 g)	Glucose (μmoles/g) Muscle with C^{14} glycogen	Untreated muscle	Difference***	Specific radioactivity** Glucose	Maltose
0	84	1.03	1.03	0		
1	4.3	14.2	9.3	4.90	44(37)	(49)
2	0	14.83	10.00	4.83	40(34)	(35)
7	-	14.80	9.70	5.10	43(37)	

* Values for muscle containing C^{14} glycogen.
** Values in brackets are for sugars eluted from paper chromatograms.
*** Calculated difference due to added glycogen = 5.05 μmoles glucose (detd. by anthrone).

After 24 hours at 0°C 25 g of the muscle containing C^{14} glycogen was extracted for ion exchange chromatography. The column "washings" (270 ml) contained 340 μmoles of glucose and 125 μmoles of ribose, the total radioactivity being 13.6×10^3 cpm. This was 28% of the "counts" added to the muscle as radioactive glycogen. It is in good agreement with the expected activity for recovery of 340 μmoles of glucose of specific activity 37 (as given in Table III), namely 12,600 cpm. The solution was concentrated to 1.0 ml and 0.1 ml chromatographed on one 17-cm-wide paper in solvent E. The radioactivity of the eluted glucose and maltose is given in Table III; the ribose was not radioactive.

The ion exchange column was eluted and the following fractions studied. Fractions 130-141 (8 μmoles G6P) were passed through a 10 x 2.8-cm (diam) Dowex 50 x 8 H^+ column and, after treating twice with methanol as usual, were made to a final volume of 2.0 ml. The G6P had a specific activity of 24. F6P was prepared similarly from fractions 196-206 (4.0 μmoles). The specific activity was 19.5.

These results suggest that glycogen is degraded post mortem at least partly via the Embden-Meyerhof pathway as well as by amylase activity.

Experiment (c) above indicated that, when a comparatively small amount of radioactive G6P was added to fish muscle, no radioactivity could be detected in the free glucose fraction. For this reason comparatively high concentrations of G6P C^{14} were added to comparatively small amounts of muscle in the following experiment. To each of 3 separate 4.0-g portions of blended salmon muscle as prepared in the previous experiment, approximately 0.1 ml of solutions of the following compounds were added, each with a total radioactivity of 250×10^3 cpm: (a) G6P C_1 C^{14}; (b) G6P C^{14} UL; (c) glucose C^{14} UL. After one day at 0°C both the glucose content and radioactivity of the treated perchloric acid extracts were determined with the following results:

(a) 37.7 μmoles of glucose of specific activity 109 recovered; 4100 cpm recovered = 1.64%.

(b) 37.2 μmoles of glucose of specific activity 91.5 recovered; 3,400 cpm recovered = 1.36%.

(c) 37.4 μmoles of glucose of specific activity 7,000 recovered; 262×10^3 cpm recovered = 105%.

This shows that only 1.36 - 1.64% of the G6P in the muscle yielded glucose in one day at 0°C. Recovery of added radioactive glucose was quantitative. Evidently nearly all glucose in fish muscle arises through amylase activity.

The Nature of the Fructose Monophosphate Fraction of Fish Muscle

Burt and Jones stated that both F6P and F1P are present in muscles of cod (Gadus callarias). In a more recent paper Burt reported that the separation of F1P and F6P in perchloric acid extracts of cod muscle was unsatisfactory due to high levels of glucose phosphates and therefore only reported changes in a single "fructosemonophosphate" fraction. The present work indicates that F1P is probably not present in fish muscle. The following experiments are presented in support of this contention.

(a) Fractions 131-133 from the "control" ion exchanged column, data concerning which was incorporated in Fig. 2 and Table I and which presumably contained F1P and F6P phosphates, were pooled. One half (10 µmoles of fructose monophosphate) the solution was used to prepare a crude lithium salt avoiding exposure to acid pH values during preparation. 5.5 Mg of crude material of approximately 20% purity were recovered (4 µmoles). The ratio of total P:fructose was 1:1.05. 0.71 µMoles were dissolved in 1 N HCl (2.0 ml volume) and hydrolysed at 100°C. The following rates of liberation of orthophosphate were observed: 5 min, 27%; 15 min, 57%; 30 min, 68%; 180 min, 78% and 300 min, 92%. These values correspond to those which would probably occur in an approximately equimolar mixture of F1P and F6P in 1 N HCl at 100°C. Under these conditions F1P is 53% hydrolysed in 5 min, 91% in 15 min and completely hydrolysed in 30 min, while F6P is only 7% hydrolysed in 15 min; 74% in 180 min and 90% in 300 min.

When an amount of the above lithium salt corresponding to 0.5 µmole of fructose monophosphate was chromatographed in solvent C, and the chromatogram developed with the Hanes and Isherwood reagent, two distinct zones corresponding to F1P and F6P were found. These results show that it is possible to distinguish between these fructose monophosphates in a mixture from an ion exchange column.

(b) Fractions 230-241 (14 µmoles F6P) from Atlantic cod (No. 9, Table II) were used to prepare a crude lithium salt. The yield was 4.8 mg of approximately 32% pure fructose monophosphate (Ratio of P:fructose was 1.0:1.10).

A small portion was hydrolysed at 100°C in 1 N HCl. In 5 min no appreciable orthophosphate was detected, while in 30 and 180 min 16% and 70%, respectively of the total P present was liberated as orthophosphate. This result indicates that F1P was absent in this material.

The above lithium salt (0.5 and 1.0 µmoles) was chromatographed in solvent C, the chromatogram being dried, sprayed with a solution of picric acid in ether and the fructose phosphate developed by alkaline-ethanol spray. Only a single zone corresponding to F6P was observed. Since this method detects 0.05 µmole of F1P or F6P the results would indicate that, if any F1P were present, the concentration would be $<5\%$.

(c) LiF6P from salmon muscle (No. 6 Table II) was chromatographed in solvent C (0.5 and 1.0 µmole). The dried chromatograms were treated as in (b) above and only F6P was observed. A similar result was obtained when 0.5 µmoles of F6P (Barium salt decomposed with Dowex 50 H^+ resin from No. 5 Table II) was chromatographed 40 hours in solvent B and sprayed by the method of Loring et al. Though this solvent separates F1P (Rf 0.39) and F6P (0.43) fairly effectively, F6P preparations always give a second spot (Rf about 0.31).

These results suggest that it is very unlikely that F1P occurs in fish muscle.

Discussion

This investigation has provided satisfactory answers to a number of hitherto unanswered questions regarding the origin of free sugars and sugar phosphates in fish muscles. Thus it now seems safe to state that glycogen is degraded post mortem by two pathways, namely by a direct amylolytic hydrolysis, via maltose to glucose and by the Embden-Meyerhof glycolytic system. Once the glycogen has entered the glycolytic pathway it appears that very little of it yields free sugars as by hydrolysis of hexose phosphates. Evidence has also been presented which indicates that the free ribose which occurs in fish muscles post mortem originates from the nucleotides (ATP, IMP, etc) and not from G6P via the hexosemonophosphate shunt system. Intravenous introduction of radioactive glucose or G6P into living fish promptly results in labelling of muscle hexose phosphates and this labelling persists for many hours. Under these conditions the liver hexose phosphates are much more radioactive than are those of the muscle. The slight radioactivity which occurred in the ATP and IMP muscle fractions of these injected fish showed that these nucleotides became labelled from glucose or G6P under the experimental conditions.

It seems almost certain that F1P is not present in fish muscles. Present evidence for occurrence of R1P post mortem would also seem unsatisfactory. It is, however, possible that this pentose phosphate may occur in some instances but, since the normal equilibrium of R5P to R1P as occasioned by fish muscle phosphoribomutase is about 9:1, it would seem rather doubtful if much R1P could be present.

Absolute proof that the natural RNA in intact fish muscles is not degraded post mortem would seem to be lacking. Present evidence strongly suggests that in intact lingcod muscle at least, RNA is not degraded. If this is so, then the sole source of ribose in intact fish muscles post mortem may be ATP and related nucleotides as the present study suggests. Present evidence also indicates that though the hexosemonophosphate shunt system is probably not of major importance in utilization of glucose for energy purposes in fish muscles, it still accounts for at least a part of the utilization. No support for participation of this shunt system in utilization of glucose has been obtained in the present work.

SUMMARY NO. 48

POSSIBLE ROLE OF THE OXIDATIVE PENTOSE CYCLE (HEXOSEMONOPHOSPHATE SHUNT SYSTEM) IN FORMATION OF SUGARS IN FISH MUSCLES

H.L.A. Tarr

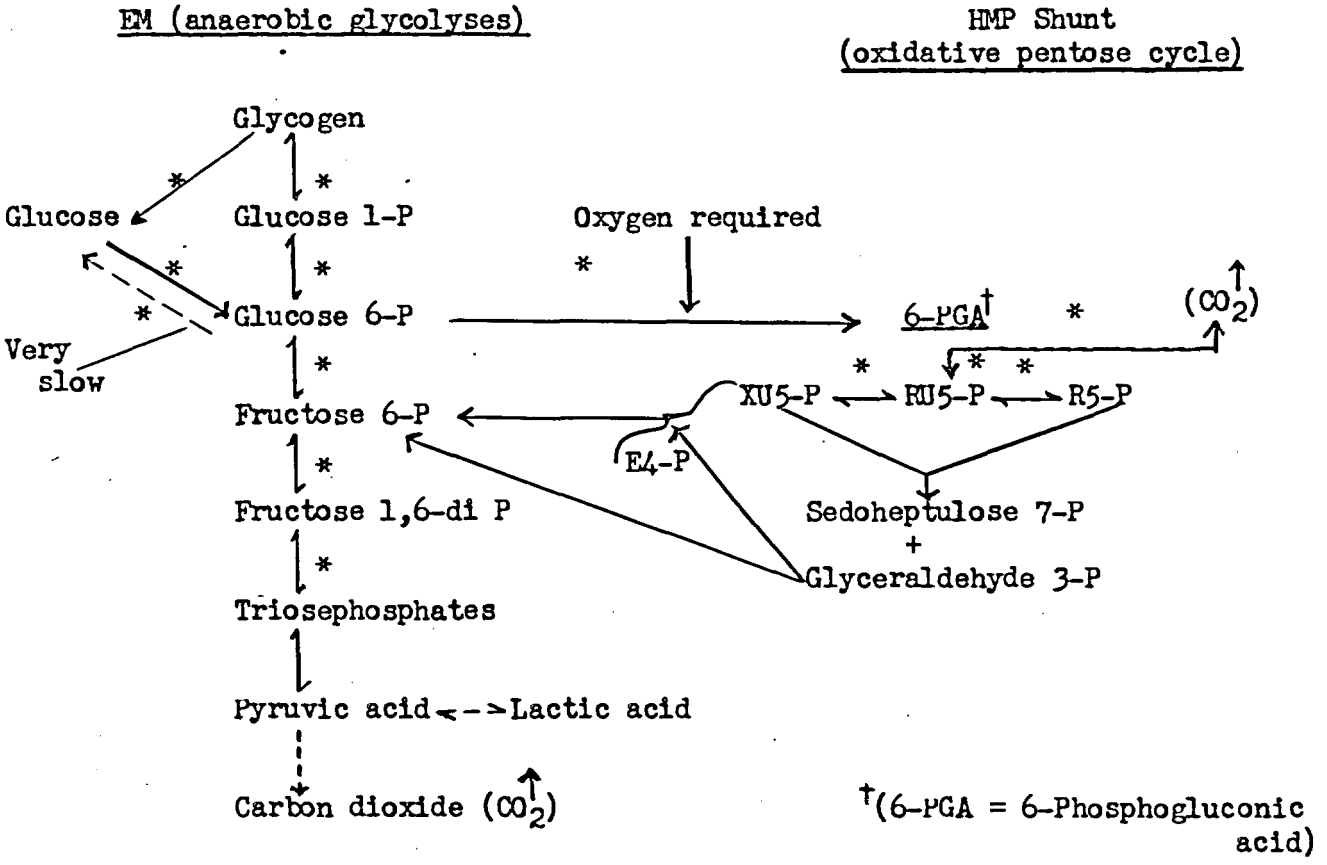
The foregoing summary and previous work at this Station indicated fairly conclusively that ribose does not normally arise from the ribonucleic acid which has been demonstrated in muscle of different species of fish (Summaries Nos 17 and 18 of this Station's Annual Report for 1959-60). It appears to originate solely from the principal nucleotides (mainly adenosinetriphosphate) and is only formed after death of the fish. At this Station, work so far indicates that free glucose is practically absent from muscles of live fish. Virtually all glucose appears to arise from action of fish muscle amylases on the natural muscle glycogen ("animal starch"), and if any arises from hydrolysis (phosphate removal) of the glucose monophosphates, it is almost certainly of negligible significance.

Two main systems by means of which glycogen in living tissues is degraded with liberation of energy are now well recognized. These are the well-known anaerobic glycolysis pathway (Embden-Meyerhof pathway = EM) and the hexosemonophosphate shunt pathway (HMP). The latter is often called the oxidative pentose

cycle since oxidation is involved in changing 6-phosphogluconic acid to ribulose 5-phosphate.

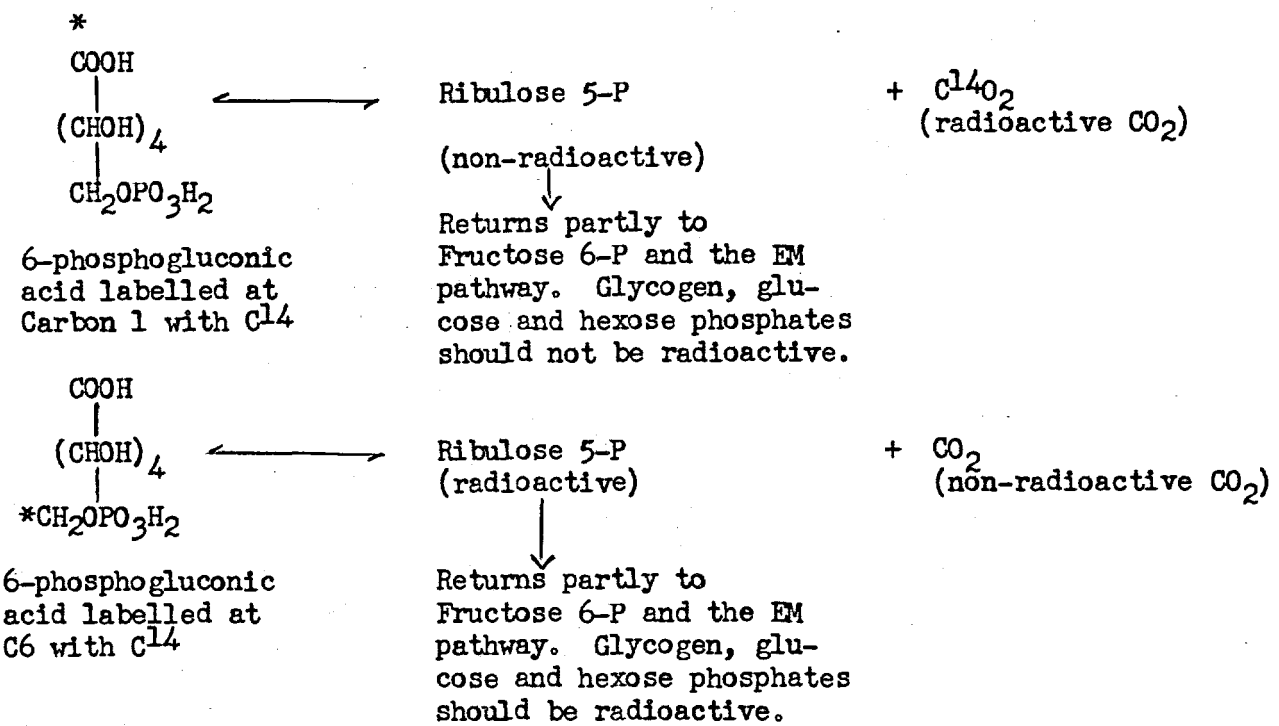
Studies in this and in other laboratories have shown that fish muscle or other organs possess all the enzymes required for successful operation of the EM pathway, and four of the enzymes required for functioning of the HMP system. However, the very limited data presently available indicate that the HMP system is probably not of primary importance in fish muscles or organs. The present work was initiated in order to investigate the possible role of the HMP system in fish using a heretofore unexplored method.

The accompanying diagram shows, in a very minimal fashion, the steps which are involved in degradation of glycogen by the EM and HMP shunt systems. In this diagram the arrows indicate the direction of a given reaction and it will be quickly seen by the number of "two-way" arrows that many of the reactions are reversible and the compounds are in equilibrium. The changes are stimulated by enzymes and an asterisk (*) indicates that the enzyme concerned has been demonstrated in fish tissues.



This diagram shows that there are two main routes by means of which glucose is used by tissues for energy. Carbon dioxide is formed at two points as indicated. By means of radioactive "tracer" compounds it is possible to determine the fate of glucose (and other radioactive or "labelled" compounds) in the animal. Work elsewhere using different forms of radioactive glucose has indicated that the HMP system is probably not of major importance in the overall metabolism of glucose by living fish (carp species). In the present experiments 6-phosphogluconic acid is being studied since it was argued that this compound in all probability must be mainly (or entirely) metabolized via the HMP system since one of the two enzymes required for formation of 6-phosphogluconic acid from glucose 6-phosphate carries out a

reaction which appears to be irreversible (or nearly so). On this assumption 6-phosphogluconate labelled with C^{14} in carbon atom 1 should yield non-radioactive ribulose and all the radioactivity should emerge as radioactive CO_2 and not enter the hexose-phosphates of the EM pathway for "recycling". If labelled in other carbon atoms (or specifically in Carbon atom 6) then all or much of the radioactivity should pass on to ribulose 5-P and eventually enter the EM pathway via fructose 6-P as indicated below.



Experimental

Radioactive 6-phosphogluconic acid was prepared as follows: the C_1C^{14} glucose 6-phosphate with bromine. The acid was isolated as the neutral potassium salt and was purified by quantitative paper chromatography and checked for radiopurity. The C_6C^{14} and uniformly labelled (all carbons C^{14} labelled) compounds were made from the corresponding radioactive glucose compounds. The glucose was phosphorylated to yield glucose 6-phosphate (using hexokinase enzyme), and the glucose 6-phosphate thus formed was oxidized to 6-phosphogluconic acid with bromine. The products (neutral potassium salts) were chromatographically homogeneous.

The work is still in progress. Steelhead (35-50 g) are used and one or two fish are kept in 12 litres of oxygenated water at 5-7°C. The fish are injected (intramuscularly or intraperitoneally) with a solution of radioactive 6-phosphogluconate and the expired CO_2 is retained and its radioactivity determined by standard procedures. In some cases at the conclusion of an experiment the fish have been stunned and the radioactivity of the glucose and hexose phosphates determined by techniques described in detail in Summary No. 47 of this Annual Report.

The results so far have shown that liberation of radioactive carbon dioxide from fish is very much greater from fish injected with either radioactive glucose or glucose 6-phosphate than with 6-phosphogluconate. Thus radioactive carbon dioxide is evolved about 40 times as rapidly with glucose-treated as with 6 phosphogluconate-treated fish. Radioactive CO_2 is liberated at about the same rate

when fish are injected with either C_1C^{14} or uniformly-labelled C^{14} phosphogluconate. Preliminary experiments have shown that the glucose (from the glycogen) from fish injected with C_1C^{14} 6-phosphogluconate is not radioactive. On the other hand the hexose phosphates (glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate) and glucose isolated from fish treated with uniformly labelled 6-phosphogluconate are all radioactive. These results indicate that 6-phosphogluconate is probably metabolized slowly via the HMP pathway. However, further work is required to verify the preliminary findings. Also, there are indications that some of the phosphogluconate may be metabolized by another route.

SUMMARY NO. 49

EXPERIMENTAL REMOVAL OF RIBOSE AND GLUCOSE FROM FISH MUSCLE — Margaret Leroux

A lyophilized culture of Pseudomonas fragi, labelled B-25 (7-16-52), was obtained from R. Weimburg, Agricultural Research Service, Peoria, Illinois. He had shown that this organism metabolizes ribose oxidatively to γ - ribonolactone rather than by a phosphorolytic pathway. When cultured in a ribose-containing medium, the bacterium caused disappearance of ribose. This was determined by testing its growth medium using the orcinol reaction.

The medium used for growth and maintenance of the culture was: NH_4Cl (0.1%), $MgSO_4.7H_2O$ (0.05%), $CaCl_2$ (0.001%), Trypticase (0.2%) and ribose (0.25%) in KH_2PO_4 - $Na_2HPO_4.12H_2O$ buffer, 0.033M, pH 6.8. The $MgSO_4.7H_2O$ and ribose were autoclaved as separate solutions and were added to the sterile medium prior to its inoculation with a 1% inoculum of cells. The cultures were incubated aerobically with shaking at 22-23°C for 16-20 hr.

Preparation of Bacterial Fractions

One litre of 16-hr cells was centrifuged. The cells were washed twice by centrifugation in 0.033M phosphate buffer, pH 6.8, at 5000 g and 0°C. The final washed pellet was resuspended in 15 ml 0.003 M phosphate buffer, pH 6.8. The cell suspension was exposed to sonic oscillation in a 10 KC Raytheon Oscillator for 15 min. The sonicate was centrifuged twice at 5000 g and 0°C to remove whole cells and cell debris. The supernatant cell free extract was centrifuged at about 140,000 g (Spinco Rotor #40 at 40,000 rpm) for 2 hr at 0°C. The resultant supernate was termed the soluble fraction; the precipitate was the particulate fraction containing cellular structures. The particulate material was suspended in 0.5 of its volume (usually 0.35 ml) of 0.003 M phosphate buffer, pH 6.8. The fractions were stored at -30°C.

Location of Ribose Oxidase Activity

TABLE I - Protocol for experiment to demonstrate removal of sugar by enzyme.

Reagent	1	2 (control)
1. sugar solution	1 part	1 part
2. 0.004 M $MgSO_4$-$7H_2O$	"	"
3. 0.1 M phosphate buffer, pH 7.0	"	2 parts
4. enzyme	"	—
5. 10% trichloroacetic acid	"	1 part

Procedure

The protocol of Table I was followed using 0.006 M ribose as the sugar and the particulate or soluble fraction of B-25 as the enzyme. The mixtures (excluding TCA) were incubated 1 or 2 hr at 30°, 4°, or 0°C. At the end of incubation the enzyme was removed by adding TCA and centrifuging at 5000 g. The controls and supernates of the enzyme-treated mixtures were examined for ribose content by the Mejbaum orcinol test. The particulate fraction of B-25 caused disappearance of ribose at all investigated temperatures in 1-2 hr. The soluble fraction was inactive at 30°C. (See Table II.)

TABLE II - The ability of the fractions of B-25 to remove ribose as shown by the Mejbaum orcinol reaction.

Enzyme fraction present	Time of incubation (in hours)	Temperature of incubation (in °C)	Optical density at 670 mμ with orcinol reagent*
Particulate	1	30	0.196
None (control)	1	30	0.930
Particulate	2	30	0.340
None	2	30	1.030
Particulate	2	4	0.140
None	2	4	1.020
Particulate	2	0	0.220
None	2	0	1.240
Soluble	1	30	0.995
None	1	30	0.980
Soluble	2	30	0.915
None	2	30	1.060

* Low optical density values indicate low ribose concentrations and vice versa.

Search for an Extracellular Ribose Oxidase

The supernate from a growing culture of B-25 was made 95% saturated with $(NH_4)_2SO_4$ at 0°C. Some precipitate was obtained which was dialyzed against 0.02 M $Na_2HPO_4.12H_2O$-KH_2PO_4 buffer, pH 6.8 at 0°C for 24 hr. The dialyzed material was used as the enzyme and 0.006 M ribose as the sugar in the protocol of Table I. No removal of ribose was effected. Thus a soluble extracellular ribose oxidase was not obtained.

Attempt to Solubilize the Ribose Oxidase

A method whereby n-butanol causes solution into water of an enzyme associated as an insoluble particle with phospholipid was used. The particulate material from a litre of cells was suspended in about 2 ml 0.85% saline and adjusted to pH 7.4 with 0.1 N NaOH and 0.02 M $NaHCO_3$ in 0.85% saline. 1.6 Ml n-butanol (40% v/v) was added and the mixture stirred frequently at 0°C for 45 min. It was centrifuged at 20,000 g and 0°C for 1 hr. Both the aqueous layer and the butanol-saturated residue were dialyzed against 0.01 M $NaHCO_3$ and were used as possible sources of

enzyme in the experiment of Table I with 0.006 M ribose as the sugar. No removal of ribose was effected by either preparation. Thus, attempts to solubilize the particulate ribose oxidase were unsuccessful.

Prevention of Browning in Lingcod Muscle by Enzymic Action of the Particulate Fraction

The effect of enzymic action on various treatments of lingcod muscle was examined. For all treatments 5 g lots of minced lingcod muscle were used. Various additions were made and the treated muscle samples were stored at 0°C for various lengths of time.

After treatment and storage at 0°C the samples were heated 1 hr at 120°C, then packed into open containers about 4 cm in diameter and 1 cm deep. Their relative percent reflectancies were measured with a reflectance attachment of a Beckman Model DU Spectrophotometer after the instrument was adjusted to read 100% reflectance at 500 mu with a container holding a standard of 2.5% $MgCO_3.3H_2O$ (cryst) and 4% Cellulose Gum (carboxymethylcellulose). Low values indicate brown samples. (See Table III.)

TABLE III - Percent reflectancies of treated muscle samples.

Additions to muscle	Time of Storage at 0°C	% Reflectancy at 500 mμ
1. None	3 days	46.0
2. Particulate of B-25, 0.165 ml	"	80.0
3. Ribose, 4 μM/g muscle	"	45.5
4. Ribose, 4 μM/g muscle + 0.165 ml particulate	"	104.5
5. Inosine, 4 μM/g muscle	"	42.5
6. Inosine, 4 μM/g muscle + 0.165 ml particulate	"	93.7
7. Ribose, 4 μM/g muscle	2 hours	59.5
8. Ribose, 4 μM/g muscle + 0.15 ml particulate	"	91.9
9. Inosine, 4 μM/g muscle	"	55.8
10. Inosine, 4 μM/g muscle + 0.08 ml particulate	"	97.5
11. Adenosine, 4 μM/g muscle	"	69.0
12. Adenosine, 4 μM/g muscle + 0.08 ml particulate	"	94.0
13. None	"	84.5
14. Particulate, 0.08 ml	"	94.5
15. None	3 days	35.8
16. Particulate, 0.1 ml	"	95.5
17. Glucose, 4 μM/g muscle	"	36.4
18. Glucose oxidase (Nutritional Biochemicals Co.), 7.5 units	"	42.8
19. Glucose, 4 μM/g muscle + 7.5 units glucose oxidase	"	39.0
20. Glucose and inosine, 4 μM of each/g muscle	"	27.2
21. Glucose and inosine, 4 μM of each/g muscle + 0.1 ml particulate + 7.5 units glucose oxidase	"	100.
22. Glucose, 4 μM/g muscle + about 0.5 mg pure glucose oxidase (Nutritional Biochemicals Co.) + drop of 30% H_2O_2	17 hours (at room temp)	35.4
23. Glucose, 4 μM/g muscle + 0.1 ml particulate	17 hours (at RT)	109.5
24. None	2½ "	68.8
25. Particulate, 0.1 ml	" "	82.5
26. Glucose, 4 μM/g muscle + 0.1 ml particulate	" "	84.5
27. Glucose, 100 μM/g muscle	" "	12.6
28. Glucose, 100 μM/g muscle + 0.1 ml particulate	" "	16.0

The pretreatment of lingcod muscle with particulate material of B-25 before storage at 0°C and subsequent heating at 120°C prevented browning. Presumably, the browning was curtailed by adding an enzymic agent which removed such free ribose as was formed from either inherent or added ribosides. Thus, the ribose oxidase of the particulate fraction was applicable to prevention of the Maillard reaction in lingcod muscle.

Since glucose also causes some browning of muscle, it was thought that removal of ribose from muscle by the particulate fraction would still leave the muscle subject to browning due to indigenous or added glucose. This was not the case. Therefore, it was surmised that the particulate material metabolized glucose as well.

The existence of an enzyme preparation having the combined ability to oxidize ribose and glucose was of interest both for the elucidation of pathways of bacterial intermediary metabolism and for the possible practical application of enzymes to prevention of Maillard browning. Moreover, a source of glucose oxidase activity independent of addition of H_2O_2 would be useful. Therefore, investigations regarding the nature of the oxidations of ribose and glucose by the particulate fraction of B-25 were deemed justifiable.

Paper Chromatographic Investigations

Solvents: (all proportions v/v)

1. n-Butanol:ethanol:water (13:8:4)
2. Ethanol:methanol:water (9:9:2)
3. n-Butanol:pyridine:water (6:4:3)
4. n-Propanol:concentrated ammonia:water (6:3:1)
5. Isobutyric acid:concentrated ammonia:water (66:1:33)

Sprays:

A. Aniline hydrogen phthalate (for ribose and glucose).
B. 0.1 N $AgNO_3$ in 5N NH_4OH. Sugars, lactones, and acids occur as dark brown spots against a tan-brown background.
C. 0.1N $AgNO_3$ in 5N NH_4OH with 0.05N NaOH. This spray develops lactones more distinctly than B.
D. (a) 0.1% $FeCl_3$ in 80% ethanol followed by:
(b) 7% sulfosalicylic acid in 75% ethanol.
Phosphates occur as white spots against a mauve background.

Paper: Whatman No. 1.

Development: Descending, 16-20 hours, 20°C.

Experiments

1. Removal of ribose and glucose by the particulate fraction of B-25

The protocol of and procedure associated with Table I were employed, using 0.006 M concentrations of ribose or glucose as the sugar and the particulate fraction of B-25 as the enzyme. The controls and supernatants of the enzyme-precipitated mixtures were spotted in 0.02 ml amounts. Solvent 1 and spray A were used. The controls yielded glucose or ribose in distinct spots. The enzyme-treated mixtures yielded no sugar spots which is further demonstration of removal of both ribose and glucose by the particulate fraction of B-25 in 2 hr at 0°C.

2. Attempt to determine whether glucose and ribose oxidations are carried out by a single enzyme or by different enzymes

The point of saturation of the enzyme with ribose was determined by using the protocol of and procedures associated with Table I. The total volume used per mixture was 0.1 ml. Ribose in varying concentrations was added and the particulate fraction of B-25 was used as enzyme. The control and enzyme-treated mixtures were incubated 2 hr at 0°C and 0.02 ml amounts were spotted for chromatographs in Solvent 1 and sprayed with Spray A. The ribose present for each application was determined approximately quantitatively by running each ribose spot through a paper strip scanner attachment of a Beckman Model DU Spectrophotometer at 400 mμ and comparing the readings to a standard graph. (See Table IV.)

TABLE IV - Results of spectrophotometric measurements on paper chromatograms of the amounts of ribose used by the particulate fraction of B-25.

Mixture	Conc. of ribose soln. added	Known no. of μg ribose/ 0.02 ml mix.	Optical density peak at 400 mμ	Exptl no. of μg ribose/ 0.02 ml mixture	Approx no. of μg ribose used/0.02 ml mixture
1. Enzyme-treated	0.006 M	3.6	0	0	2
2. Control	"	"	.275	2	-
3. Enzyme-treated	0.012 M	7.2	.018	0	6.4
4. Control	"	"	.470	6.4	-
5. Enzyme-treated	0.024 M	14.4	.098	0-1	12.6
6. Control	"	"	.700	12.6	-
7. Enzyme-treated	0.048 M	28.8	.276	2	41.0
8. Control	"	"	1.000	23	-
9. Enzyme-treated	0.1 M	60.0	1.050	25.4	24.6
10. Control	"	"	1.400	50	-

It appeared that about 0.048 M ribose in the foregoing experiment saturated the enzyme in 2 hr; that is, about 21-24 μg ribose could be oxidized by 0.004 ml of particulate fraction in this time.

The protocol of and methods associated with Table I were employed, using as the sugar a solution of 0.048 M ribose plus 0.006 M glucose and the particulate fraction as the enzyme. After chromatography in Solvent 1 and Spray A, the resultant spots were read on a paper strip scanner. (See Table V.)

TABLE V - Results of spectrophotometric measurements on paper chromatograms of ribose and glucose removal.

Mixture	Amount of application on paper (in ml)	Sugar measured	Optical density peak at 400 mμ
1. Enzyme-treated	0.02	glucose	0.170
		ribose	0.580
2. Control	0.02	glucose	0.171
		ribose	0.750
1. Enzyme-treated	0.04	glucose	0.275
		ribose	0.740
2. Control	0.04	glucose	0.325
		ribose	1.350

If the two sugars were oxidized by separate enzymes, saturation of the ribose oxidase with ribose should not prevent the glucose-oxidizing enzyme from metabolizing glucose. However, as seen above, glucose did not disappear as was previously shown when 0.006 M glucose was used as the sole source of sugar. Therefore, there is an indication that the two oxidizing processes may be functions of the same enzyme.

3. Products of glucose oxidation

TABLE VI - Protocol for obtaining enzyme-treated mixtures and controls for paper chromatography.

Reagent	1	2 (control)
1. 0.006 M glucose	1 part	1 part
2. 0.004 M $MgSO_4 \cdot 7H_2O$	"	"
3. Phosphate buffer, 0.1 M, pH 7.0	"	2.5 parts
4. Particulate fraction of B-25	1.5 parts	-
5. UL C^{14} glucose (0.835×10^6 cpm/μc/0.2 ml)	0.5 part	0.5 part
6 10% trichloroacetic acid	1 part	1 part

The mixtures of reagents 1-5 in Table VI were incubated 2 hr at 0°C. Trichloroacetic acid precipitation of enzyme followed incubation as before. The control and enzyme-treated mixtureswere examined chromatographically as follows:

(i) 1 and 2 were spotted beside known solutions of glucose, Δ-gluconolactone, K-gluconate (prepared from glucose dissolved in methanol by oxidation with I_2 and KOH) and Ca-2-ketogluconate. Solvent 3 and Spray B were used. A distinct glucose spot occurred for 2 but not for #1. A dark brown spot showed up for 1 with the same R_f as the Δ-gluconolactone and the K-gluconate, which ran at the same rates. Scanning strips of 1 and 2 with an actigraph strip recorder showed a sharp radioactive glucose peak for #2 but not for #1. No. 1 had a sharp radioactive peak in the lactone or gluconic acid region.

(ii) #1 and #2 were spotted beside known solutions of glucose, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate. Solvent 4 and Spray D were used. When the strips of 1 and 2 were run through an actigraph strip counter, a sharp glucose peak of radioactivity occurred for the control but not for the enzyme-treated mixture; nor did radioactive peaks occur in the regions of the hexose phosphates for 1. Evidently glucose is not phosphorylated.

The protocol of Table VI was employed, using distilled water in place of the phosphate buffer and the $MgSO_4 \cdot 7H_2O$ solution. Chromatography in Solvent 5 and scanning showed that glucose was metabolized to two radioactive products without the addition of inorganic phosphorus. The metabolism of glucose is evidently not by the glycolytic pathway nor any other phosphorolytic pathway.

(iii) 1 and 2 were spotted beside known solutions of glucose, ribose, Δ-gluconolactone, K-gluconate, and Ca-2-ketogluconate. Solvent 2 was used. The lactone and acids were detected with Spray C. They all had essentially equal chromatographic mobilities in this solvent. Ribose and glucose were detected with Spray A. Scanning showed that 2 had a large glucose peak of radioactivity; 1 had a smaller glucose peak, a peak in the region of ribose, one in the region of gluconate, its lactone or the 2-keto acid, and a fourth unknown peak.

(iv) 1 and 2 were spotted beside the same known solutions as in (iii). Solvent 5 and Spray B were used. The R_f's were: glucose 0.29, ribose 0.39 and Δ-gluconolactone, K-gluconate, and Ca-2-ketogluconate 0.25. 2 Showed a glucose region by colorimetric detection and by radioactive scanning. 1 Showed two radioactive peaks, the R_f of one being 0.25, the lactone region, and the R_f of the other being 0.40, approximately the ribose region.

The oxidation of glucose by the particulate fraction of B-25 yields either gluconic acid, its lactone, 2-ketogluconate, or all three. A method of separating these three compounds chromatographically has not yet been devised. Another product seems to have the mobility of ribose (ribulose?), or perhaps of ribose derivatives. It is hoped that further investigations may reveal the identities of the products of glucose oxidation by the particulate fraction.

Conclusion

It is now possible to investigate browning in fish muscles more accurately than formerly since a tool is available whereby both glucose and ribose may be removed. Hitherto only ribose could be removed by whole washed bacterial cells. Washed muscle is not satisfactory since both sugars and soluble substances possessing amino groups are both removed.

DEOXYRIBONUCLEIC ACIDS, STEROID HORMONES AND ENERGY EXPENDITURES IN SALMON

SUMMARY NO. 50

BASE COMPOSITION OF SALMON SPERM DEOXYRIBONUCLEIC ACIDS — Michael Smith

This is one phase of a new project concerned with the isolation and characterization of the nucleic acids or marine species. It is now well established that the genetic information carried by living organisms is coded in their nucleic acids, particularly the deoxyribonucleic acids (DNA). Thus Avery, McLeod and McCarty in 1944 showed that DNA isolated from one strain of bacteria was capable of transforming another strain into one which had the characteristics of the strain of bacteria from which the transforming DNA was derived. This experiment, which was the first to definitely establish the genetic character of DNA, has been extended in many directions amongst bacteria. The infectious nature of bacteriophage DNA and of the ribonucleic acid obtained from plant viruses further confirm the nucleic acids in their role as the primary carriers of genetic information.

Because of the genetic nature of nucleic acids, it is very probable that their chemical composition will reflect phylogenetic relationships. This has been demonstrated by Lee, Wahl and Barku for bacteria, and we intend to investigate the deoxyribonucleic acids of marine algae, invertebrates and fishes to see if taxonomically related species have nucleic acids of similar composition.

The present report is concerned with the isolation and characterization of the DNA from the sperm of three species of Pacific salmon.

Materials and Methods

Freshly stripped salmon sperm was collected in plastic bottles and frozen in solid carbon dioxide for transport to the laboratory. During the stripping the usual

precautions against contamination by slime and excreta were taken. The sperm samples were diluted with two volumes of 0.1 M saline, 0.05 M sodium ethylenediaminetetraacetate, pH 8.0, (saline-ETDA) containing 0.5 ml sec-octanol and the mixture homogenised at full speed in a Serval Omni-mixer for 10 seconds. This and subsequent operations were carried out at 0°C. The sperm heads were collected by centrifugation at 2000 rpm (10 minutes) in a Serval SS-1 rotor. The homogenization and centrifugation cycle was repeated five times to yield the sperm heads as a white sediment which was stored at -30°C until required.

DNA was isolated using a slight modification in the procedure of Marmur. Sperm heads (100 mg) were suspended in saline-ETDA (25 ml) and 25% sodium dodecyl sulphate (2 ml) was added with vigorous stirring. The mixture immediately became very viscous and was then kept at 60° for 10 minutes to ensure that lepsis was complete. Five molar sodium perchlorate (6.5 ml) was added and the mixture shaken vigorously to ensure complete mixing. After cooling to 0°C the mixture was extracted with an equal volume of n-butanol with shaking by hand for 5 minutes. The resultant emulsion was broken by centrifugation at 5,000 rpm for 10 minutes. Butanol was removed with a pipette and the aqueous solution shaken with an equal volume of chloroform-iso-amyl alcohol (24:1) by hand for 5 minutes. Following centrifugation, the aqueous solution of DNA was removed by pipette and retreated with chloroform-octanol until no insoluble material was present at the interface (usually three times). Sodium DNA was precipitated by addition of 95% ethanol (2 volumes) collected on a glass rod and immediately transferred to 0.015 M saline-0.0015 M sodium citrate, pH 7.0 (10 ml). When the DNA had dissolved, 1.5 M saline-0.15 M sodium citrate (1 ml) was added and the precipitation with two volumes of 95% ethanol repeated. After a further precipitation the DNA was stored in 0.15 M saline-0.015 M sodium in citrate (10 ml) at 0°C until required. The yield was approximately 3.5 mg of sodium DNA as measured spectrophotometrically.

The Tm (see below) of a sample of salmon sperm deoxyribonucleate was determined by following the change in adsorption at 260 mu with temperature of a solution of the deoxynucleate in 0.15 M saline-0.015 sodium citrate, pH 7.0, in stoppered cells of 1 cm path length. The adsorption was measured at 2 to 3° intervals, the temperature being measured by an accurate thermometer, calibrated to 0.1°C, immersed in saline-citrate solution in a blank cell. The Tm of each sample of deoxynucleate was measured three times. For reference purposes the Tm of a sample of thymidylic acid-deoxyadenylic acid copolymer was measured in the same apparatus.

Results

Tm's of the deoxynucleic acids from the sperm of *Oncorhynchus nerka*, *O. gorbuscha* and *O. kisutch* are recorded below.

	Tm
O. nerka	85.7° ± 0.0°
O. gorbuscha	85.9° ± 0.1°
O. kisutch	85.2° ± 0.1°
dAT copolymer	64.9° ± 0.1°

Discussion

Marmur and Doty have established that under standard conditions there is a linear relationship between the amount of guanine contained by a nucleic acid and the temperature at which it undergoes a transition from a double-stranded helical configuration to a disordered coil. The temperature at the midpoint of this transition, Tm, is therefore a characteristic of the base-composition of the deoxyribonucleate. In the present work Tm was measured by following the increase in ultraviolet

adsorption which occurs during the transition.

The two extremes, O. gorbuscha and O. kisutch differ in Tm by 0.7°C, which, according to the relationship of Marmur and Doty, indicates that O. gorbuscha sperm deoxyribonucleate contains approximately 1.5% more guanine than does that of O. kisutch. It is of interest to note that O. nerka is more closely related to O. gorbuscha from the point of view of the Tm of its deoxyribonucleate. This is in agreement with the relationship between these species demonstrated by Dr. H. Tsuyuki from a study of their water soluble muscle proteins.

Acknowledgments

We are greatly indebted to Dr. A. Kornberg, Stanford University, for the thymidylic acid-deoxyadenylic acid copolymer, to H. Ha vey of the International Pacific Salmon Fisheries Commission for O. nerka sperm, to F. Boyd of the Canadian Department of Fisheries for O. gorbuscha sperm and to S. Fallert of the Washington State Department of Fisheries, Green River Salmon Hatchery for O. kisutch sperm.

SUMMARY NO. 51

ISOLATION OF 20β-DIHYDROCORTISONE FROM SOCKEYE SALMON (ONCORHYNCHUS NERKA) PLASMA

D.R. Idler
P.J. Schmidt
A.M. Bernie
A.P. Ronald

During the course of the investigation of steroid hormones in salmon plasma, numerous compounds have been isolated and identified (Summary No. 53 of this Annual Report). While isolating some of these steroids it was observed that ultraviolet absorbing substances, more polar than cortisol, were present when chromatographing salmon plasma in a toluene-propylene glycol system (Summary No. 5 of this Station's Annual Report for 1960-61). This report describes the isolation and identification of one of these substances.

Reduction of the C-20 carbonyl group occurs during the metabolism of progesterone and the adrenocortical hormones in man. This reduction has been demonstrated by incubating certain adrenocorticosteroids with liver homogenates or by perfusing them through liver. 20β-Dihydrocortisol has been isolated from the urine of man, from rat plasma after the administration of cortisol, and from the urine of rats treated with cortisone acetate. Both 20α- and 20β-dihydrocortisol have been reported as urinary metabolites in guinea pigs given cortisol and 20α-dihydrocortisol has also been isolated from urinary extracts of normal human beings after the oral administration of cortisol. 20α-Dihydrocorticosterone has been identified in mouse plasma and from plasma of normal males treated with corticosterone, while 20α-dihydrocortisone has been isolated from beef and hog adrenals, and 20β-dihydrocortisone from human placenta.

We wish to report the isolation and identification of 20β-dihydrocortisone from the plasma of both male and female spawned sockeye salmon.

Experimental

Reference Standards

The 20-dihydrosteroids were synthesized by the chemical reduction of the corresponding 20-ketosteroids. Sodium borohydride (218 µg) was dissolved in 1 ml

of 80% aqueous tertiary butanol and added to 4 mg of the 20-ketosteroid. This mixture was shaken for one hour after which the reaction was stopped by the addition of 2 ml of 2.5% acetic acid and the steroids were extracted twice with 5 ml volumes of dichloromethane. The 20-dihydroepimers were then separated from their parent compounds by preliminary paper chromatography in a toluene-30% propylene glycol system for 24 hours, followed by chromatography in the same system for 9 days to separate 20α- and 20β-dihydroepimers of cortisol and cortisone and for 4 days to separate the epimers of corticosterone. Based on ultraviolet (U.V.) absorption at 240 mu the yield of the isomeric pairs from cortisol, cortisone and corticosterone was 27.8, 65.9 and 27.3%, respectively, and the respective ratios of β to α epimers were 8:1, 9.1:1, and 9.5:1. The chromatographic mobilities of these steroids are recorded elsewhere.

The spectra of the sulphuric acid chromogens of the chromatographically pure steroids were obtained by adding 1.2 ml of sulphuric acid to 10 ug of steroid and leaving at room temperature for 2 hours. The spectra were recorded using a Beckman Dk-1 spectrophotometer. The spectra, shown in Fig. 1, do not permit distinction between the α and β epimers.

Sampling and Processing

The procedure used in catching the sockeye salmon, bleeding the fish, treatment and extraction of the plasma, and finally the partitioning between 70% methanol and hexane was the same as that described previously except that ethyl acetate replaced dichloromethane for the extraction of the plasma and for the final extraction after the 70% methanol-hexane step.

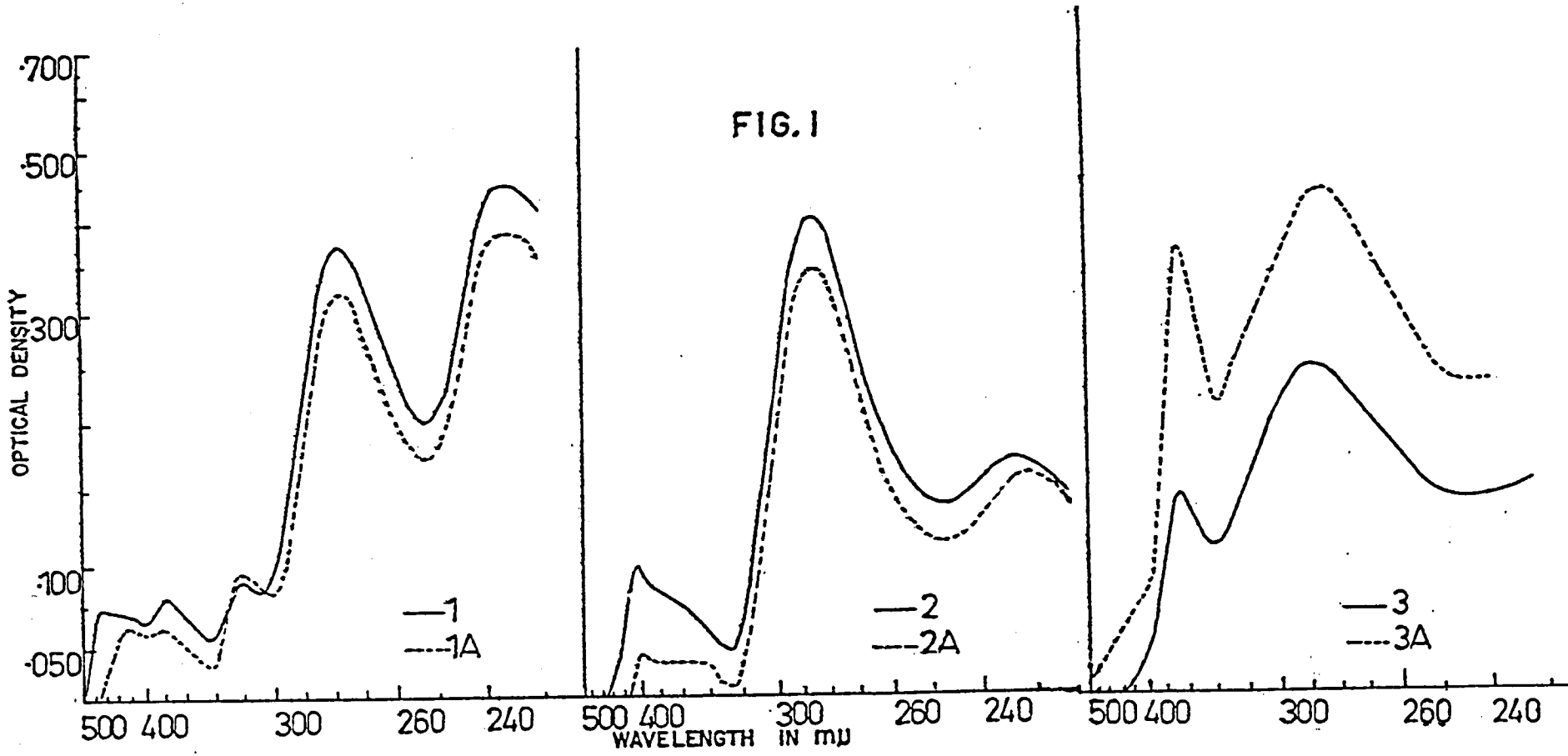
Sockeye salmon plasma was obtained from male and female post-spawned fish taken at Weaver Creek, British Columbia, in October, 1960, and from male and female post-spawned fish taken at Cultus Lake, British Columbia, in November, 1960.

All chromatograms were developed descending and were in general the same as those described previously. The plasma extracts were applied to the paper in 10-cm strips, while the spots were detected by UV light and eluted with methanol using descending chromatography.

Results and Discussion

The extracts from 820 ml of Weaver Creek female plasma and 789 ml of male plasma were separately chromatographed on paper with a toluene-70% methanol solvent system (descending) for 2 hours together with a cortisol reference standard. This chromatogram was developed for such a short period of time in order to isolate certain other steroids for another investigation. From the two plasma strips, the fractions including the origin and cortisol were eluted and re-chromatographed using the same solvent system (toluene-70% methanol) for 16 hours together with reference standards of cortisol and the 20α- and 20β-dihydroepimers of cortisol, cortisone and corticosterone. After chromatography, both the male and female plasma strips had a UV absorbing spot corresponding to 20α- and 20β-dihydrocortisol and these were eluted. The corresponding eluates of the male and female plasma were combined, evaporated, and the spectra of the sulphuric acid chromogens obtained. The spectra of the chromogen were unlike those of standard 20α- and 20β-dihydrocortisol. A single elution was made of the remainder of each of the paper chromatograms, which had UV absorbing spots in the 20β-dihydrocortisone region and the cortisol region of both male and female plasma. In order to get better separation of these compounds, the plasma eluates and standard 20α- and 20β-dihydrocortisone, 20α- and 20β-dihydrocorticosterone, and cortisol were acetylated.

FIG. 1


·700
·500
·300
·100
·050
OPTICAL DENSITY
—1
---1A
—2
---2A
—3
---3A
500 400 300 260 240
500 400 300 260 240
500 400 300 260 240
WAVELENGTH IN mμ

One ml of the acetylating mixture was used and left at room temperature for 48 hours. The samples were then taken to dryness and chromatographed using a heptane:benzene 1:1 - 70% methanol system for 2½ hours. The R_T values of the acetylated reference standards were found to be as follows: cortisol acetate, 0.180, 20α-dihydrocortisone diacetate, 0.386; 20β-dihydrocortisone diacetate, 0.315; 20α-dihydrocorticosterone diacetate, 0.695; 20β-dihydrocorticosterone diacetate, 0.692. The plasma chromatograms each (male and female) had only two spots, one corresponding to cortisol acetate and the other to the 20-dihydrocortisone diacetates. There was no evidence of the 20-dihydroepimers of corticosterone being present. The material from the plasma samples with the chromatographic mobilities of the α and B isomers of 20-dihydrocortisone diacetate were eluted, the male and female fractions were combined, and re-chromatographed with a benzene:hexane 1:2 - 80% methanol solvent system for 7 hours together with reference standards. The R_T for 20α-dihydrocortisone diacetate was 3.7 cm/hr and the R_T for 20β-dihydrocortisone diacetate was 3.2 cm/hr. The substance from plasma had the same chromatographic mobility as 20β-dihydrocortisone diacetate.

Another batch of plasma (1048 ml of post-spawned male Cultus Lake sockeye) was extracted, chromatographed with a toluene - 70% methanol solvent system, first for 2 hours and then for 16 hours as described above for the earlier plasma samples. The plasma spots corresponding to 20α-dihydrocortisol or 20β-dihydrocortisol and 20β-dihydrocortisone were detected and eluted and taken to dryness. One-fifth of the residue failed to give a Porter-Silber reaction. These residues and corresponding reference standards (10 µg) were subjected to sodium bismuthate oxidation by adding 2.5 ml of acetic acid and 0.35 g of $NaBiO_3$. After shaking for ½ hour, the reaction mixtures were filtered through paper and extracted three times with 2 ml dichloromethane, which was then evaporated. The residues and standard adrenosterone and 11β-ol-Δ^4-androstene-3,17-dione were chromatographed employing heptane - 80% methanol for 16 hours. The paper chromatogram of the oxidation products of standard 20α- and 20β-dihydrocortisol had UV absorbing spots corresponding to 11β-ol-Δ^4androstene-3,17-dione (R_T = .275) while the corresponding plasma chromatogram did not produce such spots. The plasma residues suspected of being 20α- or 20β-dihydrocortisol appeared to be still at the origin. The oxidation product of standard 20β-dihydrocortisone and its corresponding plasma oxidation product contained a UV absorbing substance corresponding to adrenosterone (R_T = 0.845 cm/hr). All spots corresponding to 11β-ol-Δ^4-androstene-3,17-dione and adrenosterone gave the typical violet colour when sprayed with Zimmerman reagent. The plasma spot corresponding to adrenosterone was eluted, evaporated and subjected to sodium borohydride reduction. The reduction product and 11-ketotestosterone were chromatographed on paper using heptane: benzene 1:1 - 70% methanol for 1-3/4 hours. The plasma and 11-ketotestosterone spots had identical mobilities (R_T = 3.43 cm/hr).

Another plasma sample (810 ml of post-spawned female Cultus Lake sockeye salmon plasma) was extracted and chromatographed with a toluene - 70% methanol system for 2 hours and then for 16 hours in the same way already described for the other samples. The plasma spot on the chromatogram corresponding to 20β-dihydrocortisone was eluted and by UV absorption at 240 mµ (corrected), its concentration was calculated to be 2.1 µg/100 ml of plasma, based on the recovery of standard 20β-dihydrocortisone from the procedures. The plasma eluate was then evaporated to dryness, 1.2 ml of sulphuric acid added and after 2 hours its spectrum was recorded. It had maximum absorption peaks at 408 and 284 mµ, the same as those shown for standard 20β-dihydrocortisone in Fig. 1.

A sample of female salmon plasma obtained from fish taken at Lytton from the 1958 Adams River run contained two UV absorbing substances which were more

polar than cortisol when chromatographed in the toluene-propylene glycol system. These substances were estimated to be present at a combined concentration of approximately one-third that of cortisol. The less polar steroid from the plasma of both male and female spawned sockeye salmon is herein identified as 20β-dihydrocortisone. The more polar steroid has the chromatographic mobility of 20α- and 20β-dihydrocortisol but could not be identified with either steroid.

SUMMARY NO. 52

HORMONES IN SALMON PLASMA DURING SPAWNING MIGRATION.
ISOLATION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF ALDOSTERONE IN POST-SPAWNED SOCKEYE SALMON PLASMA. II.

A.P. Ronald
D.R. Idler

The isolation and quantitative determination of a compound which accompanies aldosterone in three solvent systems, toluene-propylene glycol, toluene-70% methanol and ethylene glycol:n-butyl acetate:water and which, after acetylation, accompanies aldosterone diacetate in heptane:benzene 1:1-70% methanol was described in last year's Annual Report.

It was strongly suspected that this compound was aldosterone but the chromatographic proof was insufficient for positive identification. If this compound could also form the 21-mono-acetate and the 11,18-lactone, 21-acetate it was felt that this would give sufficient evidence to say that the compound was aldosterone.

Samples of chromatographically pure aldosterone, aldosterone 21-monoacetate, aldosterone 11,18-lactone, 21-monoacetate and aldosterone diacetate were prepared for use as carriers and standards. Aldosterone was prepared by chromatogramming authentic aldosterone in toluene-70% methanol and eluting with methanol. Aldosterone, 21-monoacetate was prepared by extracting a mixture of aldosterone acetates in sesame oil, removing the oil by hexane partitioning and chromatogramming the extract in heptane: benzene-70% methanol and eluting the 21-monoacetate with methanol. The 21-monoacetate was also prepared by an acid hydrolysis of the diacetate. 0.4 Ml of 0.1 N HCl was added to 10 µg of aldosterone diacetate and left overnight in a glass-stoppered test tube at 37°C. The solution was then extracted with 5 ml of methylene chloride and the remaining acid solution neutralized with sodium bicarbonate and extracted with ethyl acetate. The extract was then purified by paper chromatography.

Aldosterone 11, 18-lactone, 21-monoacetate was prepared by oxidation of aldosterone, 21-monoacetate. A solution of 10 µg of aldosterone, 21-monoacetate in approximately 0.02 ml of glacial acetic acid was cooled to 13°C and 0.02 ml of a solution of 1 g of chromium trioxide in 1 ml of water and 99 ml of glacial acetic acid at 16°C was added and then cooled to 13°C. After 10 minutes at 13°C, 0.5 ml of water and 5 ml of ethyl acetate was added. The ethyl acetate was then washed twice with 0.5 ml of water, taken to dryness in a jet of nitrogen and then chromatogrammed. The yield was 50%. Aldosterone diacetate was prepared by acetylation of chromatographically pure aldosterone, chromatogramming in heptane:benzene-70% methanol and eluting with methanol. Table I shows the relative mobilities of these compounds in heptane:benzene-70% methanol.

TABLE I - Relative mobilities of prepared standards in heptane: benzene-70% methanol, equilibrated for 2 hours.

Compound	Rf
Aldosterone	.03
Aldosterone, 21-monoacetate	.19
Aldosterone, 11, 18-lactone, 21-monoacetate	.31
Aldosterone diacetate	.57

The above measurements are approximate as the compounds were run at different times on different papers.

A sample which contained a known amount of C^{14} aldosterone diacetate was obtained by combining the samples from the 400 ml of spawned Cultus Lake sockeye plasma, used for the quantitative determination of aldosterone (Summary No. 3 of this Station's Annual Report for 1960-61). To this was added 62.8 µg of aldosterone as the diacetate. The sample now contained 64.8 µg of steroid by UV analysis at 240 mµ, E = 15,900, and had 2163 cpm established with a D-47 gas flow detector.

Therefore, the specific activity of the mixture = $\frac{2163 \text{ cpm}}{64.8 \text{ µg}}$ = 33.4 cpm/µg and the activity due to added C^{14} diacetate = 20.4% = 6.68 cpm/µg.

The mixture was then hydrolyzed to the 21-monoacetate and chromatogrammed in heptane:benzene-70% methanol. Before elution an actigraph scan of the chromatogram was run and the radioactive areas determined. The paper was also viewed under UV light.

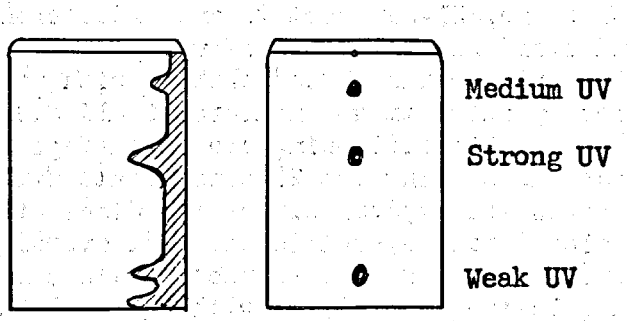


Fig. 1. On left, actigraph scan of paper chromatogram of acid hydrolysed sample. On the right, the paper chromatogram of 1. aldosterone, 2. aldosterone, 21-monoacetate.

The aldosterone, 21-monoacetate was then eluted with methanol. Unfortunately at this point a spill occurred and about half of the fraction was lost. UV analysis at 240 mµ, E = 15,900, showed the fraction to contain 28.2 µg of aldosterone, 21-monoacetate (calc. as aldosterone) which had a radioactivity of 93.2 cpm, giving a specific activity = 3.3 cpm/µg, which is the exact activity one would expect from the added aldosterone.

The eluted 21-monoacetate was then converted to the 11,18-lactone, 21-monoacetate with chromium trioxide and chromatogrammed in heptane:benzene-70% methanol. UV-absorbing spots were observed on the paper, one at the 11,18-lactone, 21-monoacetate position and a very faint one travelling just ahead. The steroid was eluted and UV analysis at 240 mµ and E = 15,900 showed the fraction to contain 9.3 µg of lactone (calcd. as aldosterone) which had a radioactive count of 20.6 cpm giving a

specific activity = 2.22 cpm/µg.

These figures seem to indicate that if aldosterone is present the level must be extremely low as this method would have detected quantities as low as 1 to 0.5 µg per litre. There is no doubt, however, that the plasma does contain steroids which travel with aldosterone and very closely with aldosterone diacetate. If one assumes that on acetylation they are diacetates because of their similar chromatographic mobilities to that of aldosterone diacetate, then it can be stated, as they do not form monoacetates on hydrolysis with 0.1 N HCl with the same chromatographic mobility of aldosterone, 21-monoacetate, neither of these steroids, which are the main components of this fraction, is aldosterone. It does appear, however, that a compound is formed which runs with aldosterone in heptane:benzene-70% methanol as can be seen from the following actigraph scan, Fig. 2, of the acid hydrolysed aldosterone diacetate fraction. A word of caution should be inserted here, however. It has been observed that when a radioactive steroid acetylated with C^{14} acetic anhydride is hydrolysed back to the free steroid, a certain amount of radioactivity is always present. A further experiment has shown that in all probability the compound in question is aldosterone.

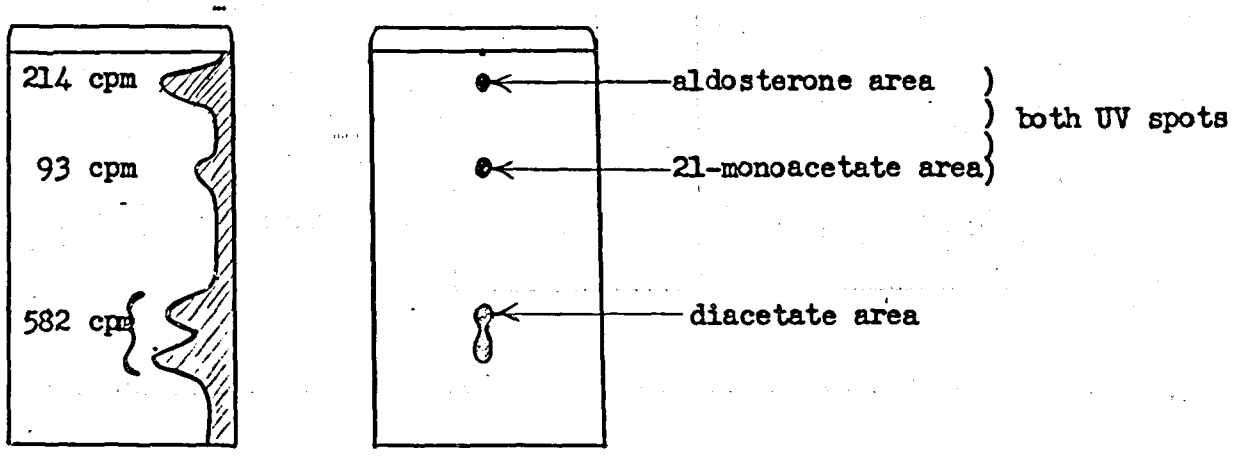


Fig. 2. On the left is the actigraph scan of the paper chromatogram of the acid hydrolysed diacetate area. On the right is the heptane:benzene-70% methanol chromatogram of the acid hydrolysed diacetate area.

At this point the argument could be presented that perhaps there was not complete hydrolysis of the aldosterone diacetate and that this would account for the high radioactivity at the diacetate area. If this were so, then the diacetate area would have contained approximately 30 µg of the aldosterone diacetate carrier, which it did not. In fact, no UV-absorbing substance was detected in this area, which would also indicate that these accompanying steroids are saturated.

Another experiment was conducted to establish, as cortisone acetate runs with aldosterone, 21-monoacetate, that cortisone was not interfering and again to try to establish the presence of aldosterone.

500 Ml of plasma from Cultus Lake post-spawned sockeye salmon containing 45.5 µg cortisone and a control containing 44.0 µg of cortisone and 0.6 µg aldosterone were chromatogrammed in toluene-70% methanol and the cortisone area which would also contain aldosterone cut out and eluted with methanol. This fraction was then acetylated with an acetylation mixture of C^{14} acetic anhydride with a specific activity of 0.327 mC/mM. Aldosterone diacetate acetylated with this mixture would have an activity

of 546 cpm/µg. The acetylated fraction was then chromatogrammed, two-dimentionally in heptane:benzene-70% methanol, and the aldosterone diacetate area and the area containing the compound which runs slightly ahead, cut out and eluted off the paper with methanol. These fractions were then hydrolysed with 0.1 N HCl at 37°C overnight, extracted in the usual manner and chromatogrammed in heptane:benzene-70% methanol. The paper was then cut into strips which were eluted with methanol, taken to near dryness in a stream of nitrogen and applied to planchets and counted with a D-47 gas flow detector equipped with a "micromil" window. The results are shown in Fig. 3.

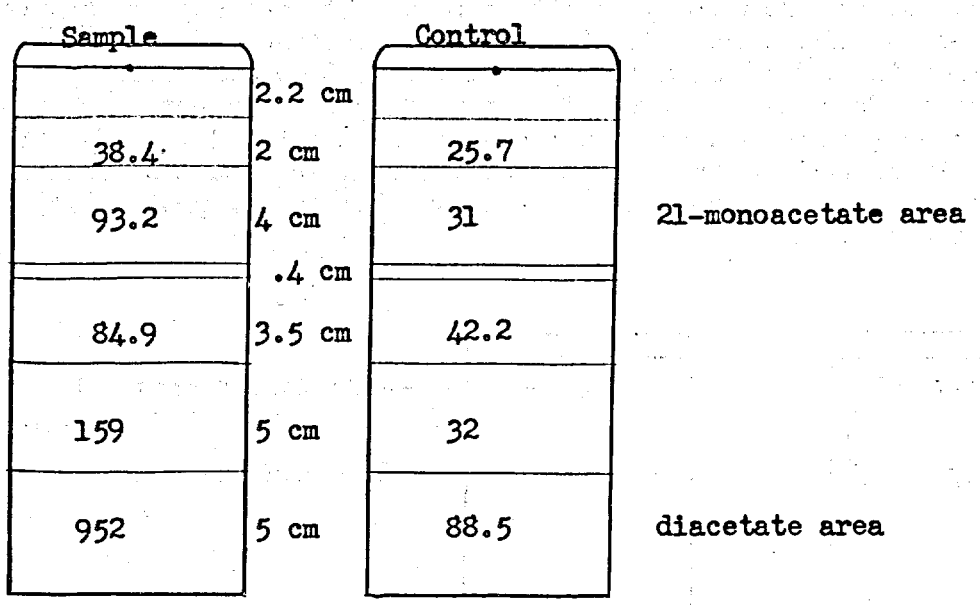


Fig. 3. Counts per minute obtained from elution of above chromatogram strips.

The above results show that this method is not refined enough to detect 0.6 µg of aldosterone (0.12 µg/100 ml) but again it has verified the presence of steroids which accompany aldosterone and aldosterone diacetate but not aldosterone, 21-monoacetate. It also shows that cortisone acetate is removed by chromatography and does not interfere with the analysis of the aldosterone, 21-monoacetate.

A similar experiment was conducted using a much more active C^{14}-acetylation mixture such that 1 µg of aldosterone would have 10,000 cpm. The experiment failed due to an unexplainable loss of activity.

Summary

Saturated steroids with the same chromatographic mobility as aldosterone in toluene-70% methanol, toluene-propylene glycol, ethylene glycol:n-butyl acetate: water and heptane-benzene-70% methanol systems, have been detected in the plasma of spawned Cultus Lake sockeye salmon. Acetylation of this fraction with radioactive C^{14} acetic anhydride and chromatography in heptane:benzene-70% methanol gives two radioactive spots, one in the same position as aldosterone diacetate and the other just slightly ahead. Acid hydrolysis and chromatography of the eluted material with the same mobility as aldosterone diacetate and the preceding area gave no detectable evidence of aldosterone, 21-monoacetate. If aldosterone is present in spawned sockeye salmon plasma it is below 0.5 µg per 100 ml.

SUMMARY NO. 53

ISOLATION AND IDENTIFICATION OF ADRENOSTERONE IN SALMON (ONCORHYNCHUS NERKA) PLASMA

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

During the course of the investigation at this Station of the steroid hormones in salmon plasma, various steroids have been isolated and characterized. (See this Station's Annual Reports: Summary No. 41, 1958-59; Summaries Nos. 4 and 5, 1959-60; Summary No. 6, 1960-61, and Summary No. 51 of this Annual Report.)

In a study involving the quantitative estimation of various steroid hormones in sockeye salmon plasma (Summary No. 15 of this Station's Annual Report for 1960-61), an unidentified ultraviolet (UV) absorbing spot was detected when plasma extracts prepared from post-spawned male sockeye were chromatographed on paper with a heptane:80% methanol solvent system for 16 hours. The polarity of the new substance was identical with adrenosterone and intermediate between 17α-hydroxyprogesterone and its 20β-dihydro-epimer, both previously isolated from salmon plasma. The Zimmermann reagent produced the violet colour, typical of 17-ketosteroids, with the new steroid.

Adrenosterone, in a crystalline form has been isolated from extracts of human adrenal cortex by other workers. It has also been identified in the perfusate obtained when blood containing added ACTH was circulated through bovine glands.

To our knowledge the presence of adrenosterone in peripheral plasma has not previously been reported.

Sockeye salmon plasma was obtained from post-spawned fish taken at Cultus Lake in November, 1960. The procedure for bleeding the fish, extracting the plasma and the chromatographic methods used have been described elsewhere (Summary No. 15 of this Station's Annual Report for 1960-61).

The extract from 500 ml of plasma was partitioned between 70% methanol and hexane. The 70% methanol fraction was then taken to dryness and chromatographed on paper using the heptane:80% methanol systems for 16 hours. The reference standard of adrenosterone and the corresponding spot on the plasma strip were detected by UV light and had identical mobilities (R_T = 0.72 cm/hr). The plasma spot was eluted with methanol using descending chromatography. By UV absorption at 240 mμ (corrected), and based on the recovery of adrenosterone through the procedures, there were 2.5 ug/100 ml of the steroid in the plasma. The plasma was eluted from the paper, evaporated to dryness and subjected to sodium borohydride reduction. The reduction product and 11-ketotestosterone were chromatographed on paper using heptane:benzene 1:1-70% methanol for 3 hours. The plasma spot and the reference standard were detected with UV light and both had identical mobilities.

The substance from the plasma was eluted from the paper, treated with 0.1 ml of acetylating mixture and left at room temperature for 24 hours. The reaction mixture was taken to dryness using N_2 at 40°. The acetylating mixture consisted of 5 volumes of pyridine, 4 volumes of benzene and 1 volume of acetic anhydride. The acetylated product was chromatographed along with 11-ketotestosterone acetate using the heptane:80% methanol solvent system for 3.75 hours and the mobilities of the steroids were identical (R_T = 4.1 cm/hr).

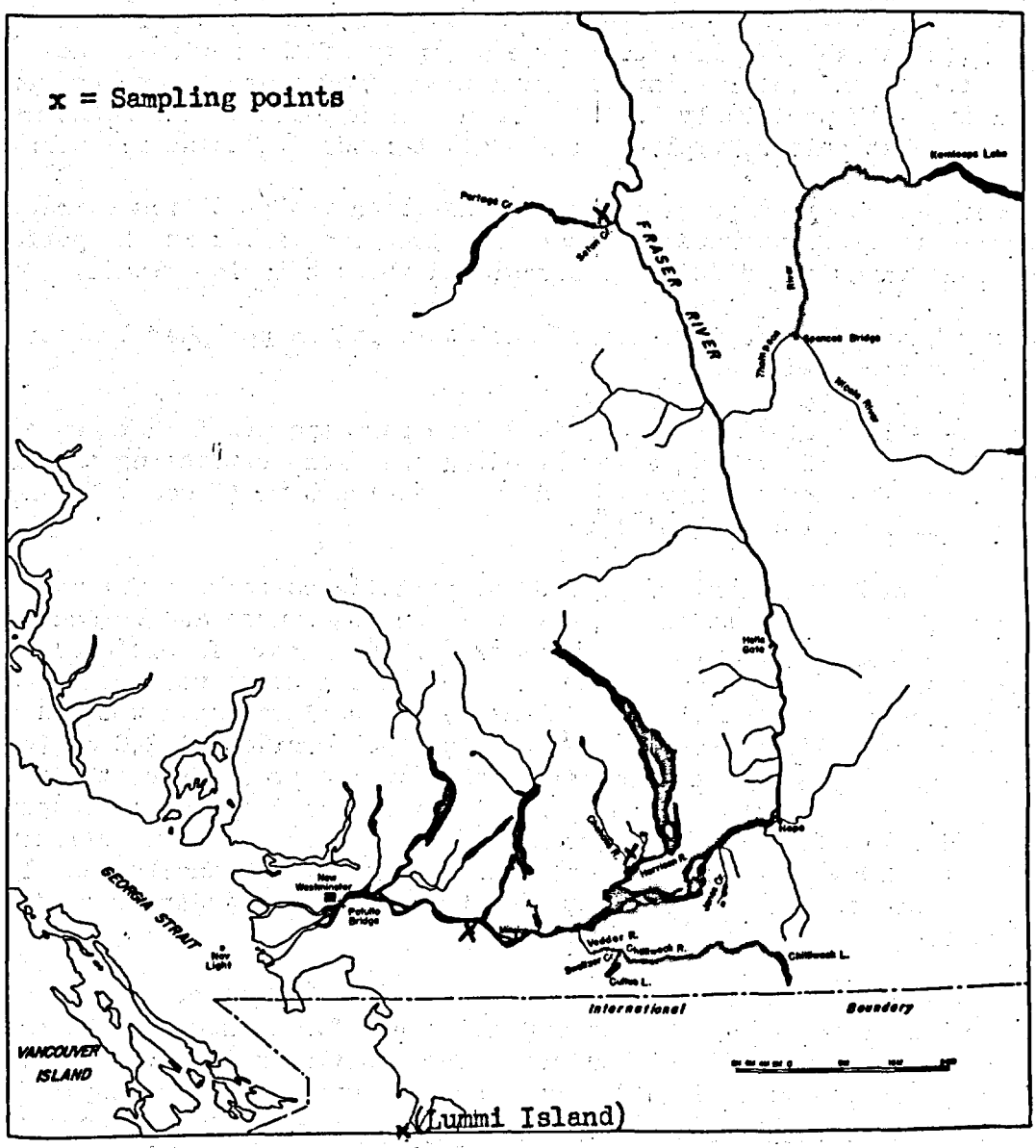
Acknowledgment

Staff of the International Pacific Salmon Fisheries Commission rendered valuable assistance in obtaining the fish.

SUMMARY NO. 54

ENERGY EXPENDITURES OF MIGRATING FRASER RIVER PINK SALMON — Michael Smith

This is a new project sponsored by the International Pacific Salmon Fisheries Commission. In principal and experimental detail the study is analogous to that recently completed on the energy expenditures of migrating Fraser River sockeye salmon. Samples of pink salmon from the 1961 runs have been collected by Salmon Commission biologists and are being analysed at the Vancouver Technological Station by Salmon Commission scientists (T. Todoroff and G. Berry). Two races of pink salmon are being studied. The first, an early run to Seton Creek which was sampled at Lummi Island, Glen Valley and at Seton Creek (see map).



Pink spawning areas of Fraser River system.

Prespawned, post-spawned and dead salmon were collected at the spawning grounds. The second race under study is a late run to the Harrison River for which analogous samples were taken at Lummi Island, Glen Valley and the spawning grounds. To date physical measurements (dimensions and specific gravities of whole fish and weights of various tissues) have been completed and protein, fat and moisture analyses are in progress prior to calculation of the energy expended by the migrating fish. On completion of the study a full report will be published by the International Pacific Salmon Fisheries Commission.

MISCELLANEOUS

SUMMARY NO. 55

MARINE STEROLS. BIOSYNTHESIS OF 24-METHYLENECHOLESTEROL IN CLAMS

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

The biosynthesis of 24-methylenecholesterol in clams was reported in Summary No. 42 of this Station's Annual Report for 1960-61. Because of the low yield of labelled 24-methylenecholesterol (0.052% of total acetone-extractable radioactivity) obtained after injection of cholesterol-4-C^{14}, another experiment was carried out incorporating some changes which were hoped to increase the rate of conversion. The conversion obtained in this experiment was 0.25%.

Five clams, 2-3 years of age, taken in the month of February were kept in sea water (aerated, but not running) at 17°. Cholesterol-4-C^{14} (S.A., 8.25×10^7 cpm/mg) was dissolved in a few drops of olive oil. The oil was injected into the digestive glands by inserting the needle between the closed shells close to the hinge. The animals apparently did not suffer any ill effects from the injections. Four clams (batch 1) were kept for 4-9 days in an aquarium, and one clam (batch 2) was kept for 11 days. The water in the aquarium was changed once, on the third day after the injection.

The procedure for extraction of the oil and for obtaining the non-saponifiable matter has been described in the above-mentioned summary. There were 7.39×10^6 cpm in the oil from batch 1. 24-Methylenecholesterol from this batch was obtained by chromatography of the non-saponifiable matter (6.83×10^6 cpm) on paper without preliminary purification. Approximately 1.3 mg (4230 cpm) was chromatographed in the 85% pyridine chromatographic system. The section containing 24-methylenecholesterol (259 cpm) was cut out. The sterol was eluted and rechromatographed in the same system. 24-Methylenecholesterol obtained from this chromatogram weighed 0.142 mg (86.7 cpm). The material was mixed with 1.265 mg of authentic 24-methylenecholesterol and crystallized twice from aqueous methanol. The S.A. dropped sharply in the first crystallization but changed only slightly in the second (18.9 cpm/mg). The diluted material was finally chromatographed on paper and the compound obtained from the 24-methylenecholesterol area was crystallized once. The S.A. was slightly higher, 20.6 cpm/mg. There was 87 mg of 24-methylenecholesterol present in the acetone extract of this batch as determined by chromatographic separation of the azoyl esters. The radioactivity carried by 24-methylenecholesterol represents 0.25% of the total radioactivity extracted from the animals with acetone.

There were 2.36×10^6 cpm in the oil fraction from batch 2. To isolate the sterols, the non-saponifiable portion (1.48×10^6 cpm) was chromatographed on alumina

of activity Grade II. The sterol fraction weighed 33.7 mg (1.29×10^7 cpm).

This fraction was ozonized and resulting ketosteryl acetates were isolated as described previously. They were chromatographed on paper in the 98% acetic acid system. The 24-ketocholesteryl acetate section was cut out and the acetate eluted and rechromatographed in the same system. Thus 0.461 mg (1150 cpm/mg) of 24-ketocholesteryl acetate was obtained. The material was mixed with authentic 24-ketocholesteryl acetate (3.163 mg) and the mixture was crystallized repeatedly from aqueous methanol. After an initial sharp drop in the first crystallization the S.A. remained constant at 38.5 cpm/mg. On the basis of this value, the S.A. of the original 24-methylenecholesterol was calculated to be 336 cpm/mg. Assuming that the ratio of the amount of total sterols to that of 24-methylenecholesterol is the same as in batch 1, the radioactivity carried by 24-methylenecholesterol from this batch represents 0.30% of the total radioactivity extracted from the animal with acetone.

Thanks are due to Dr. D.B. Quayle of the Nanaimo Station at Nanaimo, B.C. for supplying the clams.

SUMMARY NO. 56

DOGFISH GELATIN

S.E. Geiger
N. Tomlinson

Summary No. 30 of this Station's Annual Report for 1960-61 recorded the results of earlier work in this investigation. This has now been completed.

In the accompanying table results of analyses of gelatins prepared from dogfish skin and skeleton are recorded. These gelatins were prepared by methods that were found to be most suitable from the point of view of quality. As was pointed out last year, yields could be improved by extraction at higher temperatures but this was accompanied by an impairment of the gelling properties of the product.

Skin and skeleton were ashed and the ash was analyzed for calcium and phosphorus. The quantity of ash from the skin (equivalent to 19% of the dry weight, which in the skin analyzed accounted for 26% of its fresh weight) was nearly double that which has been reported from whole cod skin (Young and Lorimer). Ash from skeleton was equivalent to 21% of its dry weight which in turn accounted for 20% of the fresh weight of the skeleton analyzed. Calcium accounted for 2.4% and 1.56%, and phosphorus for 0.89% and 0.67%, respectively, of the fresh weight of the skin and skeleton.

Glue was prepared from dogfish gelatin by heating a 19% (w/v) solution for five hours at 180°F. This glue provided a sufficiently strong wood joint that wood failure resulted when attempts were made to break the bond. Glue prepared by heating gelatin solution for 2 hours at 212°F also gave a satisfactory wood joint. However, these glues were very readily soluble in water and soaking glued wood joints in water soon resulted in their destruction.

Gelatin from skin and skeleton of dogfish (Squalus suckleyi).

	% of dry weight of gelatin*	
	from skin	from skeleton
Total nitrogen	16.3	15.7
Chloride	0.46	0.45
Hydroxyproline	6.4	8.8
Tyrosine	0.28	0.29
Melting point**	° centigrade	
of 10% solution	22	23
of 2% solution	18	19
Gelling point		
of 10% solution	15	14
of 2% solution	7	8

* Prepared by two consecutive 30-min extractions at 70°C from material pretreated by method C (see Summary No. 30 of this Station's Annual Report for 1960-61). Yields, 7% from skin and 2% from skeleton based on wet weight of fresh material.

** Extracts concentrated by lyophilization for preparation of 10% and 2% (w/v) solutions.

PUBLICATIONS

VANCOUVER TECHNOLOGICAL STATION

(January 1, 1961 to December 31, 1961)

1. Cooper, D.M., J. Biely, B. March, R.A. MacLeod and H.L.A. Tarr. The reproductive performance of broad breasted bronze turkeys maintained on slatted floors. Poultry Science, Vol. 40, No. 1, pp. 242-247, 1961.

2. Fagerlund, U.H.M., and D.R. Idler. Marine sterols. VIII. In vivo transformation of the sterol sidechain by a clam. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 3, pp. 505-509, 1961.

3. Marine sterols. IX. Biosynthesis of 24-methylenecholesterol in clams. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 9, pp. 1347-1355, 1961.

4. Idler, D.R., I. Bitners and P.J. Schmidt. 11-Ketotestosterone: an androgen for sockeye salmon. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 11, pp. 1737-1742, 1961.

5. Idler, D.R., J.R. McBride, R.E.E. Jonas and N. Tomlinson. Olfactory perception in migrating salmon. II. Studies on a laboratory bioassay for homestream water and mammalian repellent. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 10, pp. 1575-1584, 1961.

6. Idler, D.R., P.J. Schmidt and I. Bitners. Isolation and identification of adrenosterone in salmon (Oncorhynchus nerka) plasma. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 10, pp. 1653-1654, 1961.

7. McBride, J.R., D.R. Idler and R.A. MacLeod. The liquefaction of British Columbia herring by ensilage, proteolytic enzymes and acid hydrolysis. J. Fish. Res. Bd., Vol. 18, No. 1, pp. 93-112, 1961.

8. Mannan, A., and H.L.A. Tarr. Preparation of deoxynucleosides, purine and pyrimidine bases and deoxyribose 1-phosphate from deoxyribonucleic acid employing salmon enzyme systems. J. Fish. Res. Bd., Vol. 18, No. 3, pp. 349-366, 1961.

9. March, B.E., J. Biely, F.G. Claggett and H.L.A. Tarr. Nutritional and chemical changes in the lipid fraction of stored antioxidant-treated and untreated herring meals. Fisheries Research Board of Canada, Vancouver Technological Station Circular No. 25, 13 pp., 1961.

10. March, B.E., J. Biely, J. Goudie, F. G. Claggett and H.L.A. Tarr. The effect of storage temperature and antioxidant treatment on chemical and nutritional characteristics of herring meal. Journal of American Oil Chemists' Society, Vol. 38, No. 2, pp. 80-84, 1961.

11. March, B.E., J.E. Biely, J.R. McBride, D.R. Idler and R.A. MacLeod. The protein nutritive value of "liquid herring" preparations. J. Fish. Res. Bd., Vol. 18, No. 1, pp. 113-116, 1961.

12. Martin, G-B., and H.L.A. Tarr. Phosphoglucomutase, phosphoribomutase and phosphoglucoisomerase of lingcod muscle. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 2, pp. 297-308, 1961.

13. Roach, S.W., J.S.M. Harrison and H.L.A. Tarr. (Appendices by W.A. MacCallum, M.S. Chan and A.W. Lantz). Storage and transport of fish in refrigerated sea water. Fisheries Research Board of Canada Bulletin No. 126, 61 pp., 1961.

14. Smith, M., G.I. Drummond and H.G. Khorana. Cyclic phosphates. IV. Ribonucleoside-3',5' cyclic phosphates. A general method of synthesis and some properties. J. Am. Chem. Soc., Vol. 83, pp. 698-706, 1961.

15. Smith, M. Adenosine-5' triphosphate and uridine-5' phosphate. Biochemical Preparations, Ed. A. Meister, J. Wiley and Sons Inc., New York, Vol. 8, pp. 1-4 and pp. 130-133, 1961.

16. Southcott, B.A., and H.L.A. Tarr. Magnesium as a factor in determining the comparative sensitivity of marine bacteria to four tetracycline antibiotics. Canadian Journal of Microbiology, Vol. 7, No. 2, pp. 284-286, 1961.

17. Tarr, H.L.A. Preservation by antibiotics and by penetrating radiations. I. Fishing News International, Vol. I, No. 1, pp. 17-20, 1961.

18. Chemical control of microbiological deterioration. Chapter 17 in "Fish as Food", Ed. Georg Borgstrom. Academic Press Inc., New York, pp. 639-680, 1961.

19. Some observations concerning experimental application of some objective quality tests to west coast fish. Canadian Fisheries Reports, No. 1, pp. 27-31, 1961.

20. Tomlinson, N., E.S. Arnold, Eve Roberts and S.E. Geiger. Observations on post-mortem biochemical changes in fish muscle in relation to rigor mortis. J. Fish. Res. Bd., Vol. 18, No. 3, pp. 321-336, 1961.

21. Tsuyuki, H., and D.R. Idler. The metabolism of inositol in salmon. III. The biochemical reactions of 2-C^{14}-myoinositol in coho liver. Canadian Journal of Biochemistry and Physiology, Vol. 39, No. 6, pp. 1039-1042, 1961.

22. Tsuyuki, H., and Eve Roberts. Muscle proteins of Pacific salmon (Oncorhynchus). I. A note on the separation of muscle proteins soluble in low ionic strength salt solutions. J. Fish. Res. Bd., Vol. 18, No. 4, pp. 637-640, 1961.

23. Wood, J.D., and J. Topliff. Dietary Marine fish oils and cholesterol metabolism. 3. The comparative hypocholesterolemic activities of fish oil and vitamin A. J. Fish. Res. Bd., Vol. 18, No. 3, pp. 377-382, 1961.

SUBMITTED FOR PUBLICATION, IN PRESS OR IN PREPARATION

1. Biely, J., J.D. Wood and J.E. Topliff. Excessive amounts of dietary vitamin A on egg production in white Leghorn hens. (Submitted to the Journal of Poultry Science.)

2. Bilinski, E. Biosynthesis of trimethylammonium compounds in aquatic animals. III. Choline metabolism in marine crustacea. (Submitted to the Journal of the Fisheries Research Board of Canada.)

3. Boyd, J.W., and B.A. Southcott. Effect of tetracycline antibiotics on objective and subjective fish quality tests. (Submitted to the Journal of the Fisheries Research Board of Canada.)

4. Buttkus, H., and H.L.A. Tarr. Physical and chemical changes in fish muscle during cold storage. (In press - Food Technology.)

5. March, B.E., J. Biely, F.G. Claggett and H.L.A. Tarr. Nutritional and chemical changes in the lipid fraction of stored antioxidant-treated and untreated herring meals. (Abstract for Proceedings of the FAO Conference on Fish in Nutrition, Washington, D.C.)

6. Nutritional and chemical changes in the lipid fraction of stored antioxidant-treated and untreated herring meals. (In press - Poultry Science.)

7. Claggett, F.G. Packaging air shipments of fresh fish. (In press - Progress Reports of the Pacific Coast Stations, No. 114.)

8. Geiger, S.E., Eve Roberts and N. Tomlinson. Dogfish gelatin. (In press - Journal of the Fisheries Research Board of Canada.)

9. Idler, D.R., P.J. Schmidt and A.P. Ronald. Isolation of 20β-dihydrocortisone from sockeye (Oncorhynchus nerka) plasma. (Submitted to the Canadian Journal of Biochemistry and Physiology.)

10. Jonas, R.E.E., Harcharan S. Sehdev and N. Tomlinson. Blood pH and mortality in rainbow trout (Salmo gairdnerii) and sockeye salmon (Oncorhynchus nerka). (Submitted to the Journal of the Fisheries Research Board of Canada.)

11. McBride, J.R., D.R. Idler, R.E.E. Jonas and N. Tomlinson. Olfactory perception in juvenile salmon. I. Observations on response of juvenile sockeye to extracts of foods. (In press - Journal of the Fisheries Research Board of Canada.)

12. Reiner, E., J. Topliff and J.D. Wood. Hypocholesterolemic agents derived from sterols of marine algae. (Submitted to the Canadian Journal of Biochemistry and Physiology.)

13. Roach, S.W., and J.S.M. Harrison. A new method for unloading fish. (In press - Progress Reports of the Pacific Coast Stations, No. 114.)

14. Schmidt, P.J., and D.R. Idler. Steroid hormones in the plasma of salmon at various stages of maturation. (Submitted to the Journal of General and Comparative Endocrinology.)

15. Smith, M., D.H. Rammler, I.H. Goldberg and H.G. Khorana. Studies on polynucleotides. XIV. Specific synthesis of the $C_{3'}$-$C_{5'}$ interribonucleotide linkage. Syntheses of uridylyl-3($C' \rightarrow 5'$)-uridine and uridylyl-($3' \rightarrow 5'$)-adenosine. (In press - J. Am. Chem. Soc., Vol. 84.)

16. Smith, M., and H.G. Khorana. Preparation of nucleotides and derivatives. (In press - Methods in Enzymology, Vol. 6, Academic Press Inc., New York.)

17. Tarr, H.L.A. Preservation by antibiotics and penetrating radiations. II. (Published in Fishing News International, Vol. 1, No. 2, pp. 46-48, 1962.)

18. Changes in nutritive values through handling and processing procedures. Chapter 6 in "Fish as Food". Ed. Geörg Borgstrom. Academic Press Inc., New York. (In press.)

19. The origin and quantitative distribution of sugars and sugar phosphates in fish muscle post mortem and the role of these in Maillard browning. (For - Proceedings of the 1st International Congress of Food Science and Technology, London.)

20. Food protection by microbial inhibitors. (For - Proceedings, Symposium on Food Protection, Iowa State University Press.)

21. Tarr, H.L.A., and M. Leroux. Acid-soluble phosphorus compounds and free sugars in fish muscle and their origin. (In press - Canadian Journal of Biochemistry and Physiology.)

22. A note concerning the origin and quantitative distribution of acid-soluble phosphorus compounds and free sugars in fish muscle. (In press - Journal of the Fisheries Research Board of Canada.)

23. Thomson, W.A.B., and F.M. Strong. The odor of cheddar cheese. I. The non-acidic compounds (Submitted to Journal of Food Science.)

24. Tomlinson, N., Shirley E. Geiger and Eve Roberts. Frozen albacore tuna. The influence of storage conditions prior to freezing. (In press - Progress Reports of the Pacific Coast Stations, No. 114.)

25. Nucleotide degradation in fish muscle in relation to glycogen concentration and rigor mortis. (Submitted to Journal of the Fisheries Research Board of Canada.)

26. Tsuyuki, H., Eve Roberts and R.E.A. Gadd. Muscle proteins of Pacific salmon (Oncorhynchus). II. An investigation of muscle protein and other substances soluble in salt solutions of low ionic strength by column chromatography. (Submitted to Canadian Journal of Biochemistry and Physiology.)

27. Muscle proteins of Pacific salmon (Oncorhynchus). III. Separation of muscle proteins soluble in low ionic strength salt solutions by starch gel electrophoresis. (Submitted to Canadian Journal of Biochemistry and Physiology.)

28. Wood, J.D. The hypocholesterolemic activity of vitamin A and related compounds as influenced by their chemical structure. (Submitted to the Canadian Journal of Biochemistry and Physiology.)

29. Wood, J.D., and S.A. Haqq. Fat hydrolysis in frozen fillets of lingcod and Pacific gray cod. (Submitted to the Journal of the Fisheries Research Board of Canada.)

STAFF OF THE VANCOUVER TECHNOLOGICAL STATION

INVESTIGATIONAL

Microbiology, Nutrition and Food Uses

Director	H.L.A. Tarr, Ph.D. (McGill; Cantab.), F.R.S.C.
Associate Scientist (Transferred to Defence Research Board effec. Nov. 1/61)	J.D. Wood, Ph.D. (Aberdeen)
Associate Scientist	Burnett A. Southcott, B.S.A. (Brit. Col.)
Associate Scientist	J.W. Boyd, B.S.A. (Brit. Col.)
Assistant Scientist	Hans Buttkus, B.S.A. (Brit. Col.)
Junior Scientist	J.E. Topliff, B.A. (Toronto)
Technician I (From May 15/61)	Margaret R. Leroux, B.S.A. (Brit. Col.)

Biochemistry

Senior Scientist	N. Tomlinson, Ph.D. (California)
Senior Scientist	H. Tsuyuki, Ph.D. (Wisconsin)
Associate Scientist (Transferred from Grande-Rivière Stn. effec. May 1/61)	E. Bilinski, Ph.D. (Louvain, Belgium)
Associate Scientist	J. R. McBride, M.A. (Brit. Col.)
Assistant Scientist	R.E.E. Jonas, B.A. (Madras)
Assistant Scientist	Eve Roberts, B.Sc. (Manitoba)
Junior Scientist	Shirley E. Geiger, B.A. (Brit. Col.)

Chemistry

Principal Scientist (Transferred to Halifax Stn. effec. May 1/61)	D.R. Idler, D.F.C., Ph.D. (Wisconsin)
Senior Scientist (From May 15/61)	Michael Smith, Ph.D. (Manchester)
Associate Scientist (From July 3/61)	W.A.B. Thomson, Ph.D. (Wisconsin)
Associate Scientist	P.J. Schmidt, B.E. (Sask.)
Associate Scientist	U.H.M. Fagerlund, M.Sc. (Åbo, Finland)
Assistant Scientist (From Oct. 26/61)	Barbara S. Findlay, B.A. (Brit. Col.)
Junior Scientist (Resigned Oct. 14/61)	Ann M. Birnie, B.Sc. (Alberta)
Technician 3	A.P. Ronald

Engineering

Associate Scientist	J.S.M. Harrison, B.A.Sc. (Brit. Col.)
Associate Scientist	S.W. Roach, B.A.Sc. (Brit. Col.)
Assistant Scientist	F.G. Claggett, B.A.Sc. (Brit. Col.)
Technician 3	E.G. Baker

Library

Librarian (Technician 1) (Resigned Feb. 24/62)	Lois M. Warren, B.A. (Brit. Col.)
Librarian (Technician 1) (From Feb. 19/62)	Anne Hall, B.Sc. (Wales)

STAFF OF THE VANCOUVER TECHNOLOGICAL STATION
(Continued)

ADMINISTRATIONAL

Director	H.L.A. Tarr, Ph.D. (McGill; Cantab.), F.R.S.C.
Assistant Director (Transferred to Halifax Stn. effec. May 1/61)	D.R. Idler, D.F.C., Ph.D. (Wisconsin)
Administrative Officer 2	P.N. MacLeod
Clerk 4	Nellie E. McBride
Clerk 4	Cecilia L. Robertson
Typist 1	Colleen C. Haskins

MAINTENANCE

Maintenance Supervisor 5	F.C. Freeman
Maintenance Craftsman 4	K.M. McLean
Maintenance Helper	P.E. Enright
Cleaning Service Woman	Agnes M. Hunter

TEMPORARY STAFF

Summer Students

Bitners, I.I. Term April 27 to October 5, 1961. (Chemistry)
Gadd, R.E.E. Term May 1 to September 12, 1961. (Biochemistry)
Gordon, G. Term May 17 to July 17, 1961. (Microbiology)
Grant, G.F. Term May 8 to September 15, 1961. (Biochemistry)
Haqq, S.A., M.S.A. (Brit. Col.). Term May 8 to Sept. 12, 1961. (Microbiology)
Sehdev, H.S., M.A. (Brit. Col.). Term May 15, to Sept. 29, 1961. (Chemistry and Biochemistry)
Wai, Wing, B.Sc. (Brit. Col.). Term May 15 to Sept. 6, 1961 (Microbiology)

Others

Berry, G.H., B.S.A. (Brit. Col.). Working under grant from International Pacific Salmon Fisheries Commission.
Todoroff, T., Ph.D. (Aachen, Germany). Working under grant from International Pacific Salmon Fisheries Commission.
Reiner, E.J., Ph.D. (London). Working under grant from National Institutes of Health, Bethesda, Maryland.