BIOCHEMICAL CHANGES WHICH TAKE PLACE IN FISH MUSCLE DURING STORAGE

by

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INTRODUCTION

PURPOSE

The purpose of this thesis is threefold. Firstly to determine whether bacteria are normally present in the living tissues of healthy haddock,(Melanogrammus aeglifinus). Secondly an investigation of the biochemical changes which take place in haddock muscle during storage, due to enzyme and bacterial action. The third section is concerned with a somewhat unrelated yet timely investigation to determine the value of certain kinds of commercial ice as a means of lowering the temperature of fish.

THE MANNER IN WHICH THE PROBLEMS WERE SELECTED

Many investigators have found that the musculature of normal healthy fish remains sterile until several hours after death. More recent work in this field has led to the conclusion that bacteria may be found in the tissues of, at least, a small percentage of appartently normal fish. After considering the more recent work the author decided that a further investigation of the sterility of fish muscle might contribute something of value.

Several investigators have carried out experiments to determine the role of enzyme action in the decomposition of haddosk muscle. Excepting the work of one investigator, toluene has been used as a disinfectant, to kill or stop further

growth of bacteria in the fish muscle. In view of the fact that certain workers have found that toluene does not inhibit bacterial growth to such a degree as to warrant its use in such a manner, it is difficult to make any conclusion as to the significance of enzymes in the decomposition of haddock muscle. It occurred to the author that, should the haddock muscle prove to be sterile, this sterile muscle might be used in an endeavour to obtain further facts concerning the role of enzymes in the decomposition of haddock muscle.

All previous researches on the problem of bacterial decomposition in fish muscle, as shown by biochemical changes, have been carried out with the use of ground muscle, in flasks. It seemed possible that such methods might give different results from those which would be obtained by taking the muscle from the fish itself, without masceration, as it is required for each observation. It was believed that the latter method would give a more representative picture of bacterial decomposition as it takes place in the dead fish.

The temperature at which fish are held is very important since both enzyme and bacterial action are involved in their decomposition. In many cases a few hours at a temperature that is too high, and such conditions are often brought about by poor iceing, may result in sufficient decomposition to make the difference between a fresh fish and a slightly stale fish. It was believed, therefore, that something of practical value might be learned from an investigation of the refrigeration value of

different kinds of commercial ice, one of which has attained usage only within recent months, as applied to fish.

LITERATURE DEALING WITH THE STERILITY OF LIVING TISSUES

The greater part of the work dealing with the sterility of muscular tissues has been carried out on warm-blooded animals.

Fedor(18), in 1884, injected saprophytic microorganisms into the blood of living animals and was unable to recover them from the blood several days after the injection. He concluded that the blood and organs of warm-blooded animals are able to destroy bacteria, and that the tissues are probably sterile.

Hauser's, (29), work in 1886 would seem to show that the musculature of healthy, warm-blooded animals is sterile.

In 1896, Neisser, (41), using mice, rabbits, and guinea pigs was unable to isolate bacteria from the flesh of the normal animals.

Opitz, (44), in 1898, found that the muscle and a large percentage of the glands, (liver, kidney, spleen and lungs), of freshly slaughtered cattle and calves are sterile.

Selter, (55), in his work of 1906, concluded that the liver, spleen and kidneys of normal animals are sterile.

Messner, although he examined 145 samples of the flesh of numerous freshly slaughtered animals, was unable to find bacteria in any of them.

In 1911, Zwick and Weichel, (80), examined 59 samples of the muscle of cattle and hogs. They found bacteria in only

one sample and concluded that the musculature of healthy animals is sterile.

Grunt, (26), in 1912, found bacteria in only 10 per cent of 540 muscle samples from slaughter animals.

Conradi, (12), in 1909 is the first reference that we have to report results contrary to the work already cited. He examined 150 samples of the muscle, liver, kidneys, lungs, lymph nodes and spleen of slaughtered cattle and hogs and obtained bacteria in 72 of these samples.

Amako, (3), 1910, using the technique of Conradi, examined 22 samples each of the muscle, liver, spleen and kidneys of cattle and obtained bacteria in 48 of these samples. Six, or 27.2 per cent of the muscle samples were found to contain bacteria.

In 1910, Bierotte and Machida, (6), found bacteria in 59 per cent of their samples from the muscle, kidneys, liver and spleen of cattle.

Bugge and Kiessig, (9), 1911, used 241 muscle samples from 66 freshly slaughtered cattle and obtained bacteria from 36 of these samples, or in 54.5 per cent of the animals.

Galippe, (22), in 1918, working with muscle samples from cattle that had been slaughtered only two or three hours, found that bacteria were present in a large percentage of such samples.

Bugge and Kiessig, (10), in 1919, repeated their work of 1911. Their experiments showed that 64 samples, out of a total of 283, contained bacteria.

In 1926 Reith, (49), carried out a very thorough research on the sterility of hog muscle. All of the samples used by Reith were obtained less than one hour after the animal had been slaughtered. Bacteria were obtained from 77 per cent of 216 samples. Cultures of the musculature of healthy living hogs, rabbits and guinea pigs showed the presence of bacteria in 83 per cent of 108 samples. Thirty-eight samples from the blood of these animals were found to contain bacteria in 84 per cent of the cultures taken.

It is difficult to draw any definite conclusion from the results of the above investigators. A number of workers have found that there are no bacteria in the musculature of normal, living animals while just as many research workers have found that bacteria are present in a large percentage of the samples examined. It would appear, however, that the more recent workers have all reported the presence of bacteria in the tissues of normal, living animals. The consensus of present day opinion would, therefore, seem to support the idea that bacteria are normally present in the musculature and tissues of normal, living mammals.

In considering the question of whether or not the muscle of fish is sterile during life we are apparently dealing with with a different problem from that in the case of warm-blooded animals. As has been already pointed out, the evidence, for and against the concept that the living tissues of mammals are sterile is very evenly divided. For fish, on the other hand, the

evidence, almost without exception, supports the idea that the muscle of living fish is sterile.

Browne, (8), in 1918, concludes that fish muscle is sterile and that autolysis, rather than bacterial action, seems to play the most important part in the initial decomposition of fish.

Hunter, (35), in 1920, working with four species of Pacific Coast salmon; the sockeye, (Onchorhynchus nerka), the humpback, (O. gorbuscha), the silver or coho, (O. kisutch), and the chum or dog salmon (O. keta); concluded that both the flesh from the dorsal and ventral sides of the fish is sterile if examined within two hours after the fish is caught.

In 1926, Harrison, Perry and Smith, (28), examined the flesh of eight haddock and found that it was sterile up to three hours after being caught.

Fellers, (19), in 1926, examined the muscle of 252 Pacific Coast salmon and found the flesh of those freshly caught to be sterile. He also found that the stomach and intestines, of those well advanced in their spawning migration, were usually sterile.

In 1930, Stewert, (59), examined 8 codlings. The fish were placed in a sterile box and conveyed to the laboratory as soon as possible after being caught. From these eight codlings 143 cultures were made and in only 5 of these cultures were bacteria obtained. These 5 organisms were found to be similar to those normally found in the slime of fishes and were regarded as accidentical contamination.

Stewart also examined the bacterial flora of the slime, gills, etc., of 19 haddock and was able to isolate a spore forming bacterium of the Mesentericus group, (see Gee), only once.

Gee, (23), (24), 1927 and 1930, is the only worker, to whom we have reference, who concluded that there are bacteria in living fish muscle. He found, in 1927, that he was consistently able to isolate a spore forming rod of the Mesentericus type, from the flesh of freshly caught and spoiled haddock.

In 1930 Gee examined the muscle of the following fish:

Species	No. of individuals
Dogfish	14
Flounder	3
Alwives	4
Mackerel	3
Squirrel hake (Urophycis chuss)	6
Silver hake (Merluccius bilinearis)	1
Pollock	1
Cod	1
Haddock	8

From the above Gee obtained bacteria from the muscle of 2 dogfish, 1 cod and 2 haddock. The number of samples taken from each fish is not given in Gee's paper so that the actual percentage of positive cultures cannot be determined. Supposing, however, that only one sample were taken from each fish, the percentage of positive cultures could not have been greater

than 12 per cent.

LITERATURE CONCERNING THE DECOMPOSITION OF FISH MUSCLE DUE TO ENZYMATIC AND BACTERIAL ACTION

Rusconi found that much more tyrosine was obtained when fish, (tench), were allowed to decompose under anaerobic conditions, than was found under aerobic conditions.

Almy, (2), in 1926, showed that decomposition in herring is chiefly due to trypsin from the pyloric caeca. He found that the flesh of "feedy" fish was also invaded by bacteria. Bursting of the stomach, which is the cause of considerable trouble in catches of small herring, was shown to be due to enzyme action rather than bacterial action.

Reed, in 1926, found that the hydrolysis of fish muscle, in which toluene had been used to inhibit the bacterial action, was at first slow and then increased rapidly. Fish muscle in which bacteria were allowed to develop showed approximately the same amount of hydrolysis for the first sixteen to eighteen hours after which it increased rapidly over that of the toluene muscle mixture.

Reed, Rice and Sinclair, (48) , in 1929, used the toluene method of Reed to follow the hydrolysis of haddock, lobster and clam muscle due to enzyme and bacterial action. They found that after 24 hours the non-coagulable nitrogen had increased 48 per cent in the lobster, 45 per cent in the clam and only 6.4 per cent in the haddock. These values were taken to be representative of the amount of hydrolysis due to enzyme action. Bacterial action was found to increase the amount of protein de-

composition greatly and was shown to be similar, in extent, in the lobster and the clam, but much slower in the haddock.

The freshly mascerated muscle of the haddock had a pH of 6.1 to 6.5 with an average of 6.3. During incubation there was no definate pH change. In the infected muscle the pH reached 7.0 in about 24 hours and only very slowly became more alkaline.

Wynne, in 1929, (78), showed that more hydrolysis took place in fresh muscle to which trypsin had been added, than in smoked, salted or dried muscle which was treated in a similar manner. He was unable to demonstrate that the size of the particles of fish muscle had any affect on the rate of hydrolysis due to enzyme action.

In 1929, (50), Rice found that certain bacteria, which are commonly found on fresh fish, (Achromobacter and Pseudomonas) when grown upon fresh and autolyzed clam muscle, bring about no increase in the amount of non-coagulable nitrogen, but rather, a marked decrease. She concluded that this type of bacteria is able to use only the products of protein hydrolysis and unable to utilize the native protein. In the case of the Bacillus, first a decrease in non-coagulable nitrogen was obtained and then an increase, which would appear to show that Bacilli are capable of splitting native protein.

In 1930, Gibbons and Reed, (25), made determinations on the ammonium nitrogen and non-coagulable nitrogen formed in aseptically collected muscle and in infected muscle. They

found that in the sterile muscle the non-coagulable nitrogen increased slightly during the first 24 hours, followed by a similar increase in 48 hours. The increase in ammonium nitrogen was found to be proportionally greater in the sterile muscle in 24 hours than was the increase in non-coagulable nitrogen. After 48 hours the increase in ammonium nitrogen was noticeably greatthan at the 24 hour period.

The increase in both ammonium nitrogen and non-coagulable nitrogen in the infected muscle was found to be much greater than in the case of the sterile muscle. After 48 hours this difference was much more noticeable than at the 24 hour period.

Wynne, (79), in 1932 found that practically no autolysis took place at temperatures from 30 to 36 deg., F. He found that bacterial decomposition and bacterial growth rate at 30°, F., was about half of that at 36 deg., F.

LITERATURE CONCERNING THE REFRIGERATION OF FISH

Stiles, (60), in an excellent report dealing with the refrigeration of foods, has brought our many important points which are essential to a thorough investigation of the subject. The factors considered by Stiles to be important in the refrigeration of fish are outlined below: internal factors; depending upon the thermal conductivity, specific heat, density, and specific surface of the body to be cooled. Exernal factors; dealing with the temperature, conductivity, specific heat, and density of the cooling medium.

Fish muscle is composed of approximately 80 per cent water and due to that fact the thermal conductivity of fish muscle does not differ appreciably from that of water.

The specific heat of a substance is defined as the quantity of heat required to raise the temperature of a unit mass of the substance 1^{o} C. It is obvious, then, that when dealing with two substances which differ in their specific heat values, the temperature of the substance with the lower specific heat will fall further, under the same identical conditions of refrigeration, than will the temperature of the substance with the higher specific heat.

The rate at which heat is taken from a substance is inversely proportional to the density of that substance. Therefore, the greater the density of the substance to be cooled, the greater the amount of refrigeration required to lower the temperature of that substance to a given temperature.

The specific surface, that is, the amount of surface per unit mass of the substance to be cooled, is an important factor upon which the cooling time always depends. The greater the amount of exposed surface the greater the area from which heat can be taken; similarly, the greater the surface the shorter the distance to the center of the body to be cooled. Under the same conditions of refrigeration substances with large surface areas cool more quickly than those with smaller surface areas, provided that all are of the same material and weight.

The thermal conductivity of the cooling medium is vastly important due to the fact that there are great variations in the thermal conductivities of cooling media. Gases have a low thermal conductivity and therefore carry away a comparatively small amount of heat. Cooling by air is an inefficient process due to the low thermal conductivity of air. Solids, especially those which contain much water, and metals, on the other hand, have a high thermal conductivity. For this reason liquids and metals make the most efficient refrigeration mediums.

Contrary to the case of the body to be cooled, the higher the specific heat of the cooling medium the more efficient is the medium as a refrigerant.

This is explained by the fact that the temperature of that part of the cooling medium immediately surrounding the body to be cooled is raised less, if this medium has a high specific heat, than it is if the specific heat of this medium is low.

The greater the specific gravity of the cooling medium the smaller the rise in temperature of this medium due to the absorption of a given amount of heat. For this reason it is well to choose a cooling medium with a high specific gravity.

Heat must be carried away from a body by conduction, convection, radiation, or some combination of these three factors. When a warm body is placed in a colder medium, heat is withdrawn from this body until the temperature of the whole system becomes alike throughout. Conditions at the surface of the body to be cooled differ from those in the interior of the body. The conductivity of the external, cooling medium governs the rate at which heat is carried away from the surface of the body to be cooled, while the conductivity of the cooled body itself governs the rate at which heat is carried away from the interior of that body.

When a body is cooled from one temperature to another without a change in phase, supposing the temperature to be constant throughout the body at the outset, the temperature gradually falls, but, the rate at which the temperature of the cooled body approaches that of the external cooling medium diminishes progressively.

LITERATURE DEALING WITH PLASTEIN FORMATION

It is generally believed, to-day, that enzymes are capable of synthesizing complex compounds from the hydrolytic products of these compounds. Many enzyme reactions have been shown to be reversible and it is quite possible that all enzyme reactions are reversible reactions.

Danilewski, (14), in 1866, is said to be the first investigator to report the formation of plastein by enzymes, Sawjalow, (54), is said to be the first to give the phenomenon the name of "plastein formation".

Robertson, (51), in 1907, was able to obtain a solid substance from a peptic digest of casein which he named paranuclein formation. He believed this to be an enzyme synthesis.

Taylor in 1907, (62), described the synthesis of the protein protamin through the action of trypsin on the hydrolytic products of protamin hydrochloride. He obtained the trypsin from the livers of the soft shell California clam.

Henrique and Gjaldbak, (31), were able to show that a solid substance, a typical plastein which did not gelatinize, is formed by the action of pepsin and trypsin on peptic and tryptic digests. They found that the amino acid content of these digests decreased during plastein formation while the free ammonia content of the same digests did not change. They, therefore, concluded that ammonia takes no part in the synthesis.

Wasterneys and Borsook, (71), in 1924, confirmed the work of Henrique and Gjaldbak. They concluded that the sub-

stance synthesized by peptic action is of the order of complexity of native protein. They consider peptic activity to be reversible, a linkage being synthesized in concentrated solution which is hydrolysed in dilute solution.

Wasterneys and Borsook, (72), in a later article showed that up to the point of destruction of the enzyme, (pepsin), increasing amounts of protein are formed with increasing temperatures at the optimum pH of 4.0.

In their third paper on the subject Wasterneys and Borsook, (73), showed that the amount of protein formed in a pepsin synthesis is partially dependent upon the concentration of the enzyme.

In 1925, Wasterneys and Borsook, (74), showed that no synthesis takes place when the concentration of the enzyme lies between 4.0 and 0.05 per cent. The addition of synthesized protein or of native protein to the solution of the digest and pepsin was found to inhibit the synthesis as would be expected according to the laws of mass action.

Rona and Chrometzka, (52), in 1927, found that when a protein splitting enzyme is incubated with its hydrolysate a plastein is formed and that there is a corresponding decrease in amino nitrogen. They are of the opinion that no true protein synthesis takes place since they found that ammonia increases simultaneously with the increase in amino nitrogen. They believe, therefore, that the decrease in amino nitrogen is due to the deaminization of the amino acids. In their experiments the

number of carboxyl groups was found to remain unchanged.

Cuthbertson and Tompsett, (13), found that there is no appreciable liberation of ammonia during the formation of plastein. They showed that there is a corresponding decrease in the amino and carboxyl groups of the amino acids during the process of plastein formation. Their work, like that of Henriques and Gjaldbak's , would tend to nullify the results of Rona and Chrometzka.

The consensus of scientific opinion appears to support the idea that enzyme syntheses of protein do take place. In view of the ease with which certain carbohydrates,(b-methyl-glucosides, etc.) and esters, (butyl acetate, etc.) may be synthesized in the laboratory with the help of specific enzymes, it seems logical to conclude that protein syntheses are just as possible when optimum conditions for temperature, enzyme concentration, substrate concentration, pH, etc., are afforded.

LITERATURE DEALING WITH THE USE OF TOLUENE AS A DISINFECTANT

In 1912, Wells and Cooper, (75), showed that spore forming bacteria, such as Bacillus subtilus, held in pure toluene, were still viable after a period of 10 days.

Benians, (4), in 1913, stated that the tubercle bacillus is killed if shaken for 1 hour in a solution containing 10 per cent of toluene. B. coli was killed in 4 hours by the same method. It required 17 days of this treatment to kill Staphlococcus aureus.

Dewitt and Sherman, (15), in 1914, found that toluene has only slight disinfecting powers.

Waksman and Davidson, (69), state that toluene is a good disinfectant to use as a means of inhibiting bacterial growth during enzyme hydrolyses provided that the containers used in the process are sterile.

Parker, (45), in 1928, found toluene to be useless as a disinfectant to inhibit bacterial growth during enzyme hydrolyses due to the fact that the enzyme preparations were heavily contaminated with different types of bacteria.

Reed, in 1926, and Reed, Rice and Sinclair, (48), in 1929, used toluene as a means of inhibiting bacterial growth in ground fish muscle. They found that while all the bacteria were not killed by this method, there was no great increase in the number of bacteria during several days incubation.

LITERATURE CONCERNING THE EFFECT OF LOW TEMPERATURES ON ENZYMES

According to Hepburn, (32), enzymes survive low temperatures and are able to carry out their usual hydrolyses when the temperature is raised the right amount and other conditions are favorable. He kept lipase for 89 months at a temperature of -9.4° to -12° C., pepsin, trypsin, and rennin at a temperature of -191° C., for 45 minutes or more and was unable to show that there was any inactivation of these enzymes.

LITERATURE DEALING WITH THE ASEPTIC METHODS OF ISOLATING FISH MUSCLE

In 1926, Hunter, (35), used the following method to isolate fish muscle aseptically. The body of the fish was thoroughly washed in alcohol and the alcohol was burned off. With instruments sterilized by flaming in alcohol, a small flap of skin just posterior to the dorsal fin was carefully lifted and pinned back. A portion of muscle weighing approximately one gram was cut out with a sterile scalpel and transferred to a sterile flask. Sterile glass and sterile physiological salt solution were then added and the whole shaken until the muscle was disintegrated. The suspension of tissues was then diluted and plated out. Samples of flesh were taken from the belly in the same manner.

Harrison, Perry, and Smith, (28), in 1926, used a method which differed considerably from that of Hunter. The room was shut up during operations. Tables were washed with a 1/1000 solution of mercury bichoride, clean aprons were worn by the operator and the hands and arms of the operator were washed with alcohol or mercury bichoride solution. All instruments and utensils were sterilized with flaming alcohol. The fish was wiped with alcohol and flamed. It was then placed on the table and the skin was cut and fastened back. 5 grams of the flesh were then removed to a sterile mortar, 5 grams of sterile sand Were added and the flesh was mascerated with a sterile pestle. 90 cc. of water were now added and carefully mixed with the

muscle. Plates and dilutions were made from this material.

Gee, (23), in 1930, used live fish brought to the laboratory in a tub of salt water. The fish were stunned with a blow on the head. The area from which the sample was to be taken was charred with a blowtorch and removing the burned surface tissues, pieces of muscle about 1/10 grams in weight were cut out with sterile scalpels and transferred to 5 cc. portions of sterile fish broth in tubes. Counts were made from the incubated broth tubes on fish agar.

Gee first sterilized his instruments by dipping them in alcohol and then burning the alcohol off, he found, however, that this treatment did not always sterilize the instruments and was forced to abandon this method.

Stewert, (59), in 1930, had his fish transported to the laboratory in a sterile box shortly after they were caught. The exterior of the fish was sterilized with a solution of 0.5 per cent crystal violet and 0.5 per cent brilliant green in 50 per cent alcohol. The fish were immersed in this solution for 20 minutes and then removed and allowed to dry slightly. An incision was then made close to the vertebral column with a sterile scalpel and a flap of tissue was turned back. A portion of flesh, approximately 1 gram in weight, was removed to sterile dilution water in tubes which also contained broken glass. Dilutions and plates were made from this muscle mixture. The procedure of isolating the muscle was carried out in a glass box to prevent air-borne contamination.

LITERATURE CONCERNING METHODS TO DETERMINE THE BIOCHEMICAL CHANGES IN PROTEIN SUBSTRATES

The Van Slyke Method. In 1911, Van Slyke, (63), described a method to determine proteolytic products which is, today, commonly known as the Van Slyke method to determine amino nitrogen. This method depends upon the reaction of primary aliphatic amines with nitrous acid as shown by the following equation: $R.NH_2 + HNO_2 = H_2O + N_2$. The nitrogen formed leaves the system in gaseous form and the reaction therefore proceeds from left to right.

In order to carry out the Van Slyke determination a complicated apparatus is used. Firstly glacial acetic acid and secondly a 30 per cent aequous solution of sodium nitrate are measured into the apparatus in definite amounts. This forms nitrous acid which, in solution, spontaneously decomposes to form nitric acid and nitric oxide. $2\ HNO_2 = HNO_2 + NO$. The latter reaction is utelized to displace all the air in the apparatus. The deaminizing bulb is now shaken until the liberated nitrous oxide forces the level of the reacting solution back to a calibration on the deaminizing bulb and by closing a stop-cock, the sodium nitrate-acetic acid mixture is held at this level. The unknown amino acid solution is then introduced, with the result that nitrous oxide and nitrogen gas is evolved. The deaminization bulb is shaken, always for the same period of time, to facilitate the action of the nitrous acid upon the amino acid. After deaminization, the nitric oxide is absorbed by an alkaline

permanganate solution and the pure nitrogen gas is thereafter measured by means of a special gas burette.

Since proteins are composed of different amino acids, all of the amino acids of which a protein is composed are not primary amino acids. Now nitrous acid reacts more slowly on amino acids which are not primary acids, that is, on amino acids which have an amino group in some other position than the alpha. Lysine, $CH_2(NH_2).CH_2.CH_2.CH_2.CH(NH_2).COOH$, for instance, requires thirty minutes to react completely with nitrous acid while ordinary primary aliphatic amino acids require only three minutes to complete this reaction. It is probable that the decomposition products of fish muscle would not be made up of primary amino acids alone. Nevertheless, if the same deaminization time is used for all determinations, the results obtained should be comparable, since we are dealing with the same type of protein in all cases.

Pure proteins, egg albumin, edestin, etc., also react with nitrous acid but the amount of nitrogen evolved in each case is only a very small fraction of that contained in the protein molecule. Soluble proteins in fish muscle, then, would have little effect upon the result obtained.

Ammonia reacts with nitrous acid to give nitrogen gas. Ammonia is said to require from one and one-half to two hours to react quantitatively. In the case of fish muscle where the decomposition is far advanced, ammonia would be present in large quantities and might be the source of some error.

Urea, methyl amine and other simple amines require long periods to react quantitatively with nitrous acid so that it is probable that they would be of little significance in proteolytic determinations made upon fish muscle.

Van Slyke concluded that the nitrous acid method is appropriate to follow the quality and extent of proteolysis.

In 1911, Van Slyke, (64), used the above method to determine the amount of proline in amino acid mixtures and found the method to be very satisfactory for this purpose.

Van Slyke and White, (65), used the Van Slyke method to follow the digestion of protein in the stomach of the dogfish. They concluded that their results were not absolutely accurate due to the fact that much urea is present in the stomach of the dogfish. The error involved, however, was believed to be small enough as to have little affect upon the results.

Following the suggestion of Van Slyke, White and Crozier, (73), used the Van Slyke method to follow the digestion of boiled beef, boiled cod and bolied dogfish muscle, with trypsin. They obtained concordant results and found the method to be excellently suited to this type of work.

In 1912, Van Slyke, (66), suggested that certain modifications be made in the apparatus and that ammonia should be removed from proteolysed solutions before the determination is made.

In 1912, Van Slyke, (67), suggested that still further changes be made in the apparatus and at the same time introduced the micro apparatus which uses only 1 or 2 cc. of the unknown

solution instead of the 10 cc. used by the macro apparatus.

Sturges and Rettger, (61), found that the Van Slyke method yields valuable results provided that no reliance is placed on a single determination. They stated that at least two determinations should always be made.

In 1922, Bushnell, (11), found that the results of Van Slyke determinations, made on a substrate which had been broken down by a saprophytic anaerobe, were thrown off by the presence of large amounts of ammonia. Their work showed that different amounts of nitrogen are evolved at different temperatures when the same amount of ammonia is present.

Dunn and Schmidt, (17), in 1922, found that in Van Slyke determinations the rate of deaminization of the alpha and gamma amino groups of amino acids is markedly influenced by temperature.

De Bord, (16), in 1923, was unable to obtain control analyses on a one per cent peptone solution using the micro Van Slyke apparatus. The failure to obtain consistent control analyses was found not to be due to the apparatus or reagents used, since control blanks were always obtained.

Morrow concludes that the Van Slyke method is limited in its application to pure proteins, solutions of proteins, or proteins free from carbohydrates, fats, fibers, etc.

Wilson, (77), in 1923, drew attention to a statement by Northrop to the effect that the Van Slyke method is more accurate than the formol titration method for absolute deter-

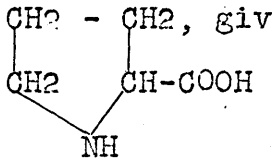
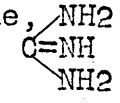
minations of amino acids.

In 1924, Wagner, Dozier and Meyer, (63), found that the Van Slyke method is sufficiently accurate to follow the amount of amino nitrogen formed by bacteria in 2 per cent peptone cultures.

From the above literature cited, it is clear that all research workers do not agree that the Van Slyke method is the best method by which to determine the amount of proteolysis in protein solutions. The majority of workers, on the other hand, seem to believe that the Van Slyke is a very accurate method of determining the extent of proteolysis.

The Formol Titration. In 1908, Sorensen, (55), introduced the formol titration as a means of titrating amino acids. In this type of titration the free amino nitrogen of the amino acids is allowed to act with formaldehyde to form a methyl derivitive. The acidity of the carboxyl group is then neutralized with a standard alkali solution. The theory behind this titration, at this time, was that the carboxyl group is released from the neutralizing powers of the free amino group, since the amino group is bound by the formaldehyde, and is therefore free to be titrated. $R.\underset{\underset{COOH}{|}}{CH}.NH_2 + OHCH = R.\underset{\underset{COOH}{|}}{CH}.NCH_2 + H_2O$

Jodidi, in 1918, (37), called attention to certain abnormalities in the formol titration. He showed that the formol titration yielded accurate results only in the case of amino acids which have amino, (NH_2), and carboxyl, (COOH), groups in

their molecule. Amino acids which have an imino group, (NH), in their molecule, such as proline,  which is too low when titrated by the Sorensen method. Irregular results were also obtained with amino acids which contain phenol or guanidine,  groups in their molecules in addition to the regular amino and carboxyl groups. Too low results were obtained with diamino acids such as lysine, $CH_2(NH_2).CH_2.CH_2.CH_2.CH(NH_2).COOH$.

In 1919, Brickner, (7), noted that amino acids, which in aequous solutions are nearly neutral to phenolphthalein, are distinctly acid in alcoholic solutions.

Foreman, (21), in 1920, confirmed the results of Jodidi and was further unable to obtain quantitative results with the formol titration when using certain monoamino monobasic acids.

Foreman, following the findings of Brickner, developed the following titration which he found to be the most efficient in estimating the acid value of amino acids: (1) A portion of an aequous solution of the amino acid mixture is titrated with N/10 aequous sodium hydroxide, using phenolphthalein as an indicator. (2) A similar portion is titrated with N/10 alcoholic potassium hydroxide in the presence of 85 per cent, by weight, of alcohol. (3) Neutral formaldehyde is added to the alcoholic titration mixture and the solution is titrated to the

same endpoint with alcoholic potassium hydroxide. Phenolphthalein is used as an indicator in both stage 2 and stage 3.

Harris, (27), in 1923, pointed out that the idea that the amino group is bound by the formaldehyde, in the formol titration did not give a proper explanation of the facts since the amino group of amino acids acts as a base only in acid solutions while the carboxyl group acts as an acid only in alkaline solutions. In aequous solutions the carboxyl groups are neutralized at pH 11.75 while in the presence of formaldehyde neutralization takes place at about pH 8.70. He concluded that the formol titration depends upon the formation of methyl amino derivitives which have a dissociation constant about 1000 times greater than that of the amino acids from which they are derived.

Harris showed that the acid value of amino acids can be accurately estimated by either of the following methods: (a) By titrating in the presence of 80 per cent, by volume, of ethyl alcohol and 5 per cent, by volume, of neutral formaldehyde, using N/10 sodium hydroxide to neutralize the acid and phenolphthalein as an indicator. (b) By titrating to a blue color with N/10 sodium hydroxide in the presence of 80 per cent by volume, of ethyl alcohol, using thymophthalein as an indicator.

Folin, (20), in 1922, used a colorimetric method to determine the amino acids in blood and in urine. The samples are made alkaline with a sodium carbonate solution after which a dedefinite amount of a solution of the sodium salt of betanaphthoquinone sulfonic acid is added. The mixture is allowed to stand

for from 19 to 30 hours after which acetic acid and sodium thiosulfate are added, the sample is then diluted and compared colorimetrically against a known standard.

De Bord, (16), utelized the method of Folin successfully to study the nitrogenous metabolism of certain bacteria.

In 1924, (70), Wasterneys and Borsook, used the following methods to determine the products of protein hydrolysis: The total nitrogen of the hydrolysate is first determined. 10 cc. of a 10 per cent solution of trichloracetic acid are then added to 40 cc. of the hydrolysate. After standing for 1 hour the solution is filtered and the total nitrogen is determined. The difference between the values of the first and the second total nitrogen determinations shows the quantity of protein and metaprotein nitrogen. The filtrate is then boiled for three hours to decompose the trichloracetic acid and then made up to its original volume. 20 grams of sodium sulfate are now added and the solution is left at 33° C., for $\frac{1}{2}$ hour. This precipitates the proteose. The solution is then filtered and another total nitrogen determination is made to obtain the value of the proteose nitrogen. The peptone is now precipitated with tannic acid and another total nitrogen determination is made, the difference between the last two total nitrogen determinations is the value of the peptone nitrogen.

These methods are probably the most accurate of any for the determination of decomposition products, they are, however, far too long to be of any use in a research where time is

at all limited.

Rona and Kleinmann, (53), in 1924, used the Nephelometer to follow the rate of disappearance of casein in a tryptic digest. This method is applicable only when turbid solutions are being used.

Northrop and Hussey, (43), used a viscometer to follow the hydrolysis of gelatin by trypsin and pepsin. This method can be used only with viscous solutions.

Reed, Rice, and Sinclair, (48), in 1929, used the method of Allen and Davidson, (1), to determine the amount of ammonia produced by enzyme and bacterial action in fish muscle. In this method the samples are weighed out into 100 cc. volumetric flasks, the volume is made up to 1000 cc. with distilled water, the flask is allowed to stand for 1 hour. After this time a definite portion is pipetted off into a 100 cc. volumetric flask. 10 cc. of Nessler's solution are then added and the volume is made up to 100 cc, with distilled water. The solution is then compared colorimetrically with a known standard and the value tabulated. The authors found this method to give good results and to be easily carried out.

Non Coagulable Nitrogen. Along with the ammonium nitrogen test, Reed, Rice and Sinclair used the non-coagulable nitrogen determination. The protein of the hydrolysate is precipitated with tannic acid (9 parts of tannic acid to 1 part of glacial acetic acid). The precipitate is filtered off and a Kjeldal determination is made on the filtrate to obtain the value of the

non-coagulable nitrogen. This method is considerably longer than the ammonium nitrogen method and it seems to give no better results.

Volatile Basic Nitrogen. In 1932, Hess, (30), used the volatile basic nitrogen determination as a means of following decomposition in fish muscle at different temperatures. This method is said to give a value for ammonia and volatile nitrogenous bases. 3 to 10 grams of muscle are ground and transferred with 20 cc. of distilled water to the distilling flask. The sample is then distilled with 80 cc. of alcohol after liberating the ammonia with MgO. 50 cc. of the distillate is collected in N/10 HCl and the excess HCl is titrated with N/50 NaOH. Hess found this method to be very dependable and excellent for this type of work.

The Electrometric Titration Method. In 1933, Lemon and Stansby, (37), worked out the electrometric titration method for the detection of relative freshness of haddock muscle. This method depends upon the theory that enzymatic, or primary changes, which take place in fish muscle, can be detected by titrating with 0.0165 N. HCl from a pH of 6.0 to a pH of 4.3. Enzyme action breaks down the protein molecule, the protein molecule acts as a buffer between the pH values of 6.0 and 4.3, therefore the greater the amount of enzyme action the greater the disappearance of protein molecule, and consequently, the smaller the amount of buffer action between pH 6.0 and pH 4.3. The more acid used, then, between the indicated pH range the better the

fish since this indicates much buffer action and denotes that little of the protein has been disintegrated by enzyme action.

In order to determine the amount of bacterial decomposition or secondary change, that takes place in fish muscle the electrometric titration method uses 0.0165 N. HCl to titrate the fish muscle to a pH of 6.0. The value obtained indicates the amount of basic products present in the fish muscle and hence the amount of secondary decomposition.

In order to carry out the electrometric titration the muscle is ground and 5 grams are transferred to a 150 cc. bottle, 70 cc. of distilled water are added and the bottle is shaken for 10 minutes. The contents of this bottle are transferred to a 250 cc. flask, the bottle is rinsed with 30 cc. of distilled water which is also transferred to the Erlenmeyer flask and an excess of quinhydrone is added. The flask is now shaken for 2 minutes after which its contents are transferred to a 250 cc. beaker. The titration is then made as outlined above, using the 0.0165 N. HCl. A qiunhydrone electrode is used to determine the pH.

Lemon and Stansby used the electrometric titration method on numerous haddock. The haddock were brought to the laboratory a few hours after they were caught, determinations were then made on fish held in ice water, packed in ice, and held at various temperatures. The A value (measure of enzyme action) was found to corredpond very colsely for different fish held at the same temperature for the same length of time. The B

value (measure of bacterial decomposition) was found, as the A value, to give good results.

Lemon and Stansby conclude that a fresh haddock probably has a B value between 7 and 8 (cubic centemeters of 0.0165 N. acid used) when first caught and that this value falls to 5 when lactic acid forms if no pronounced bacterial decomposition sets in. Any value less than 8, then, for B is an indication of little or no bacterial decomposition. If the value exceeds 8 suspicion is raised that the fish, at some previous time, was allowed to stand at too high a temperature. Haddock with a B value of 12 or greater is said to be stale. The amount of primary change is said to be pronounced when the A value is less than 18 and extreme when the value is less than 16.

The Biuret Method. Nickerson, (42), in 1932 showed that the biuret test is quantitative for pure proteose or pure peptone fractions. This test is carried out by adding caustic soda and dilute copper sulfate solution to the protein fraction. A violet color is given with compounds which contain two CO-NH groups in the molecule. This test is of little use to determine proteolytic products since protein and metaprotein must be precipitated before it can be carried out.

EXPERIMENTAL

METHODS USED TO DETERMINE WHETHER OR NOT BACTERIA ARE PRESENT IN FISH MUSCLE AND IN FISH BLOOD

The author was unable to make arrangements whereby live fish could be brought to the laboratory. The nature of this research necessitated that the fish should arrive at the laboratory before any biochemical changes had taken place in the muscle, and also before the bacteria of the slime, gills, etc., had an opportunity to penetrate into the muscle of the fish. It was believed, provided that the fish were quickly frozen, immediately after being caught and kept in the frozen state until they arrived at the laboratory, that the required conditions would be fulfilled. It was also believed that the frozen condition of the muscle would facilitate the ease with which the fish muscle could be removed aseptically.

Muscle Samples. The fish, melanogrammus aeglifinus, three haddock and three scrod, (small haddock), were caught on a beam trawler. As soon as they were caught they were thrown into an ice-box containing "dry ice" (solid CO_2). which has a temperature of $-70^{o}C$. They were transported to the cold storage plant in the frozen condition. They were stored in the cold storage plant at 0^{o} F., for from 1 to 4 weeks until they were used. The fish,were then brought to the laboratory in the frozen state. The temperature of the muscle was approximately 0^{o} F at this time. An area of the skin was first stripped from the

flesh, a flap of the muscle was then cut away from the surface of the muscle with a sterile scalpel and a portion of the frozen muscle, approximately 5 grams in weight, was cut out with a sterile gouge. The muscle was then transferred to a wide mouth Erlenmeyer flask containing 50 cc. of sterile dilution water and broken glass. The flask was stoppered with a sterile rubber stopper and then shaken in a mechanical shaker for 15 minutes in order to disintegrate the muscle. Dilutions were made from this mixture. 1 gram samples were removed from the same area of the fish and placed directly in broth tubes and petri dishes wihtout disintegrating the muscle.

The scalpels and gouges used were sterilized by flaming thoroughly with a Bunsen burner, cooling in 95 per cent alcohol and burning off the alcohol.

Samples Taken. Portions of flesh were taken from near the head on each side of the fish, from the middle portion on each side of the fish and from near the tail on each side of the fish when the haddock were used. A total number of 90 samples were taken from three haddock.

The scrod were small enough so that the portions of muscle were taken only from the middle section of the fish on each side. 30 samples were, therefore, taken from the scrod.

Dilutions. Dilutions were made from the muscle mixture in the wide mouth 100 cc. Erlenmeyer flask. All dilutions were covered by duplicate agar plates and broth cultures. The dilutions were carried up to 1/1,000,000 in order to rule out

any antibodies from the muscle which might have an inhibiting effect on the growth of bacteria.

Medium. Fish broth and fish agar, as used by Gee, (23), were used as a medium of growth for bacteria from fish muscle. 500 grams of the ground haddock muscle were added to 1000 cc. of tap water. This mixture was stirred while cold for 1 hour. The mixture was then heated for 20 minutes until it came to a boil and then boiled for 5 minutes. This was filtered and the filtrate was autoclaved at 15 pounds pressure for 20 minutes to bring down the remaining coagulable protein. After the second filtration the medium was tubed and sterilized. Solid media was made by adding 1.5 per cent agar-agar, filtering the resulting mixture and sterilizing.

All sterilizations by autoclave were carried out at 20 pounds pressure for a period of 20 minutes.

Incubation Period Of Cultures. All cultures, both agar plates and broth tubes, were incubated for 168 hours at 20° C.

RESULTS
Table 1.

Haddock No. 1.

Head Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,1mold	0,0	0
1/1000	0,0	0,0	1 bacterium
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Head Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Middle Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 1.

Haddock No. 1.

Middle Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	1 bacterium,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,1 mold	0
1/100	0,0	0,0	0
1/1,000	0,1 bacterium	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 2

Haddock No. 2.

Head Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,1 bacterium	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Head Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	1 bacterium
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,1 bacterium	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Middle Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Plates	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 2

Haddock No. 2.

Middle Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0, bacteria	0
1/10	4,1 (bacteria)	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 3

Haddock No. 3.

Head Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Head Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Middle Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 3

Haddock No. 3.

Middle Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 1.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tail Portion Side No. 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Table 4

Scrod No. 1.

Middle Portion Side No. 1 and 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Scrod No. 2.

Middle Portion Side No. 1 and 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Scrod No. 3.

Middle Portion Side No. 1 and 2.

Bacteriological and Mold Count

Dilution	Agar Plates	Broth Tubes	Control Plates
0	0,0	0,0	0
1/10	0,0	0,0	0
1/100	0,0	0,0	0
1/1,000	0,0	0,0	0
1/10,000	0,0	0,0	0
1/100,000	0,0	0,0	0
1/1,000,000	0,0	0,0	0

Tests Made to Determine the Sterility of Muscle Used to Determine Proteolysis. Excepting the first week, a small piece of muscle was taken from each portion of muscle used and placed aseptically in sterile, nutrient broth before proteolytic tests were made at the start of each day's run. Agar plates were made from these broth tubes at the end of the 120 period, thus the presence of any infected muscle was indicated. During the first week, a sterile loop was drawn over the surface of the muscle in the flasks and then streaked over agar slants. This method was discontinued thereafter due to the fact that the above method was preferable. Three broth tubes from the last weeks work were incubated anaerobically.

Table 5

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
4	24 hours	0
4	48 hours	0
4	72 hours	0
4	96 hours	0
4	120 hours	0

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
5	24 hours	0
5	48 hours	bacteria
5	72 hours	0 present
5	96 hours	0
5	120 hours	0

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
6	24 hours	0
6	48 hours	0
6	72 hours	0
6	96 hours	0
6	120 hours	0

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
7	24 hours	0
7	48 hours	0
7	72 hours	0
7	96 hours	0
7	120 hours	0

Table 5

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
8	24 hours	0
8	48 hours	0
8	72 hours	0
8	96 hours	0
8	120 hours	0

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
9	24 hours	0
9	48 hours	bacteria
9	72 hours	0 present
9	96 hours	0
9	120 hours	0

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
10	24 hours	0
10	48 hours	0
10	72 hours	0
10	96 hours	0
10	120 hours	bacteria present

Haddock Number.	Sterile Muscle Sample	Bacteria found in Broth Tubes
11	24 hours	0
11	48 hours	0
11	72 hours	bacteria
11	96 hours	0 present
11	120 hours	0

The Counts on Fish Blood. Blood specimens were taken from two silver hake (Merluccius Bilinearis) and one squirrel hake (Urophycis chuss). The samples were taken on the fishing ground and as soon as the fish were taken from the water they were wiped off along the ventral portion, covered with 95 per cent alcohol in this region and the alcohol burned off. An incision was then made from the anus to the gills with sterile scissors. A sterile hypodermic needle was then inserted into the still beating heart and a $\frac{1}{2}$ cc. sample of blood was drawn off into the sterile syringe. The blood was then transferred to 5 cc. of sterile physiological salt solution.

Several hours later, when the blood samples arrived at the laboratory cultures were made from them. 1 cc. of the 1/10 blood dilution was used to make higher dilutions while duplicate nutrient agar plates and duplicate nutrient broth cultures were made from the remaining 4 cc. Dilutions up to 1/1,000,000 were made on all three blood samples and nutrient agar plates and nutrient broth cultures were made on these dilutions in duplicate. One half of all the broth tubes were incubated anaerobically. All cultures were incubated for 96 hours at 25 degrees, C., before observations were made.

Table 6

Squirrel Hake No. 1.

Blood Sample

A

Dilution	Agar Plates	Anaerobic	Aerobic	Control Plates
0	0,0	0	0	0
1/10	0,0	0	0	0
1/100	0,1 bacterium	0	0	0
1/1,000	0,0	0	0	0
1/10,000	0,0	0	0	0
1/100,000	0,0	0	0	0
1/1,000,000	0,0	0	0	0

Silver Hake No. 1.

Blood Sample

B

Dilution	Agar Plates	Anaerobic	Aerobic	Control Plates
0	0,0	0	0	0
1/10	0,0	0	0	0
1/100	0,0	0	0	0
1/1,000	0,0	0	0	0
1/10,000	0,1 mold	0	0	0
1/100,000	0,1 mold	0	0	0
1/1,000,000	0,0	0	0	0

Silver Hake No. 2.

Blood Sample

C

Dilution	Agar Plates	Anaerobic	Aerobic	Control Plates
0	0,0	0	0	0
1/10	0,0	0	0	0
1/100	0,0	0	0	0
1/1,000	0,0	0	0	0
1/10,000	0,0	0	0	0
1/100,000	0,0	0	0	0
1/1,000,000	0,0	0	0	0

METHODS USED TO DETERMINE THE PROTEOLYTIC CHANGES THAT TAKE PLACE IN FISH MUSCLE DURING STORAGE

The Fish Used. The fish used in these experiments were haddock. The haddock were placed in an ice-box containing "dry ice", (solid carbon dioxide), as soon as they were caught. After the fishing boat had arrived in Boston Harbor with the ice-box, the fish were transported to a cold storage plant, while still frozen. The fish were stored at 0° F., until they were taken to the laboratory to be used.

The Method of Isolating Sterile Muscle. Since the toluene method of Reed was not used in this research, it was necessary to isolate portions of the fish muscle in such a manner that they would not be infected. These portions of muscle were all taken from the same side of the fish as soon as the fish had arrived at the laboratory. The fish being in the frozen state, an area of the skin was first stripped away from the fish. A flap of muscle was then cut away from the surface of the flesh with a sterile scalpel and a piece of the muscle, about 15 grams in weight, was taken from this area with a sterile gouge. The gouge was sterilized, in each case, by heating it thoroughly in the flame of a Bunsen burner, cooling it in 95 per cent ethyl alcohol and burning off the alcohol. The portion of muscle was then, with the aid of sterile forceps, transferred to a sterile flask. After six to eight of these portions of muscle had been taken from the same side of the fish, the cotton plugs, of the flasks containing the flesh, were cov-

ered with lead foil to prevent evaporation of water from the muscle. The flasks were then placed in the refrigerator at which temperature they were to be incubated.

This aseptically removed fish muscle was that upon which proteolytic tests were made in order to follow decomposition due to enzyme action.

The Infected Muscle. Since the author wished to have the conditions under which the experiments were conducted as nearly natural as possible, the infected muscle was not ground up and placed in flasks. The fish, after the sterile muscle had been taken from it, was intact on all but the one side. The intestines were now removed from the fish, the gills being left undisturbed. The fish was then left at room temperature until the muscle had thawed, after which it was placed in a metal tray in the refrigerator with the sterile samples.

A portion of infected muscle, about 15 grams in weight, was removed daily from the intact side of the fish. On this sample, the regular daily determinations were made. The infected muscle was removed from the fish by taking a portion, about 1 cm. in width, running from just below the range of the anal fins to one inch below the pectoral fins. The first sample was always taken on the dorsal side of the fish, thereafter they were taken at a distance of about 1 cm. from the previous sample, always further towards the ventral side. The fact that these samples were taken a distance of 1 cm. apart excluded the possibility of excessive bacterial contamination.

Temperatures at Which the Fish were Incubated. The incubation period lasted for 120 hours for all fish excepting those which were incubated at + 15° C., or above. The latter were incubated only for 96 hours, due to the fact that they were far along in the stages of decomposition at the end of this time.

Each fish was incubated at a definite temperature. The incubation temperatures were as follows: First and fifth week 0° C., second and sixth weeks + 5° C., third and seventh weeks + 10° C., fourth week + 17° C., eighth week + 15° C., 17° C., was used instead of 15° C., for the fourth week because of the fact that the electric refrigerator was out of order and it was necessary to use an ordinary ice box. The temperature of these refrigerators was found to vary not more than + or - 0.5° C., from that of the stated incubation temperatures.

Proteolytic Determinations Used During the First Four Week Period. The determinations for proteolysis made daily on the fish samples were identical during the first four week period. Identical determinations were made daily on both the infected and the sterile muscle. All of the determinations used, excepting the Van Slyke method, and the electrometric titration method, were chosen because of their general reliability and because of the ease in which they can be carried out.

The Van Slyke Method. 5.0000 grams of fish muscle were weighed out, in small pieces, on a cover glass. They were then transferred to a 100 cc., wide mouth Erlenmeyer flask con-

taining broken glass. 25 cc. of distilled water were added and the flask was stoppered with a rubber stopper. After shaking the flask by hand for 10 minutes to disintegrate the muscle the contents were poured into a beaker. The flask was then rinsed with 25 cc. of distilled water and this was added to the above beaker. The muscle-water mixture in the beaker was now filtered through an ordinary 24 cm. folded filter, the mixture was allowed to filter for one hour. Both the filtering funnel and the filtrate were refrigerated with ice water during and after the filtration in order to prevent further decomposition due to enzyme action. After filtering, determinations were made for aliphatic amino nitrogen using the micro Van Slyke apparatus. 1 cc. of the solution was used for each determination. Three blanks were run on the apparatus for each unknown estimated. No blank values were accepted which gave more than 0.300 cc. of gas. No values were accepted, either in the case of the blanks or the muscle filtrate, which did not check within 10 per cent of each other, that is, the value of the lowest and that of the highest did not differ from one another by more than 10 per cent. Three determinations that checked within 10 per cent of each other were obtained for all blanks and all unknowns. The average of three determinations was taken to be the true value for each blank and each unknown muscle sample.

The Electrometric Titration Method. 5.0000 grams of fish muscle were weighed out in small pieces on a watch glass. The muscle was then transferred to a wide mouth 100 cc. Erlen-

meyer flask containing broken glass, 25 cc. of distilled water were added and the flask was shaken by hand for 10 minutes to disintegrate the muscle. The mixture was then transferred to a narrow mouth 250 cc. Erlenmeyer flask, the first flask was washed out with 75 cc. of distilled water which was transferred to the mixture in the 250 cc. flask. An excess (more than 0.3) grams of quinhydrone were added to the muscle-water mixture and the flask was shaken for two minutes. The contents of the flask were now poured into a 250 cc. beaker. The Electrodes of the quinhydrone pH indicator were placed in the beaker with the fish muscle, the standard cell of the indicator was adjusted until the potentiometer showed no deflection, the millivolt scale was then adjusted until the galvanometer showed no deflection and a reading was taken. Successive portions of 0.0165 N. HCl were then added from a burette until the galvanometer showed no deflection when the millivolt scale stood at 0.100 (E=0.100), 0.140, 0.170, and 0.200. During and after the addition of the acid the contents of the beaker was thoroughly stirred and time was allowed for an equilibrium to be reached between each addition of acid. Readings were taken from the burette when equilibrium had been reached at each of the above E. M. F. values.

Proteolytic Determinations Used During the Second Four Week Period. Since the worth of the Van Slyke method for proteolytic determinations on fish muscle was not known, this test was discontinued during the second four week period. The author wished, moreover, to determine the value of the elect-

rometric titration method, as compared to that of known, tried methods. The following methods were used in the second four week period: Volatile Basic Nitrogen. 3.0000 grams of muscle were weighed out on a cover glass and transferred to a 200 cc. Erlenmeyer flask containing broken glass. 20 cc. of distilled water were added and the flask was shaken for five minutes by hand. The contents of the flask were then transferred to a 500 cc. ring neck flask and the Erlenmeyer flask was washed with 80 cc. of ethyl alcohol into the ring neck flask. 2 grams of MgO were then added to the flask and 50 cc. of the alcohol from this mixture was distilled over into 25 cc. of N/10 HCl, the tip of the condenser was kept below the level of the acid during the distillation. The excess acid was then titrated against N/10 NaOH and the amount of acid neutralized by the distillate was determined. Blank determinations were made on 50 cc. of alcohol in each case and the value of the blank was subtracted from the amount of alkali used.

This method was discontinued after the first week since the values were too small to be of any significance.

Ammonium Nitrogen. After the first week of the second four week period the ammonium nitrogen test as used by Reed, Rice and Sinclair (48) in 1929, was substituted for the Volatile Basic Nitrogen test. 3.0000 grams of fish muscle were weighed out on a watch glass and then transferred to a 100 cc. graduated flask. The volume was brought up to the graduation with distilled, ammonia free water and the flask was allowed to stand for 1

hour. The contents were then poured out into a clean beaker which had been washed with distilled water and 20 cc. of this solution were pipetted off into another 100 cc. graduated flask. 10 cc. of Nestler's solution were then added to this flask and the volume was made up to 100 cc. with distilled water. The flask was allowed to stand for 15 minutes after which a portion of the contents was compared colorimetrically, by means of a colorimeter, with a known standard. The amount of ammonium nitrogen was figured from the reading obtained.

The Harris Modification of The Formol Titration. 3.0000 grams of fish muscle were weighed out on a cover glass and transferred to a 200 cc. Erlenmeyer flask containing broken glass. 30 cc. of ethyl alcohol (80 per cent by volume) were then added and the flask was shaken for five minutes in order to disintegrate the muscle. 2 drops of a 0.1 per cent solution of thymophthalein were added and the mixture was titrated with alcoholic KOH. A blank was run on 30 cc. of the alcohol used in the titration and the blank was subtracted from the value of the muscle titration.

The electrometric titration method was used along with the above tests.

Bacteriological Counts. Bacteriological counts were made on the infected muscle during the last period of four weeks, time did not allow for this to be done during the first four week period. The skin was cut away from the flesh with sterile scissors and scalpels. A piece of the muscle was then taken at a dis-

tance of approximately $\frac{1}{2}$ cm. from the surface and the muscle was cut into small pieces, the pieces falling into a sterile beaker. 1 gram of this muscle was then weighed into a test tube containing 9 cc. of dilution water and broken glass, the tube and its contents having been previously sterilized. The tube was stoppered with a sterile rubber stopper and shaken for 10 minutes. Dilutions were made from this mixture and the counts were made, using nutrient agar as a medium of growth. The plates were incubated for 48 hours at 25° C., before counts were made.

Table 7

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperatures of incubation: 0 deg., C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations made: (a) Van Slyke (b) electrometric titration.

Sterile Muscle X

Time	Van Slyke mgm amino nitrogen	elect. titration A value	B value	pH at start
At start	0.7437	32.37	6.11	6.65
24 hours	0.7519	34.85	6.49	6.64
48 hours	0.7728	23.36	7.25	6.64
72 hours	0.7311	24.35	7.14	6.80
96 hours	0.6164	21.34	6.79	6.65
120 hours	0.6369	18.98	8.40	6.77

Infected Muscle Y

Time	Van Slyke mgm amino nitrogen	elect. titration A value	B value	pH at start
At start	0.7437	32.37	6.11	6.65
24 hours	0.8123	27.85	5.95	6.64
48 hours	0.7607	35.97	5.72	6.64
72 hours	0.9779	17.51	10.91	6.64
96 hours	1.0337	24.03	11.05	6.74
120 hours	1.1977	18.98	8.40	6.65

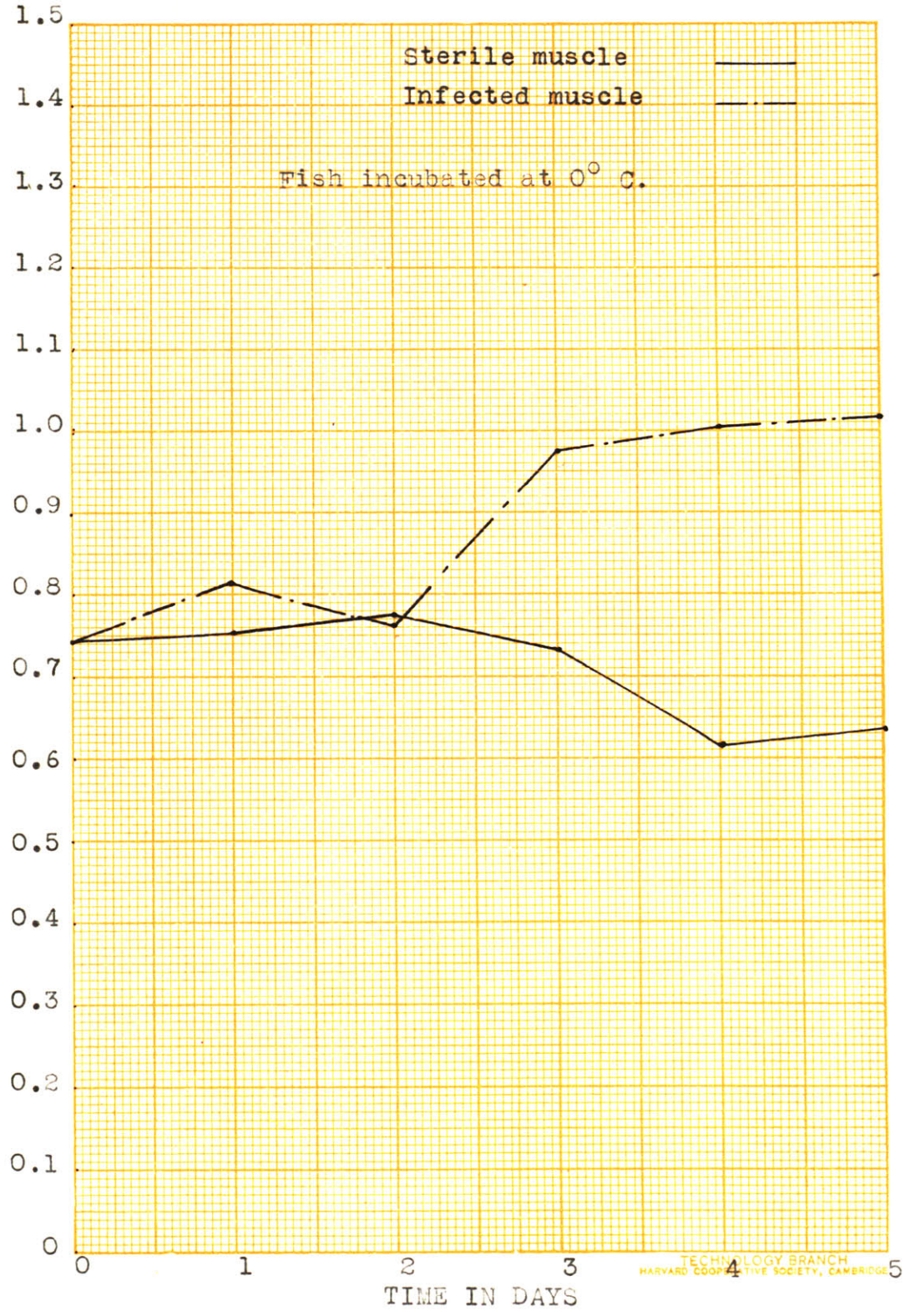

Sterile muscle
Infected muscle
Fish incubated at 0° C.
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
TIME IN DAYS
Milligrams of Amino Nitrogen per Gram of Muscle
Van Slyke Method
TECHNOLOGY BRANCH
HARVARD COOPERATIVE SOCIETY, CAMBRIDGE

Table 8

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 5 deg., C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations made: (a) Van Slyke (b) electrometric titration.

Sterile Muscle X

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.8109	34.44	7.20	6.58
24 hours	0.8499	31.90	9.20	6.63
*48 hours	0.7110	23.90	9.32	6.65
72 hours	0.9880	23.04	9.03	6.55
96 hours	0.8643	26.42	8.89	6.55
120 hours	08214	23.90	9.92	6.55

Infected Muscle Y

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.8109	34.44	7.20	6.58
24 hours	0.8445	21.47	8.92	6.65
48 hours	0.9250	22.83	11.00	6.73
72 hours	1.0742	22.07	11.13	6.65
96 hours	0.8525	27.00	11.79	6.65
120 hours	0.6377	25.82	12.08	6.65

* The values for the 48 hour period in the sterile muscle are void due to the fact that the muscle was infected.

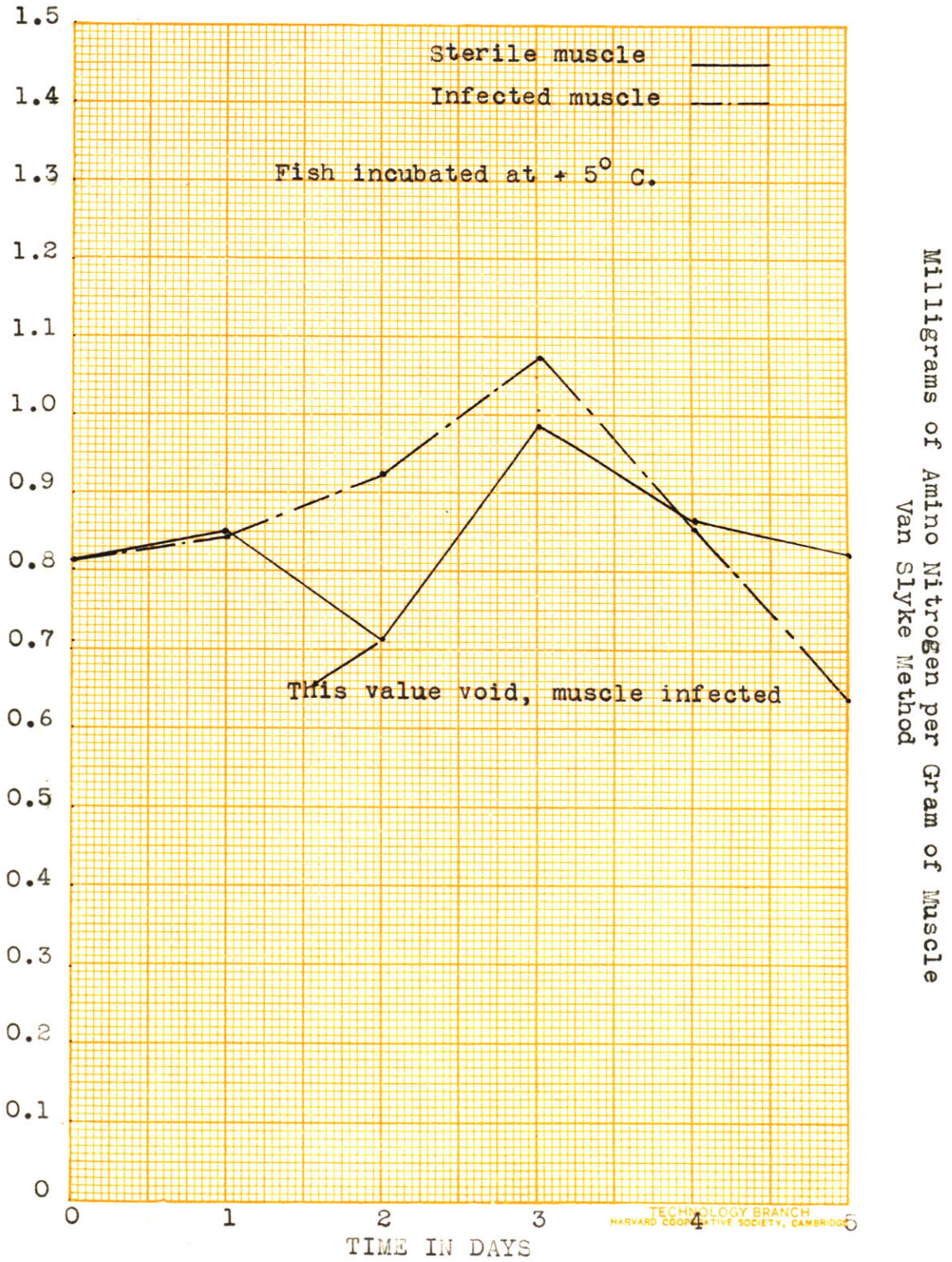

Sterile muscle
Infected muscle
Fish incubated at + 5° C.
This value void, muscle infected
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
TIME IN DAYS
Milligrams of Amino Nitrogen per Gram of Muscle
Van Slyke Method
TECHNOLOGY BRANCH
HARVARD COOPERATIVE SOCIETY, CAMBRIDGE

Table 9

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperatures of incubation: + 10 deg., C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations made: (a) Van Slyke (b) electrometric titration.

Sterile Muscle X

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.7684	28.38	12.50	6.60
24 hours	0.8897	37.80	10.15	6.60
48 hours	0.8989	26.12	10.90	6.70
72 hours	0.8036	21.48	10.28	6.70
96 hours	06947	21.38	8.19	6.55
120 hours	0.6814	23.39	9.05	6.50

Infected Muscle Y

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.7684	28.38	12.50	6.60
24 hours	0.9347	27.35	10.80	6.65
48 hours	0.6351	19.73	13.11	6.75
72 hours	0.8266	20.50	11.04	6.75
96 hours	0.8303	19.42	10.55	6.70
120 hours	0.9179	20.41	10.91	6.65

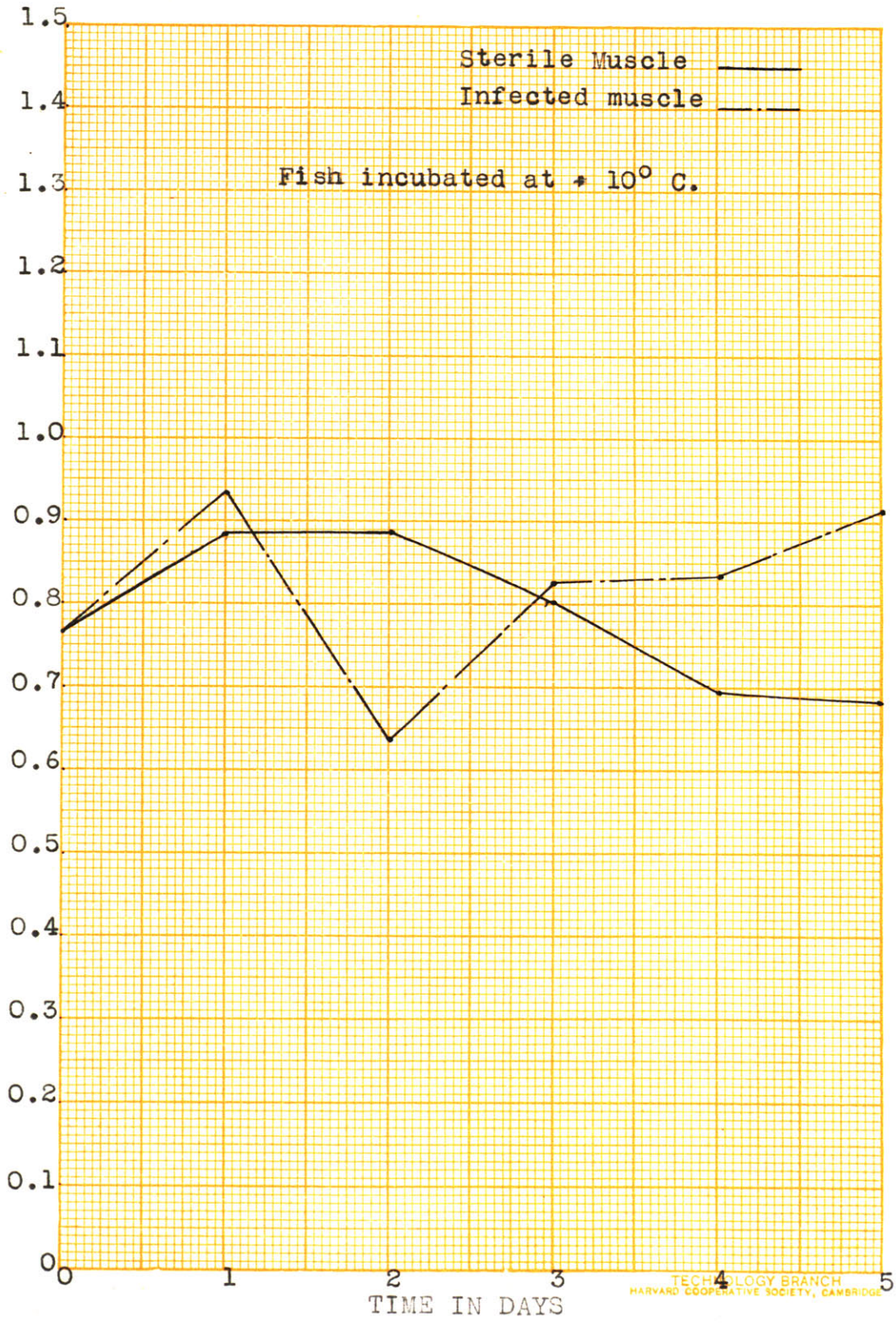

Sterile Muscle
Infected muscle
Fish incubated at + 10° C.
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
TIME IN DAYS
Milligrams of Amino Nitrogen per Gram of Muscle
Van Slyke Method
TECHNOLOGY BRANCH
HARVARD COOPERATIVE SOCIETY, CAMBRIDGE

Table 10

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 17 deg., C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations : (a) Van Slyke (b) electrometric titration.

Sterile Muscle X

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.5324	24.39	12.95	6.65
24 hours	0.7425	22.36	14.50	6.68
48 hours	0.5990	26.02	12.89	6.70
72 hours	0.5871	23.95	12.88	6.70
96 hours	0.5891	24.16	11.61	6.60

Infected Muscle Y

Time	Van Slyke mgm. amino nitrogen	elect. titration A value	B value	pH at start
At start	0.5324	24.39	12.95	6.65
24 hours	0.6233	20.43	15.92	6.68
48 hours	0.6961	20.02	13.80	6.75
72 hours	0.6586	25.17	13.63	6.70
96 hours	0.9527	23.87	15.95	6.80

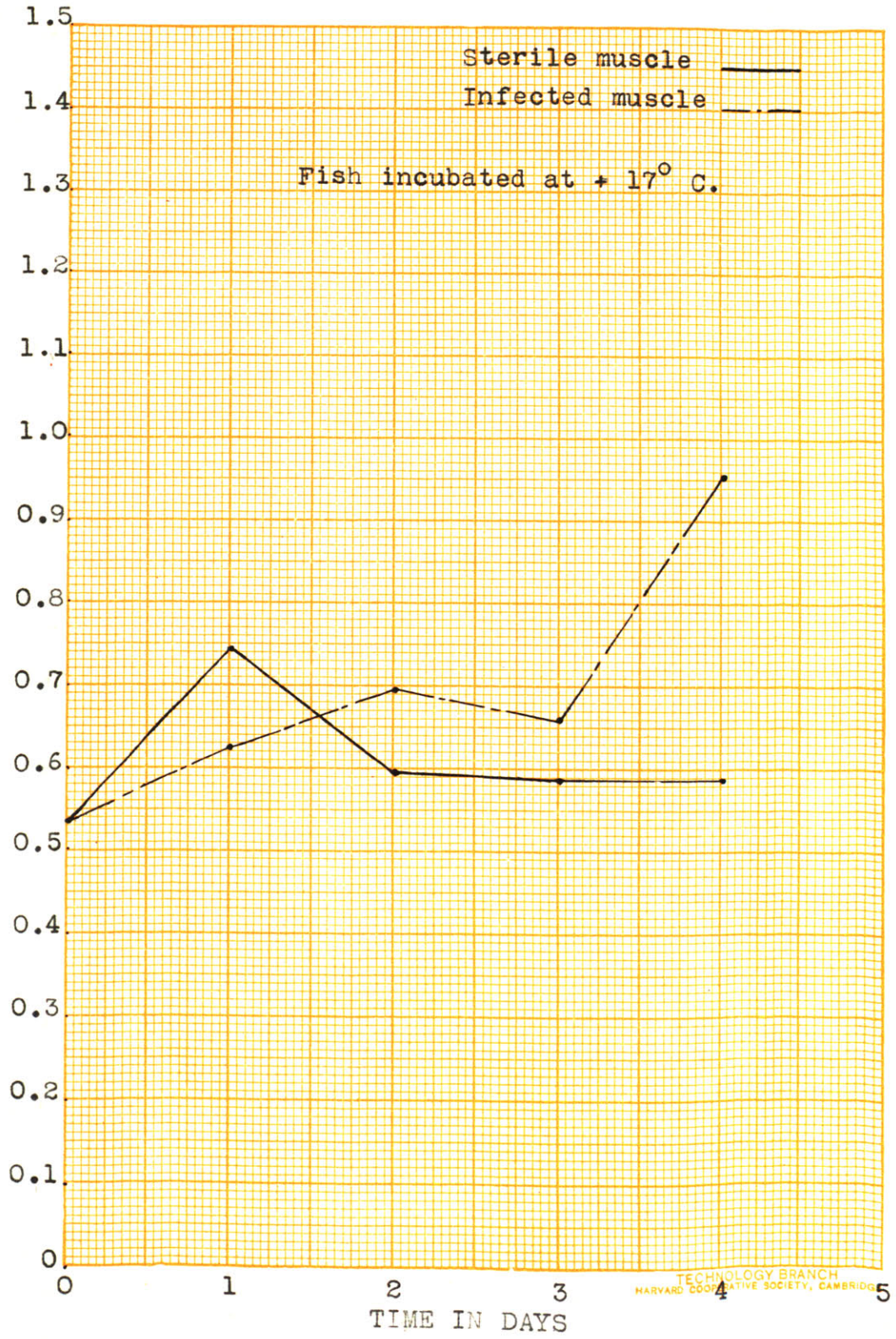


Table 11

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: 0 deg. C.

Determinations made on (a) sterile muscle (b) infected muscle.

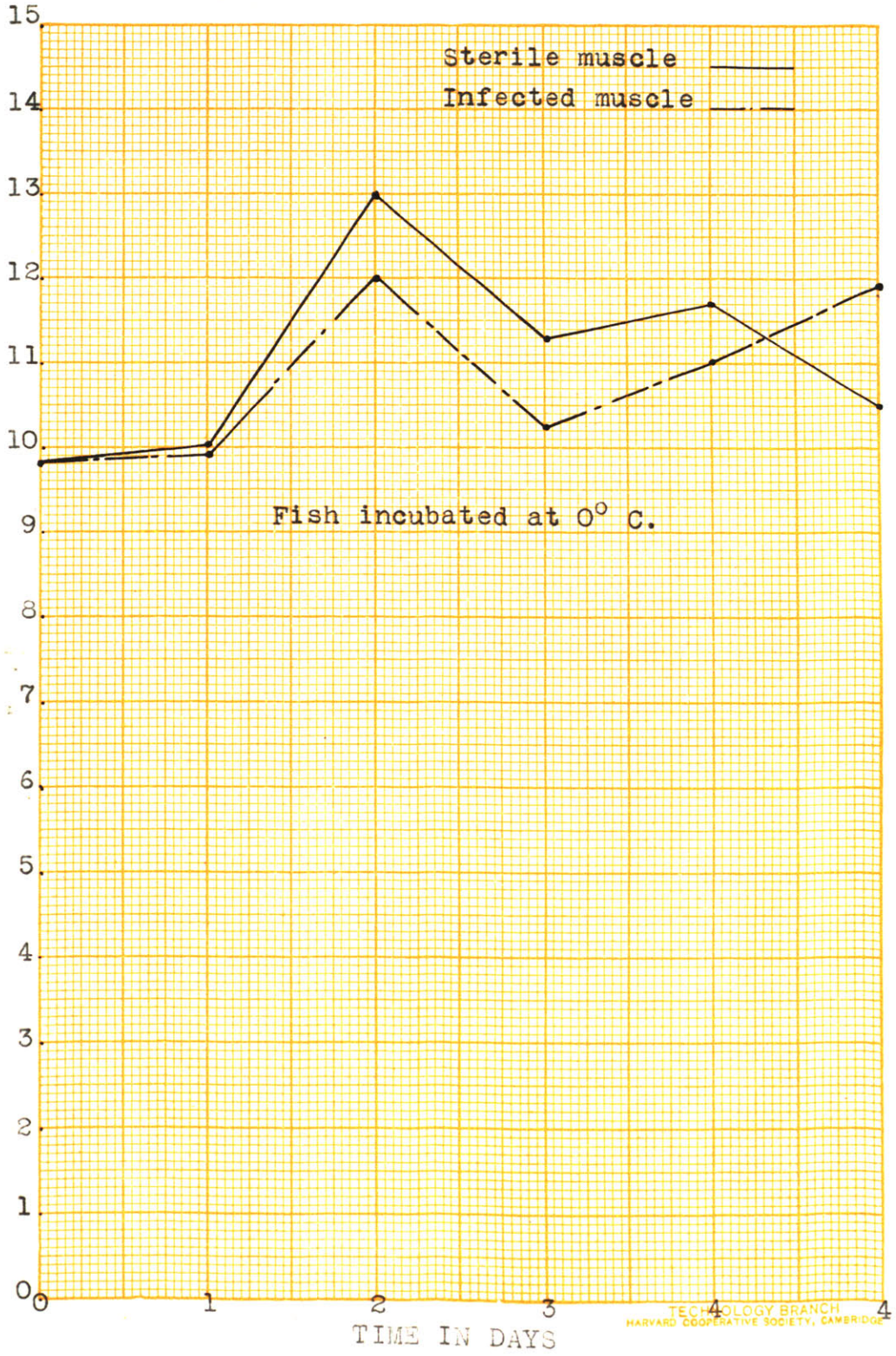
Determinations made: Formol titration (b) electrometric titration (c) ammonium nitrogen.

Sterile Muscle

Time	Formol Titration cc.alc.KOH per 10 g. Muscle	elect. titration A value	B value	pH at start
At start	9.8	24.92	12.58	6.75
24 hours	10.0	24.30	13.94	6.90
48 hours	13.0	24.70	13.17	6.90
72 hours	11.3	24.92	13.30	6.90
96 hours	11.7	25.86	14.90	6.90
120 hours	10.5	25.43	14.40	6.90

Infected Muscle

Time	Formol Titration cc.alc.KOH per 10 g. Muscle	elect. titration A value	B value	Bacteriological count Bacteria per gram	pH at start
At start	9.8	24.92	12.58	----------	6.75
24 hours	9.9	21.15	12.68	120 & 130	6.90
48 hours	12.0	26.69	12.92	139 & 146	6.70
72 hours	10.25	23.37	13.32	500 & 500	6.90
96 hours	11.00	22.34	14.08	800 & 920	6.90
120 hours	11.90	22.06	14.82	2900 &3200	6.90



cc. of Alcoholic KOH per 10 Grams of Muscle
Harris Modification of Formol Titration

Table 12

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 5° C.
Determinations made on: (a) sterile muscle (b) infected muscle,
Determinations made: (a) Formol titration (b) electrometric titration (c) ammonium nitrogen.

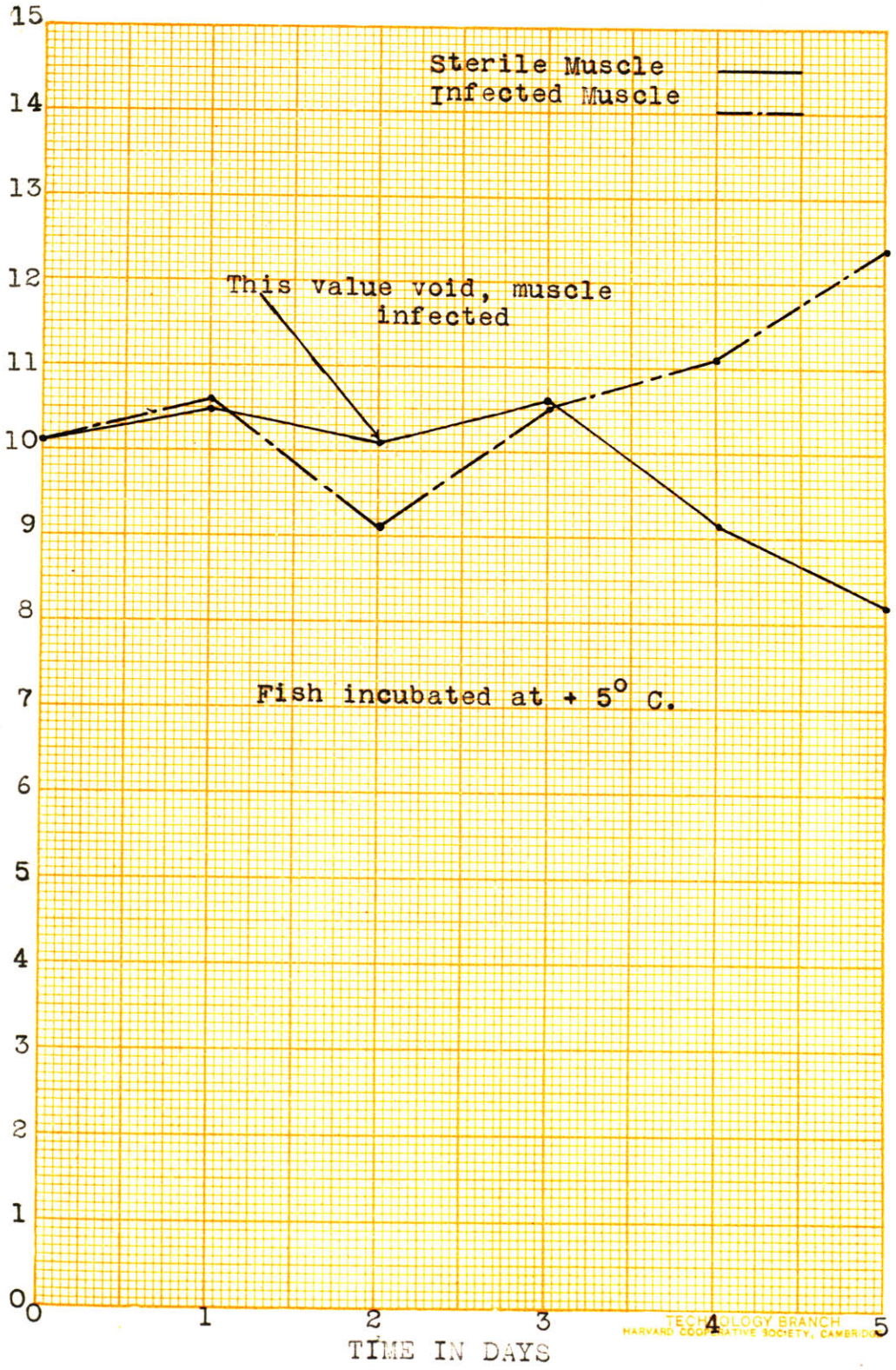
Sterile Muscle

Time	Formol Titration cc. alc.KOH per 10 gms. of muscle	elect. titration A value	B value	pH at start
At start	10.10	25.97	13.90	6.90
24 hours	10.50	25.67	12.60	6.85
*48 hours	10.10	26.54	13.49	6.87
72 hours	10.60	26.01	12.60	6.80
96 hours	9.15	26.23	12.48	6.85
120 hours	8.20	26.30	12.30	6.75

Infected Muscle

Time	Formol Titration cc.alc.KOH per 10 gms. of Muscle	elect titration A value	B value	Bacteriological count Bacteria per gram	pH at start
At start	10.10	25.97	13.90		6.90
24 hours	10.60	22.88	12.67	810,760	6.85
48 hours	9.10	25.00	13.00	1210,1090	6.93
72 hours	10.50	22.20	14.99	3000,3800	6.95
96 hours	11.10	23.09	14.34	4700,4200	6.95
120 hours	12.40	20.56	12.95	7200,8900	6.95

* The values for the RI hour period in the sterile muscle are void due to the fact that the muscle was infected.



cc. Alcoholic KOH used per 10 Grams of Muscle
Harris Modification of Formol Titration

Table 13

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 10 deg. C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations made: (a) Formol titration (b) electrometric titration. (c) Ammonium nitrogen test.

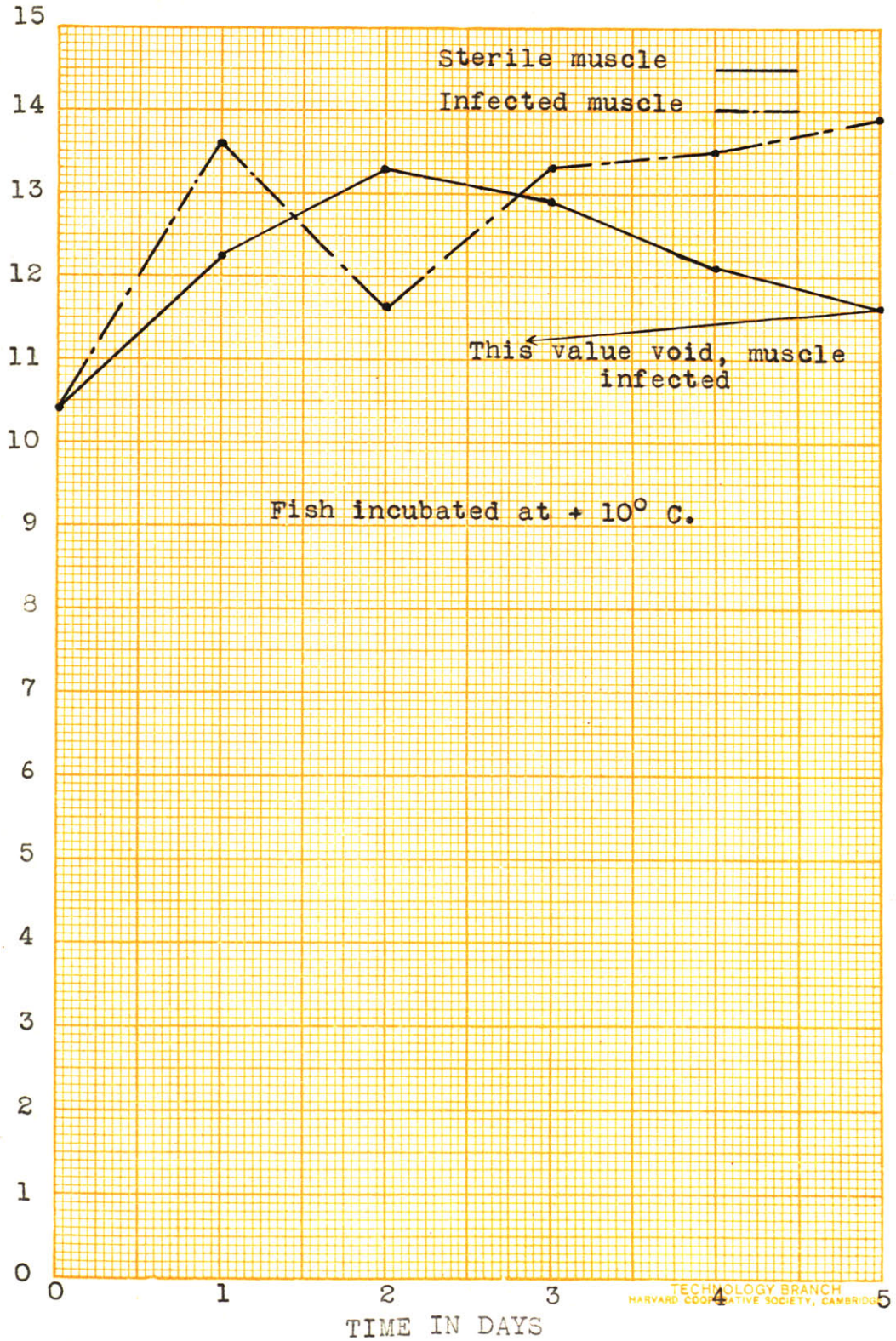
Sterile Muscle

Time	Formol Titration cc.alc.KOH per 10 gms. of muscle	elect.titration A value	B value	pH at start
At start	10.40	25.01	12.60	6.80
24 hours	12.30	25.99	11.54	6.81
48 hours	13.30	26.56	11.14	6.82
72 hours	12.90	24.50	10.81	6.85
96 hours	12.10	24.61	10.96	6.75
*120 hours	11.60	25.32	11.50	6.75

Infected Muscle

Time	Formol Titration cc.alc.KOH per 10 gms. of muscle	elect.titration A value	B value	Bacteriological count Bacteria per gram		pH at start
At start	10.40	25.01	12.60			6.80
24 hours	13.60	23.90	13.08	300,	330	6.80
48 hours	11.60	22.80	11.70	560,	490	6.83
72 hours	13.30	22.12	12.34	4,700,	3,800	6.87
96 hours	13.50	23.12	12.06	106,000,	98,000	6.84
120 hours	13.90	19.86	11.99	390,000,	460,000	6.95

* The values for the 120 hour period in the sterile muscle are void due to the fact that the muscle was infected.



cc. Alcoholic KOH used per 10 Grams of Muscle
Harris Modification of Formol Titration

Table 14

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 15 deg. C.

Determinations made on: (a) sterile muscle (b) infected muscle.

Determinations made: (a) formol titration (b) electrometric titration (c) ammonium titration.

Sterile Muscle

Time	Formol Titration cc.alc.KOH per 10 gms. of muscle	elect.titration A value	B value	pH at start
At start	12.20	26.04	12.47	6.75
24 hours	13.10	25.30	13.40	6.77
48 hours	14.50	24.48	11.00	6.75
*72 hours	13.40	26.05	9.50	6.70
96 hours	12.60	26.02	11.86	6.74

Infected Muscle

Time	Formol Titration cc.alc.KOH per 10 gms. of muscle	elect.titration A value	B value	Bacteriological count Bacteria per gram	pH at start
At start	12.20	26.04	12.47		6.75
24 hours	13.10	24.01	13.65	46,000, 52,000	6.76
48 hours	12.50	22.62	13.03	132,000, 135,000	6.82
72 hours	14.80	25.55	13.35	206,000, 218,000	6.87
96 hours	16.50	21.62	16.65	3,100,000, 3,900,000	6.95

* The values for the 72 hour period in the sterile muscle are void due to the fact that the muscle was infected.

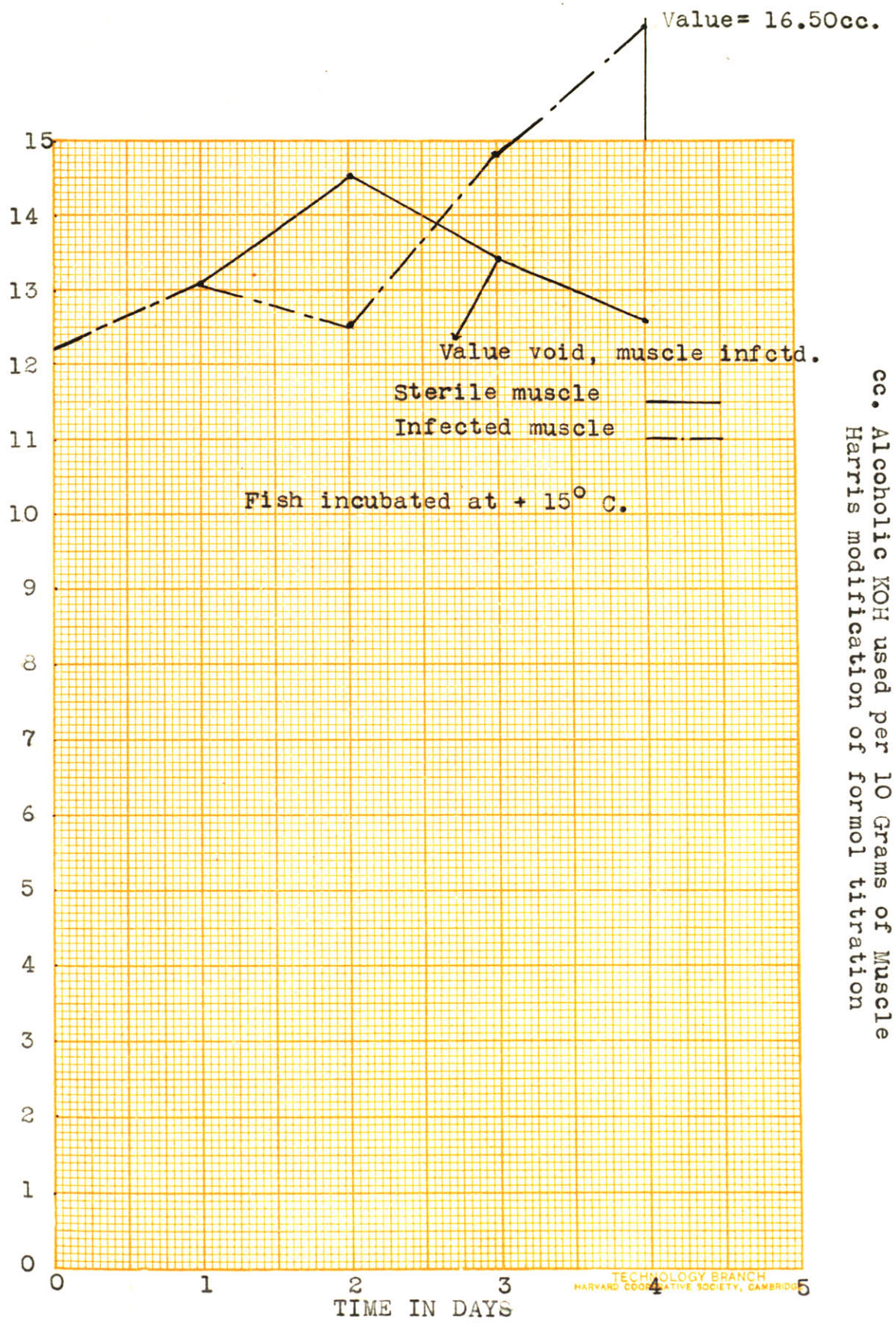

Value= 16.50cc.
Value void, muscle infctd.
Sterile muscle
Infected muscle
Fish incubated at + 15° C.
cc. Alcoholic KOH used per 10 Grams of Muscle
Harris modification of formol titration
TIME IN DAYS
0
1
2
3
4
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
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Table 15

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 5 deg. C.
Determinations made on: (a) sterile muscle (b) infected muscle.
Determinations made: (a) formol titration (b) electromatric titration (c) ammonium nitrogen test.

Sterile Muscle X

Time	Ammonium nitrogen per 100 gms.muscle	elect.titartion A value	B value	pH at start
At start	0.00054	25.97	13.90	6.90
24 hours	0.00062	25.67	12.60	6.85
*48 hours	0.00089	26.54	13.49	6.87
72 hours	0.00094	26.01	12.60	6.80
96 hours	0.00092	26.23	12.48	6.85
120 hours	0.00090	26.30	12.30	6.75

Infected Muscle Y

Time	Ammonium nitrogen per 100 gms.muscle	elect.titration A value	B value	Bacteriological count Bacteria per gram	pH at start
At start	0.00054	25.97	13.90		6.90
24 hours	0.00064	22.88	12.67	810, 760	6.85
48 hours	0.00091	25.00	13.00	1,210, 1,090	6.93
72 hours	0.00098	22.20	14.99	3,000, 3,800	6.95
96 hours	0.00108	23.09	14.34	4,700, 4,200	6.95
120 hours	0.00128	20.56	12.95	7,200, 8,900	6.95

* The values for the 48 hour period in the sterile muscle are void due to the fact that the muscle was infected.

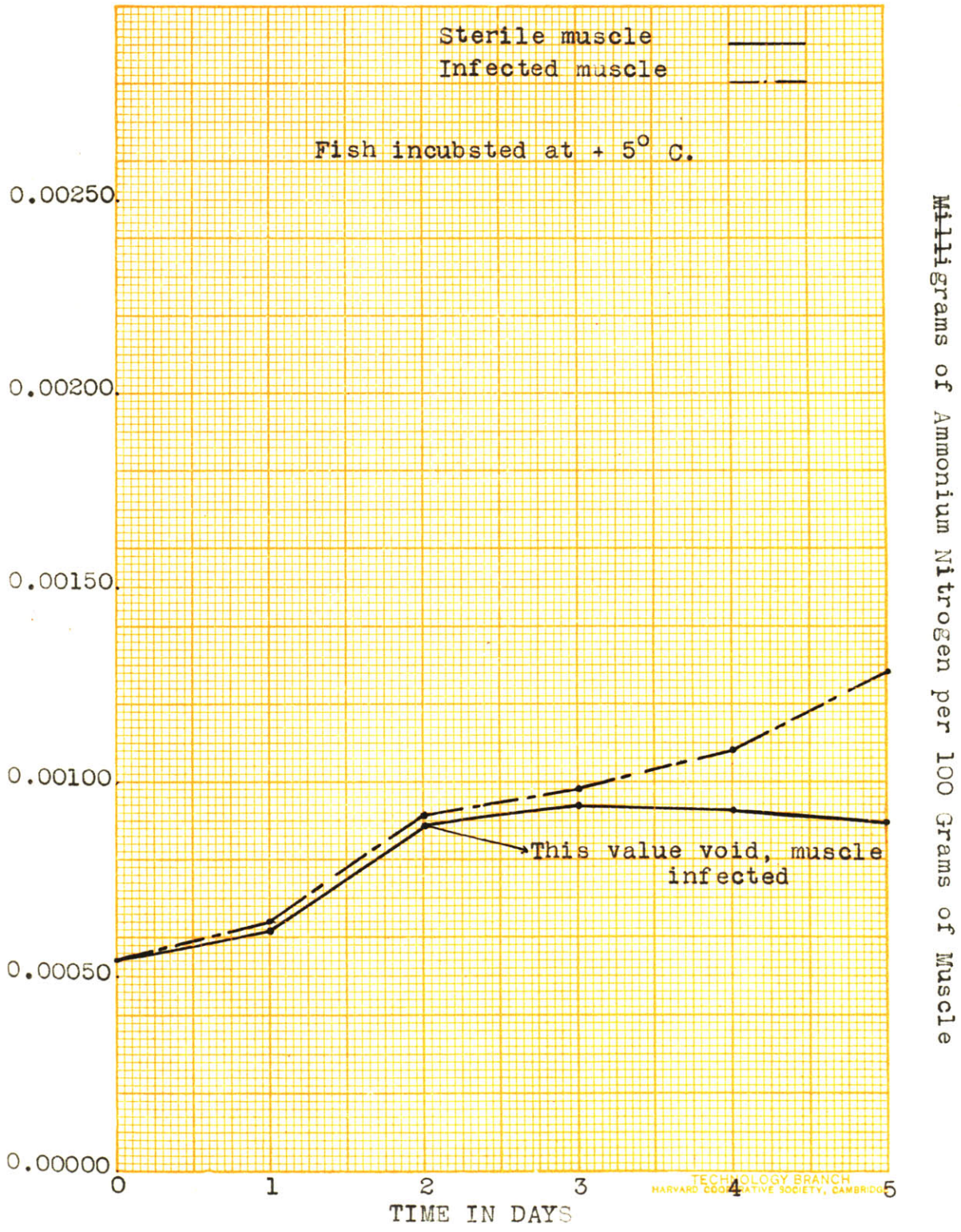

Sterile muscle
Infected muscle
Fish incubsted at + 5° C.
0.00250.
0.00200.
0.00150.
0.00100.
0.00050.
0.00000
0
1
2
3
4
5
TIME IN DAYS
This value void, muscle infected
Milligrams of Ammonium Nitrogen per 100 Grams of Muscle
TECHNOLOGY BRANCH
HARVARD COOPERATIVE SOCIETY, CAMBRIDGE

Table 16

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYMES AND BACTERIAL ACTION

Temperature of incubation: + 10 deg. C.
Determinations made on: (a) sterile muscle (b) infected muscle.
Determinations made: (a) formol titration (b) electrometric titration (c)ammonium nitrogen

Sterile Muscle X

Time	Ammonium nitrogen gms. per 100 gms. of muscle	elect.tiration A value	B value	pH at start
At start	.0.00060	25.01	12.60	6.80
24 hours	.0.00089	25.99	11.54	6.81
48 hours	.0.00091	25.56	11.14	6.82
72 hours	.0.00085	24.50	10.81	6.85
96 hours	.0.00081	24.61	10.96	6.75
*120 hours	.0.00086	25.32	11.50	6.75

Infected Muscle Y

Time	Ammonium nitrogen gms. per 100 gms. of muscle	elect.titration A value	B value	Bacteriological count Bacteria per gram	pH at start
At start	.0.00060	25.01	12.60		6.80
24 hours	.0.00087	23.90	13.08	300, 330	6.80
48 hours	.0.00103	22.80	11.70	560, 490	6.83
72 hours	.0.00122	22.12	12.34	4,700, 3,800	6.87
96 hours	.0.00176	23.12	12.06	106,000, 98,000	6.84
120 hours	.0.00185	19.86	11.99	390,000, 460,000	6.95

* The values for the 120 hour period in the sterile muscle was void due to the fact that the muscle was infected.

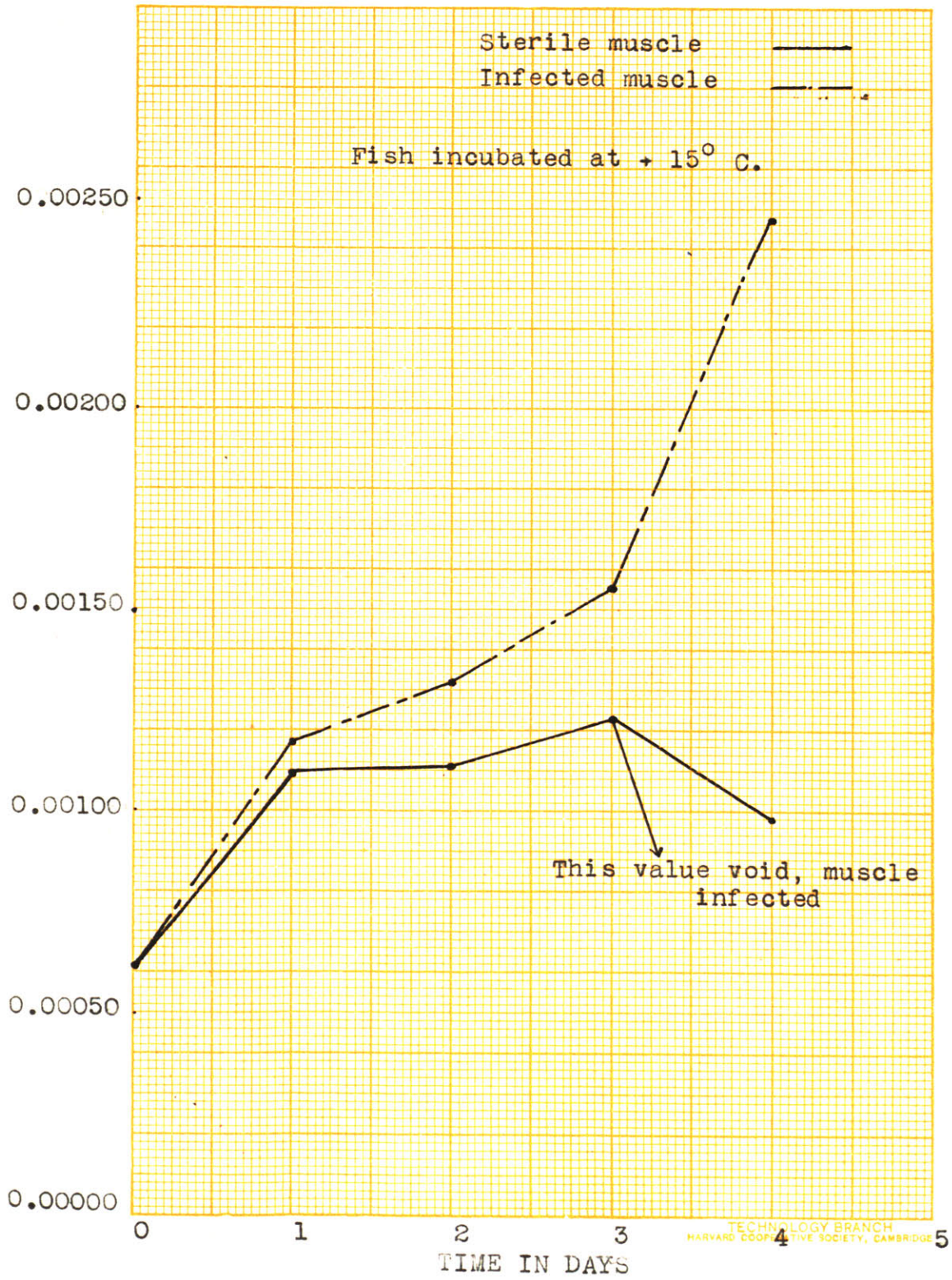


Table 17

PROTEOLYTIC CHANGES IN FISH MUSCLE DUE TO ENZYME AND BACTERIAL ACTION

Temperature of incubation: + 15 deg. C.
Determinations made on: (a) formol titration (b) electrometric titration (c) ammonium nitrogen.

Sterile Muscle X

Time	Ammonium nitrogen gms. per 100 gms. of muscle	elect.titration A value	B value	pH at start
At start	0.00061	26.04	12.47	6.75
24 hours	0.00110	25.30	13.40	6.77
48 hours	0.00111	24.48	11.00	6.75
*72 hours	0.00123	26.05	9.50	6.70
96 hours	0.00098	26.92	11.86	6.74

Infected Muscle Y

Time	Ammonium nitrogen gms. per 100 gms. of muscle	elect.titration A value	B value	Bacteriological count Bacteria per gram		pH at start
At start	0.00061	26.04	12.47			6.75
24 hours	0.00117	24.01	13.65	46,000,	52,000	6.76
48 hours	0.00132	22.62	13.03	132,000,	135,000	6.82
72 hours	0.00155	25.55	13.35	206,000,	218,000	6.87
96 hours	0.00247	21.62	16.65	3,100,000,	3,900,000	6.95

* The values for the 72 hour period in the sterile muscle are void due to the fact that the muscle was infected.

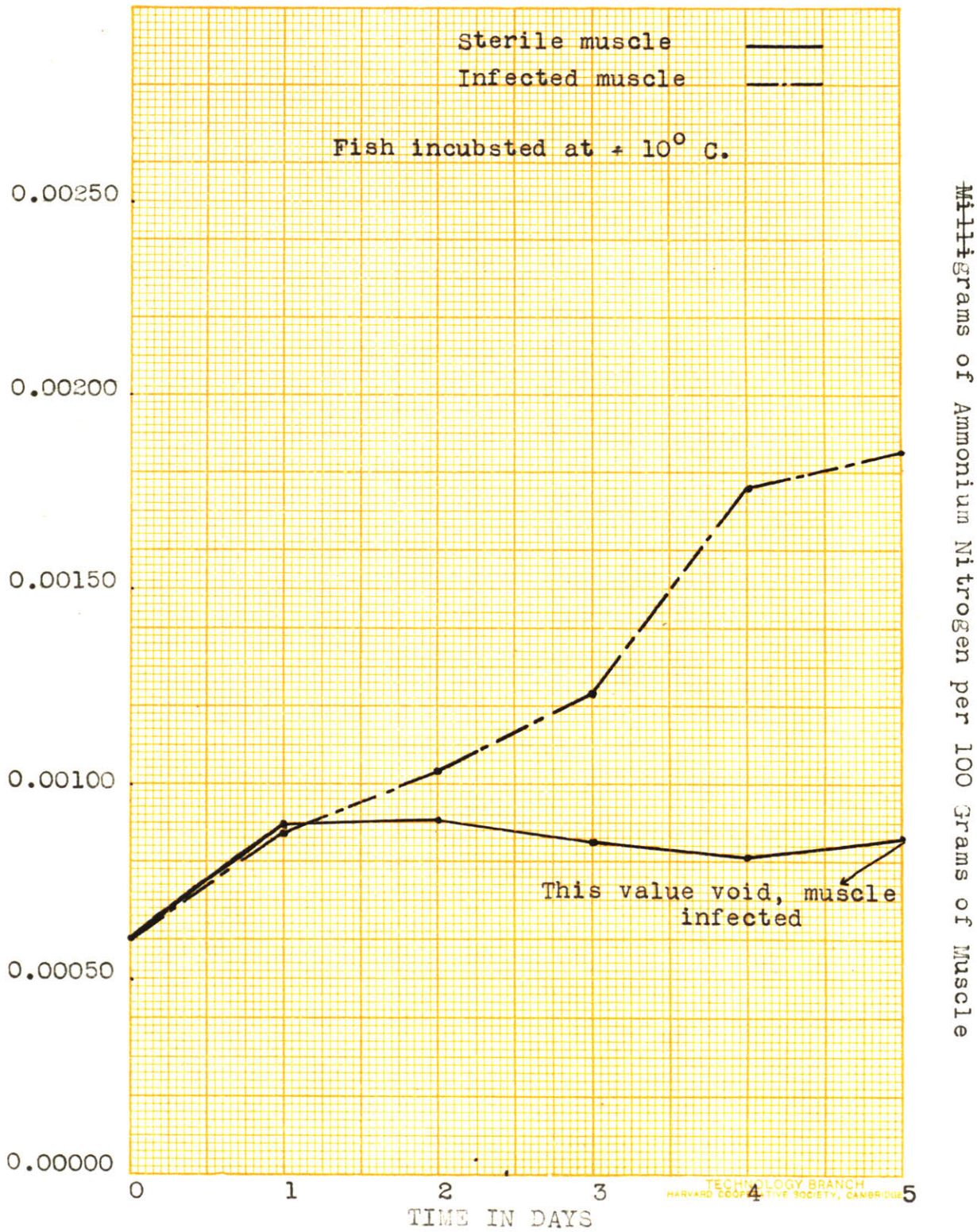

Sterile muscle
Infected muscle
Fish incubsted at + 10° C.
0.00250
0.00200
0.00150
0.00100
0.00050
0.00000
This value void, muscle infected
0
1
2
3
4
5
TIME IN DAYS
Milligrams of Ammonium Nitrogen per 100 Grams of Muscle
TECHNOLOGY BRANCH
HARVARD COOPERATIVE SOCIETY, CAMBRIDGE

Table 18

PER CENT OF CHANGE IN PROTEOLYTIC PRODUCTS OF FISH MUSCLE DUE TO ENZYME ACTION

Temperature of incubation: 0 °C., + 5 °C., +10 °C., and + 17 °C.
Determinations made on: sterile muscle.
Determinations made: Van Slyke
Values figured from table 7X, table 8X, table 9X and table 10X
Values at start taken as 100 per cent.

Time	At 0 °C. Van Slyke values taking 0 hr. determinations to be 100 %	Time	At + 5 °C. Van Slyke values taking 0 hr. determinations to be 100 %
At start	100.0	At start	100.0
24 hours	101.1	24 hours	104.8
48 hours	103.9	48 hours	contaminated
72 hours	98.2	72 hours	121.8
96 hours	82.9	96 hours	106.6
120 hours	85.6	120 hours	101.3

Time	At + 10 °C. Van Slyke values taking 0 hr. determinations to be 100 %	Time	At + 17 °C. Van Slyke values taking 0 hr. determinations to be 100 %
At start	100.0	At start	100.0
24 hours	115.8	24 hours	139.5
48 hours	116.9	48 hours	112.5
72 hours	104.3	72 hours	110.3
96 hours	90.4	96 hours	110.6
120 hours	88.6		

Percentage Curves for Van Slyke Values of Enzyme Decomposition of Sterile Muscle

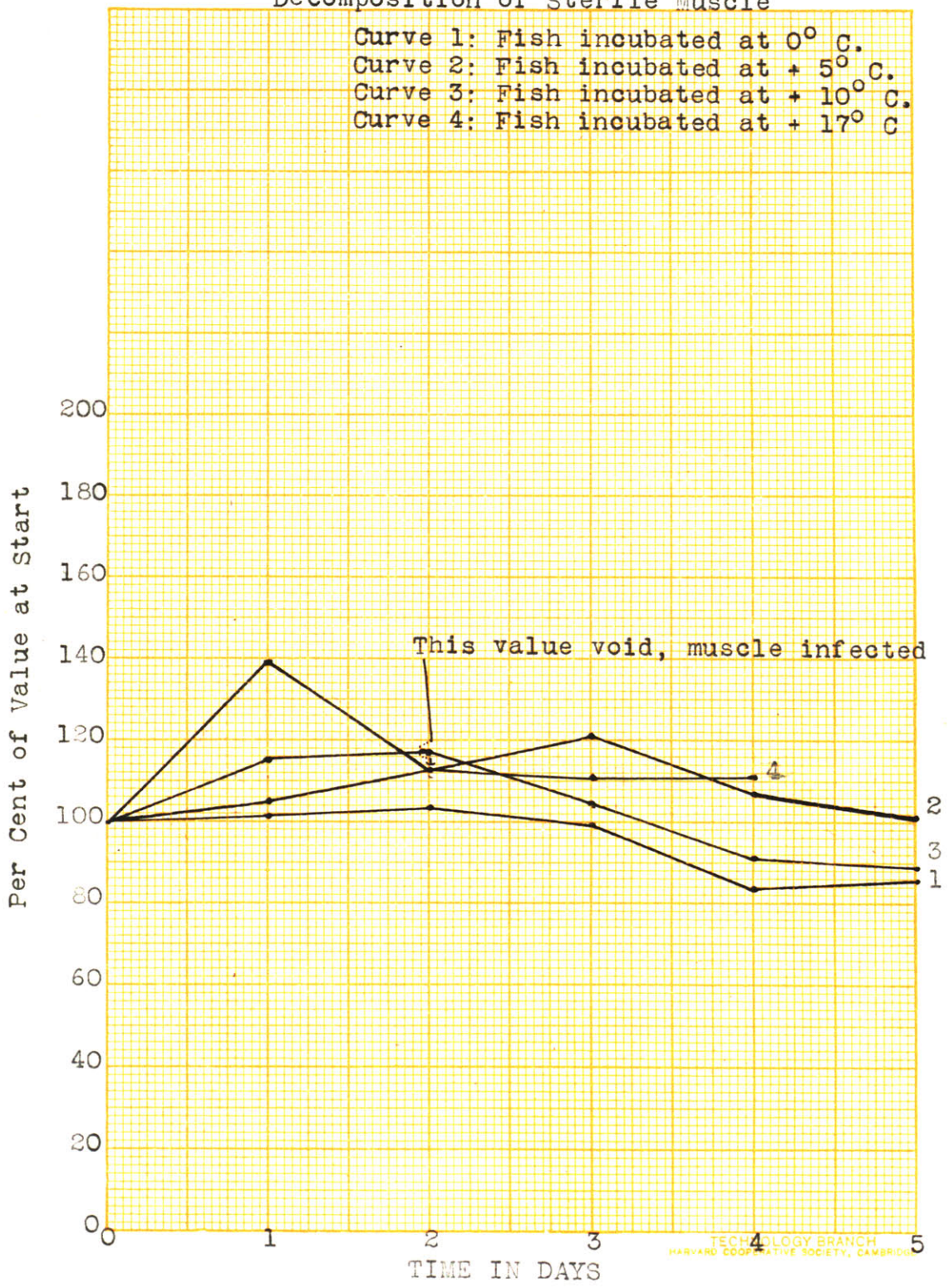


Table 19

PER CENT OF CHANGE IN PROTEOLYTIC PRODUCTS OF FISH MUSCLE DUE TO BACTERIAL ACTION

Temperature of incubation: 0° C., + 5° C., + 10° C., and + 15° C.
Determinations made on: infected muscle.
Determinations made: Van Slyke
Values figured from table 7Y, table 8Y, table 9Y and table 10Y
Values at start taken as 100 per cent.

Time	At 0° C. Van Slyke values taking 0 hr. determinations to be 100 %	Time	At + 5° C. Van Slyke values taking 0 hr. determinations to be 100 %
At start		At start	
24 hours	109.2	24 hours	104.1
48 hours	102.4	48 hours	114.1
72 hours	131.5	72 hours	132.5
96 hours	138.9	96 hours	105.1
120 hours	161.0	120 hours	78.6

Time	At - 10° C. Van Slyke values taking 0 hr. determinations to be 100 %	Time	At - 15° C. Van Slyke values taking 0 hr. determinations to be 100 %
24 hours	121.6	24 hours	117.0
48 hours	82.7	48 hours	130.8
72 hours	107.6	72 hours	123.7
96 hours	103.0	96 hours	178.9
120 hours	119.4	120 hours	

Percentage Curves for Van Slyke Values of Enzyme plus Bacterial Decomposition of Infected Muscle

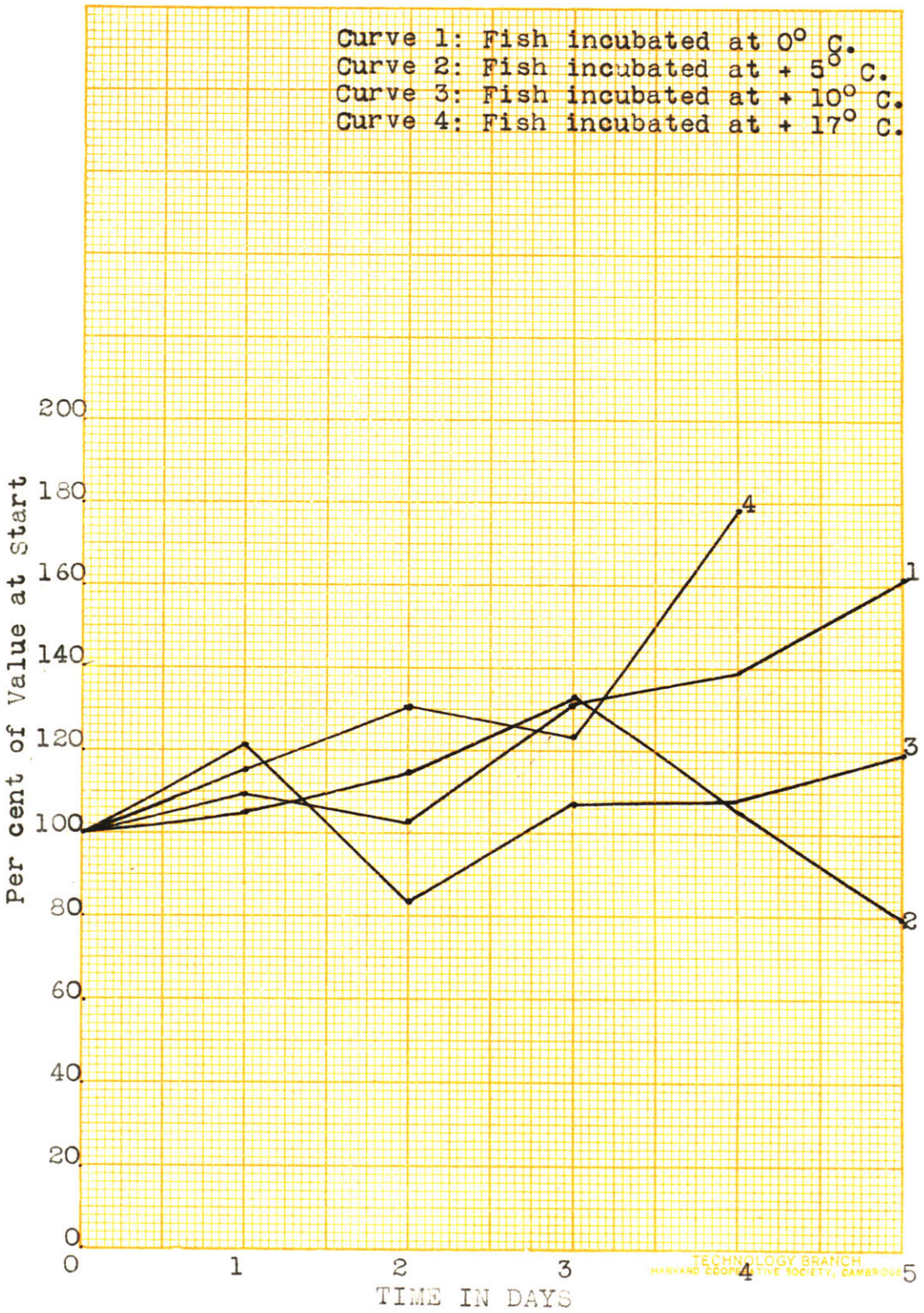


Table 20

PER CENT OF CHANGE IN PROTEOLYTIC PRODUCTS OF FISH MUSCLE DUE TO ENZYME ACTION

Temperature of incubation: + 5 °C., + 10 °C., and + 15 °C.
Determinations made on: sterile muscle.
Determinations made: Ammonium nitrogen test.
Values figured from table 11X, table 12X, and table 13X.
Values at start taken as 100 per cent.

At + 5 °C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100%
At start	100.0
24 hours	114.9
48 hours	contaminated
72 hours	174.1
96 hours	170.4
120 hours	166.6

At + 10 °C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100 %
At start	100.0
24 hours	148.0
48 hours	151.7
72 hours	141.7
96 hours	135.0
120 hours	contaminated

At + 15 °C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100 %
At start	100.0
24 hours	180.3
48 hours	181.9
72 hours	contaminated
96 hours	160.7
120 hours	

Percentage Curves for Ammonium Nitrogen Values of Enzyme Action in Sterile Muscle

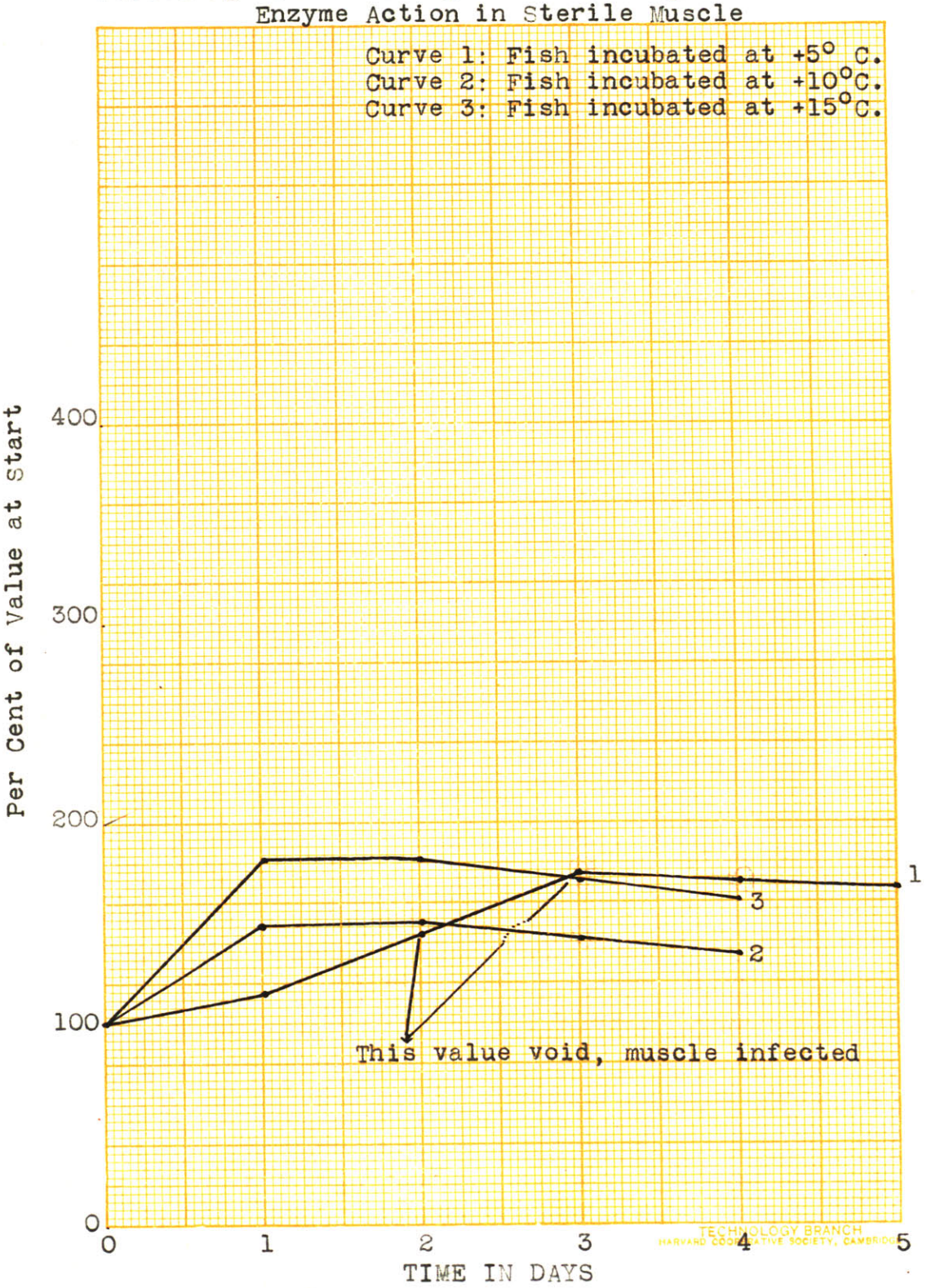


Table 21

PER CENT OF CHANGE IN PROTEOLYTIC PRODUCTS OF FISH MUSCLE DUE TO BACTERIAL ACTION

Temperature of incubation: + 5° C., + 10° C., + 15° C.
Determinations made on: infected muscle.
Determinations made: ammonium nitrogen test.
Values figured from table 11Y, table 12Y and table 13Y.
Value at start taken as 100 per cent.

At + 5° C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100 %
At start	100.0
24 hours	118.5
48 hours	167.9
72 hours	181.5
96 hours	200.0
120 hours	237.0

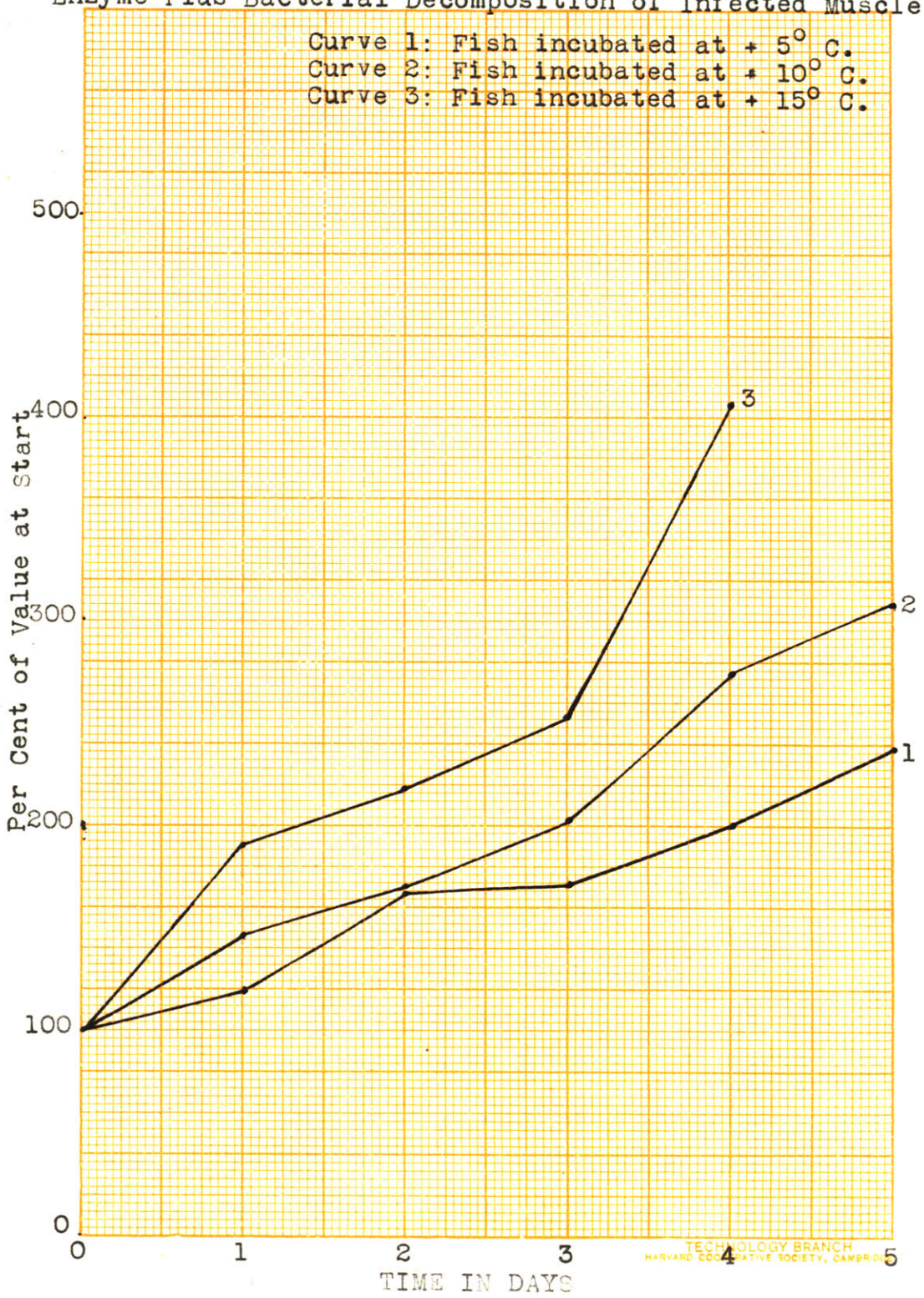
At + 10° C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100 %
At start	100.0
24 hours	145.0
48 hours	171.7
72 hours	203.3
96 hours	293.3
120 hours	308.3

At + 15° C.

Time	Ammonium nitrogen percentage values taking 0 hr. determinations to be 100 %
At start	100.0
24 hours	191.8
48 hours	216.4
72 hours	254.1
96 hours	404.9

Percentage Curves for Ammonium Nitrogen Values of Enzyme Plus Bacterial Decomposition of Infected Muscle



EXPERIMENTAL

TO DETERMINE THE RELATIVE VALUES OF CERTAIN KINDS OF COMMERCIAL ICE AS REFRIGERATING AGENTS

In the cooling experiments, the haddock, Melanogrammus aeglifinus, was used as the material to be cooled. The haddock used were ordinary market fish, the intestines of which had been removed. The head and gills were intact.

Ordinary chopped ice and "flake ice" were used as refrigerating agents in these experiments. "Flake ice" differs from ordinary ice only in that it is frozen in a thin shell and then broken up into flat flakes which are approximately one inch x one inch x one-quarter inch in size.

A description of the thermocouple-galvanometer arrangement used to measure the temperature of the fish will be found in the appendix.

At the outset of each determination the fish was placed in a wooden box with holes in the bottom through which the water from the melting ice could flow, thus preventing any water from collecting around the fish. A layer of ice, about six inches deep, was then placed over the bottom of the box, the fish was placed upon the ice and a slight pressure put upon it to settle it into the ice. The couples of the cold junction were then led into the box through a small hole in the side. These were then inserted, three on either side of the backbone of the fish, at the middle of the dorsal fin. Each couple was inserted for a distance of 1 1/4 inches. The couples of the hot junction

were placed in water which was kept constantly boiling. The first reading was then taken on the galvanometer after which the fish was thoroughly covered with ice until the box was filled. After the fish had been covered with ice, readings were taken on the galvanometer at every five minute period until the temperature of the fish had reached 0^{o} C. The same fish was used for both kinds of ice. After the temperature of 0^{o} C. had been reached the fish was taken out of the ice and allowed to stand at room temperature until the galvanometer readings were the same as it was at the beginning of the experiment. The fish was then placed in the box as before and the experiment repeated, the only difference being that the ice used in this case was different from that of the first determination, that is, if "flake" ice was used for the first run, ordinary chopped ice was used for the second and vice versa.

Three fish were used in these experiments, in all. In two of these experiments the "flake" ice was used for the first determination while the ordinary ice was used for the second, while in the third experiment the ordinary ice was used first and the "flake" ice for the second determination.

Table 22

TIME TAKEN BY DIFFERENT KINDS OF COMMERCIAL ICE TO COOL FISH TO 0° C.

Fish used: Haddock

Weight of fish: 1012 grams

Condition of fish: intestines removed

Time	Temperature using Crushed ice	Flake ice
At start	21 deg., C	21 deg., C.
5 minutes	14.8 " "	12.4 " "
10 "	10.0 " "	6.2 " "
15 "	7.4 " "	2.0 " "
20 "	5.8 " "	1.3 " "
25 "	4.6 " "	0.9 " "
30 "	3.7 " "	0.2 " "
35 "	3.0 " "	0.0 " "
40 "	2.0 " "	
45 "	1.0 " "	
50 "	0.5 " "	
55 "	0.3 " "	
60 "	0.0 " "	

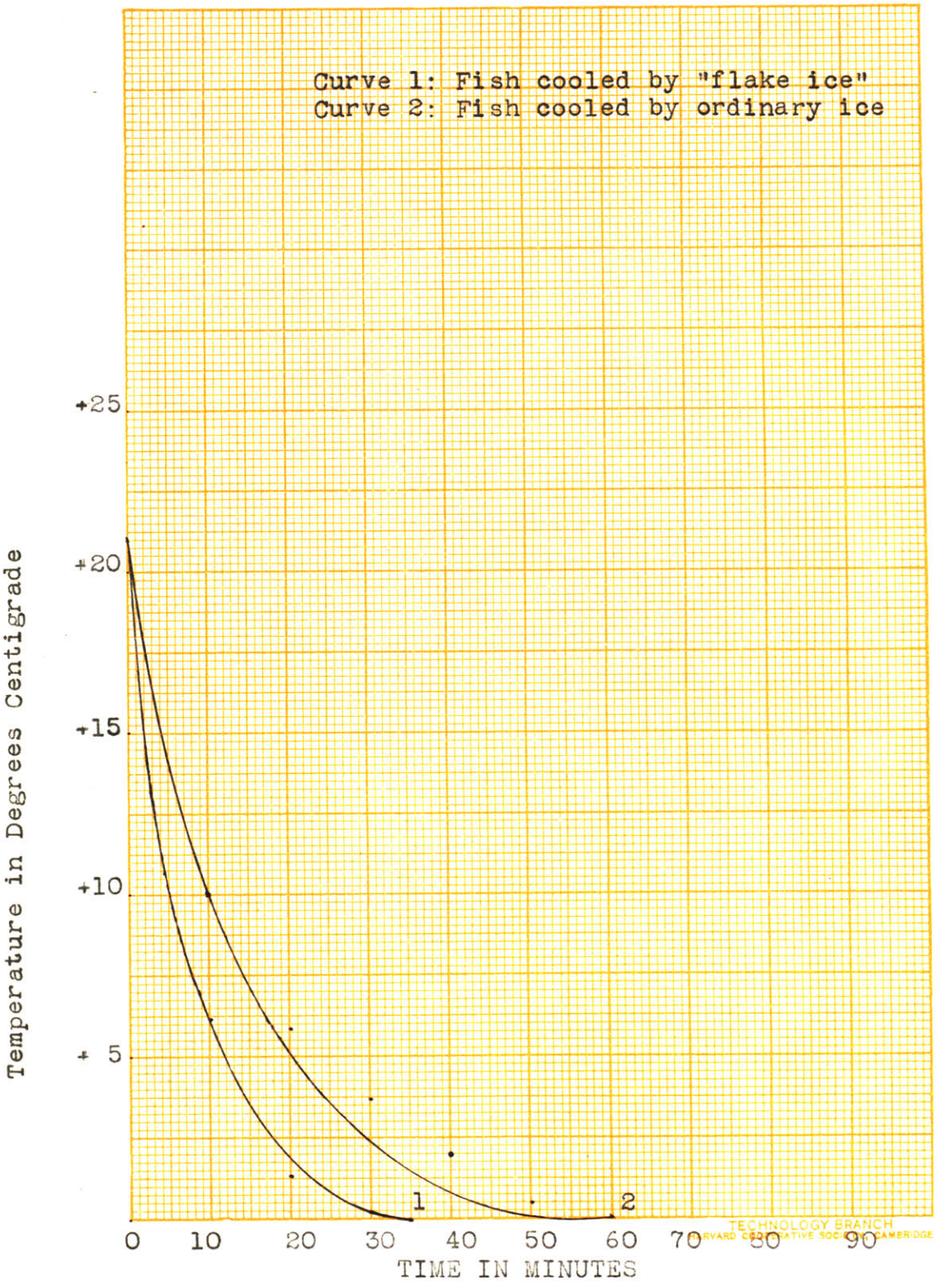

Curve 1: Fish cooled by "flake ice"
Curve 2: Fish cooled by ordinary ice
Temperature in Degrees Centigrade
+25
+20
+15
+10
+ 5
0
10
20
30
40
50
60
70
80
90
TIME IN MINUTES
1
2
TECHNOLOGY BRANCH

Table 23

TIME TAKEN BY DIFFERENT KINDS OF COMMERCIAL ICE TO COOL FISH TO 0° C.

Fish used: haddock

Weight of fish: 1116.7 grams

Condition of fish: intestines removed, head intact

Time	Temperature using Crushed ice	Flake ice
At start	22.2 deg., C.	22.2 deg., C.
5 minutes	15.2 " "	12.8 " "
10 "	10.6 " "	6.6 " "
15 "	7.8 " "	2.4 " "
20 "	6.1 " "	1.8 " "
25 "	5.0 " "	1.3 " "
30 "	4.1 " "	0.7 " "
35 "	3.2 " "	0.2 " "
40 "	2.2 " "	0.0 " "
45 "	1.3 " "	.....
50 "	0.9 " "	.....
55 "	0.7 " "	.....
60 "	0.5 " "	.....
65 "	0.2 " "	.....
70 "	0.0 " "	.....

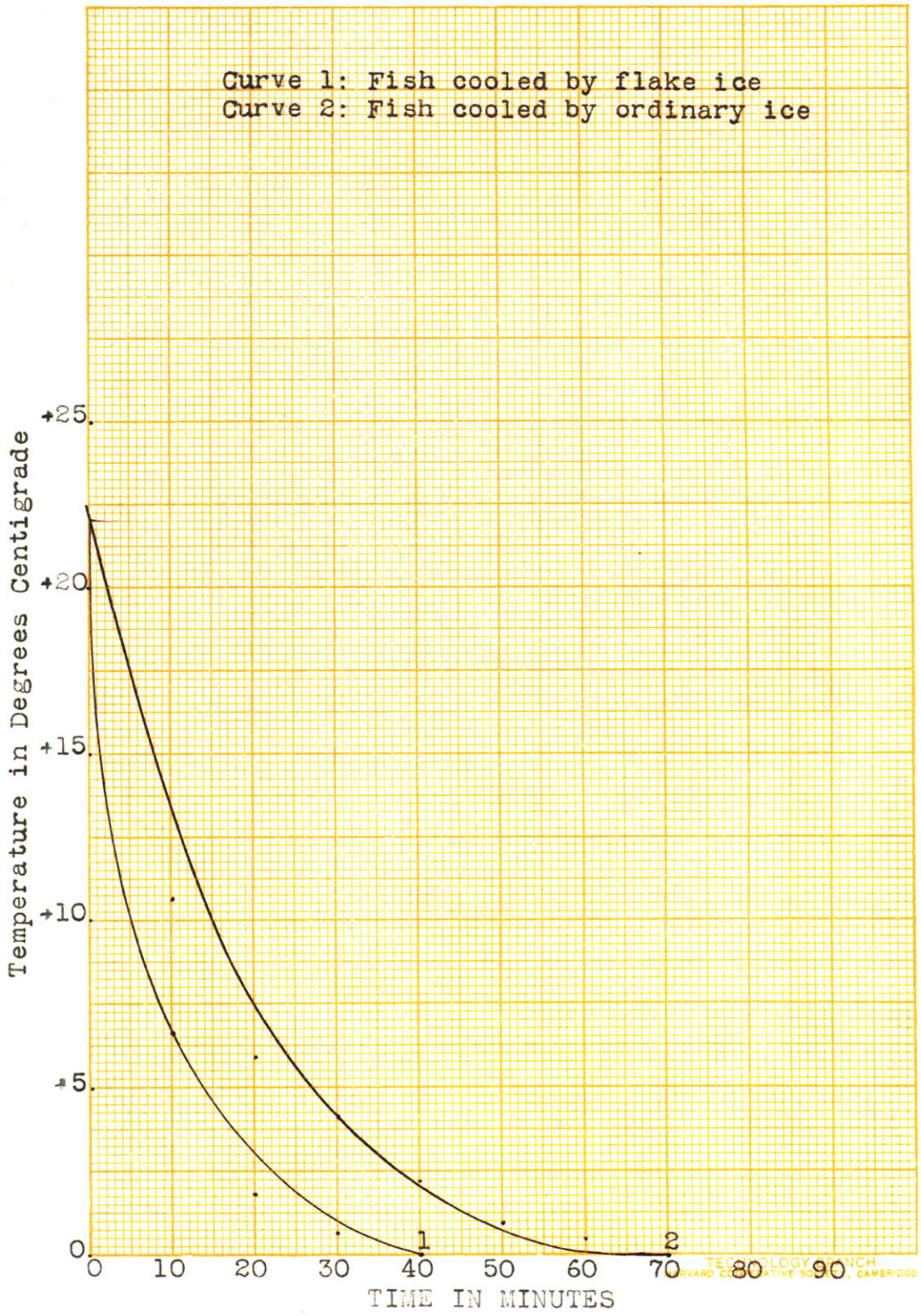

Curve 1: Fish cooled by flake ice
Curve 2: Fish cooled by ordinary ice
Temperature in Degrees Centigrade
+25.
+20.
+15.
+10.
+5.
0.
0
10
20
30
40
50
60
70
80
90
TIME IN MINUTES
1
2

Table 24

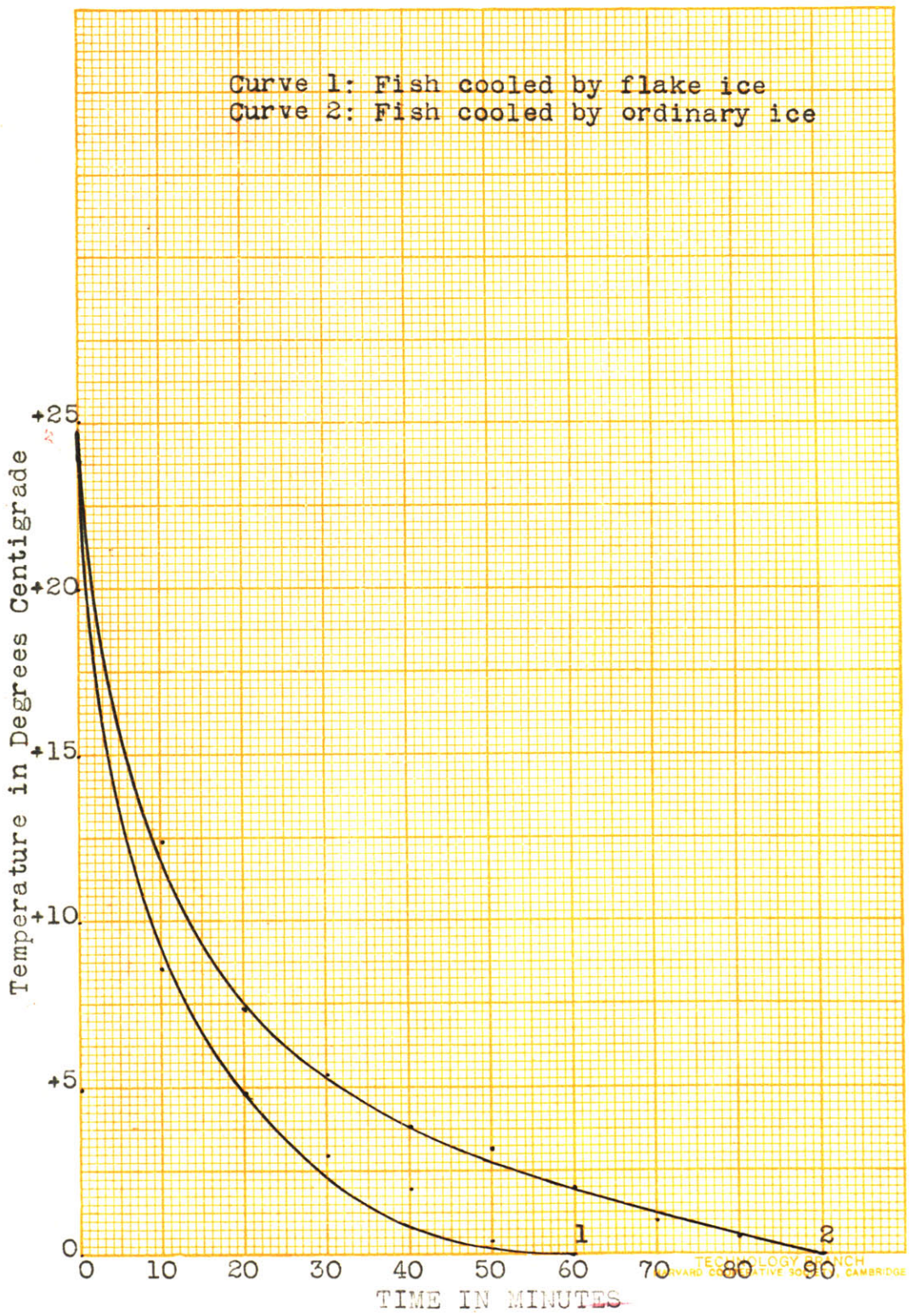
TIME TAKEN BY DIFFERENT KINDS OF COMMERCIAL ICE TO COOL FISH TO 0° C.

Fish used: haddock

Weight of fish: 1536.7 grams

Condition of fish: intestines removed, head intact

Time	Temperature using Crushed ice	Flake ice
At start	24.8 deg., C	24.6 deg., C.
5 minutes	16.0 " "	14.8 " "
10 "	12.4 " "	8.6 " "
15 "	8.8 " "	6.5 " "
20 "	7.4 " "	4.8 " "
25 "	6.2 " "	3.7 " "
30 "	5.4 " "	3.0 " "
35 "	4.8 " "	2.5 " "
40 "	3.8 " "	2.0 " "
45 "	3.4 " "	1.0 " "
50 "	3.2 " "	0.4 " "
55 "	2.8 " "	0.2 " "
60 "	2.0 " "	0.0 " "
65 "	1.6 " "	"
70 "	1.0 " "	"
75 "	0.8 " "	"
80 "	0.5 " "	"
85 "	0.2 " "	"
90 "	0.0 " "	"


Curve 1: Fish cooled by flake ice
Curve 2: Fish cooled by ordinary ice
Temperature in Degrees Centigrade
+25
+20
+15
+10
+5
0
0
10
20
30
40
50
60
70
80
90
TIME IN MINUTES
1
2

METHODS USED TO DETERMINE THE EFFECT OF LOW TEMPERATURES ON ENZYMES

In order to determine whether or not low temperatures have an effect on enzymes the following experiment was carried out: 400 cc. of nutrient gelatin,(1.2 grams of Bacto beef extract, 2 grams of Bacto peptone and 48 grams of Bacto gelatin, in 400 cc. of distilled water) were melted up. The mixture was allowed to cool and 1 gram of trypsin, (Cenbral Scientific C9.) was added. The enzyme was thoroughly mixed with the substrate and then 50 cc. of this mixture was placed in each of four test tubes; the tubes were stoppered with rubber stoppers and placed in a mixture of ice and HCl to cool. While the gelatin in the tubes was solidifying, a formol titration was made on 5 cc. of the remaining gelatin-trypsin mixture. The formol titration was carried out by adding 25 cc. of distilled water to 5 cc. of the gelatin-trypsin mixture, then adding 1.5 cc. of neutral formaldehyde and two drops of phenolphthalein and titrating against N/10 aequous NaOH. After the gelatin in the tubes had solidified two of the tubes were placed in a cold storage room at 0 ^{o} F. The remaining two tubes were placed in a water bath at 37 ^{o} C., where they were held for 3 hours. A formol titration was then run on 5 cc. of the contents of each of the tubes held at 37 ^{o} C.

The tubes which were placed in cold storage were left there for 21 days. They were then placed in a water bath at 37 ^{o} C., for three hours. Formol titrations were then made on

5 cc. of the contents of each tube as outlined above.

Table 25

EFFECT OF LOW TEMPERATURES ON ENZYMES

cc. N/10 NaOH at start		cc. N/10 NaOH used after cooling and then incubating at 37° C.		cc. N/10 NaOH used after cooling, holding at 0° F., for 21 days and then incubating at 37° C.	
1	2	1	2	1	2
4.32	4.26	10.91	11.04	10.89	10.95
Average: 4.29			10.97		10.92
		Minus blank	6.68		6.63

PLASTEIN FORMATION

Since the results of the determinations on sterile muscle suggested the possibility that an enzyme synthesis was taking place, in the later stages of the incubation, in the fish muscle, it was believed that something might be learned from an experiment to demonstrate the presence or absence of plastein formation.

12 grams of the incubated, sterile muscle were ground up with 6 cc. of water and the mixture was filtered through a sterile Mandler bacteriological filter into a sterile test tube. The test tube was then stoppered with a sterile rubber stopper and incubated at 35° C. Observations were made hourly to determine whether or not any solid substance was formed.

This experiment was carried out on one portion of sterile muscle that had incubated for 24 hours at 15° C., and also on another portion that had incubated for 48 hours at 15° C.

The biochemical results show that enzyme action in sterile muscle has reached its maximum at or between the 24 and 48 hour periods. A maximum of amino acids, the product of enzyme action, are necessary in order to obtain a plastein formation. It was believed, therefore, that either the muscle which had incubated for 24 hours or that which had incubated for 48 hours at 15° C,, would give the best demonstration of plastein formation, if plastein formation could be demonstrated by this method.

Table 26

PLASTEIN FORMATION

Time of incubation	Observations made on 24 hour filtrate	Observations made on 48 hour filtrate
At start	clear	clear
1 hour	opalescent	cloudy
2 hour	cloudy	cloudy
3 hour	cloudy	solid formation
4 hour	solid formation	solid formation
5 hour	about ½ solid	about ¾ solid

EXPERIMENTS MADE WITH TOLUENE

In order to determine the efficiency of toluene as a disinfectant, when used to stop bacterial growth in a mash of fish muscle, bacteriological counts were made on fish muscle mixtures containing different amounts of toluene.

In the first two experiments infected fish muscle was ground, distilled water was added and then 5 per cent, by volume, of toluene. More toluene was placed over the surface of the muscle in the tubes and the tubes were stoppered with rubber stoppers. Bacteriological counts were made thereafter at definite periods. The counts were made on the muscle in the tubes by allowing the the water and the toluene to drain away from the the flesh, and weighing out 1 gram of the drained muscle into 10 cc. of sterile dilution water. Dilutions were made from this mixture. Nutrient agar was used as a medium for bacterial growth. Plates were incubated for 72 hours at 25° C.

The second set of experiments differed from the first only in that 20 per cent, by volume, of toluene was added to the muscle mixture in the tubes.

Table 27

BACTERIOLOGICAL COUNTS MADE ON FISH MUSCLE MIXTURES CONTAINING TOLUENE

Experiment No. 1: 5 per cent of toluene by volume mixed with water and muscle. Muscle mixture placed in tubes and a layer of toluene placed over mixture. Tubes incubated at 25° C.

Time	Bacteriological count	
48 hours	24,000,	25,000
120 hours	2,800,000,	2,500,000

Experiment No. 2: same as No. 1

Time	Bacteriological count	
48 hours	16,100,	13,000
120 hours	1,760,000,	1,810,000

Experiment No. 3: 20 per cent of toluene by volume mixed with water and muscle. Muscle mixture placed in tubes and a layer of toluene placed over mixture. Tubes incubated at 25° C.

Time	Bacteriological count	
At start	60,	90
24 hours	20,	20
48 hours	40,	45
72 hours	80,	120
96 hours	4,900,	5,300
146 hours	97,000,	106,000

Experiment No. 4: Same as No. 3

Time	Bacteriological count	
At start	98,	111
24 hours	51,	41
48 hours	56,	48
72 hours	184,	162
96 hours	23,000,	31,000
146 hours	270,000,	350,000

DISCUSSION OF RESULTS

THE PRESENCE OR ABSENCE OF BACTERIA IN LIVING FISH TISSUES

In order to facilitate a logical discussion of the results of the experimental work concerning the sterility of fish muscle, a summary of these results is given below.

(1) 158 muscle samples from 11 haddock and 3 scrod were examined.

(2) 336 agar plates and 374 broth tubes were innoculated with material from the 158 muscle samples while 168 agar plates were made for controls.

(3) 5 of the 336 agar plates innoculated with material from the 158 muscle samples were found to contain bacteria.

(4) 1 of the 336 agar plates innoculated with material from the 158 muscle samples was found to contain molds.

(5) 2 of the 168 control plates were found to contain bacteria.

(6) 5 of the 374 broth tubes innoculated with material from the 158 muscle samples were found to contain bacteria.

(7) 1 of the 374 broth tubes innoculated with material from the 158 muscle samples was found to contain molds.

(8) The 5 agar plates which were found to contain bacteria, (included in (3)), were the plates of the following dilutions: 1/100,000, 1/10,000, 1/100, and 1/10, 1/10. Of these, the 1/10 and 1/10 dilutions were duplicate plates.

(9) The agar plate in which molds were found, (included in (4)), was one of the 1/100 dilution plates.

(10) The 5 broth tubes which were found to contain bacteria, (included in (6)), all were those in which the muscle sample had been placed in the broth tube without dilution.

(11) The broth tube which was found to contain molds, (included in (7)), was one of the 1/10 dilution tubes.

In considering the significance of the above results it should be pointed out that in a research of this kind, where it is necessary to pour a large number of agar plates, it is not unlikely that a small number of these plates would be accidently contaminated. For this reason, any case in which the presence of bacteria or molds is indicated only by one of two duplicate plates, excepting those cases where plates or tubes are made from undiluted samples, would appear to indicate a case of accidental contamination. It is believed that the two agar plates from the 1/10 dilution, (see (8)), are the only ones in which bacteria have been found in such a manner as to allow the possibility that these bacteria were present in the fish muscle.

Gee, (23),(24), obtained bacteria from fish in certain instances and concluded that a spore forming rod may be present in healthy living fish tissues. There would seem to be some question as to the accuracy of Gee's conclusions since he obtained bacteria from not more than 12 per cent of his samples and considering the fact that the method, used in his first research to sterilize the instruments, was found to be at fault.

Stewart, (59), examined 143 cultures of muscle from 8 codlings and obtained bacteria from something over 3 per cent of the samples. He concluded that the muscle of normal fish is sterile.

As has already been pointed out, it is believed that the results of this research indicate that bacteria were obtained from only 6 of 86 haddock muscle samples. The methods used to isolate fish muscle are not without fault. The conclusions of the above authors and those of other investigators would seem to designate that the results of this investigation support the conception that the muscle of healthy living haddock is sterile.

THE PRESENCE OR ABSENCE OF BACTERIA IN THE BLOOD OF LIVING FISH

Tables, (6A to 6C) show that bacteria and molds were found in the plates made from fish blood in the following instances:

(1) 3 blood samples, one each from 2 silver hake and 1 squirrel hake, were examined.

(2) 42 agar plates and 36 broth tubes were innoculated with material from 3 blood samples. 18 agar plates were made for controls.

(3) 1 of the agar plates innoculated with the fish blood material was found to contain a bacterium.

(4) 2 of the agar plates innoculated with the fish blood material were found to contain molds.

(5) The broth tubes innoculated with fish blood material, both those incubated aerobically and those incubated anaerobically, all remained sterile.

(6) The agar plate found to contain the bacterium, (included in (3)), was one of the plates of the 1/100 dilution mixture from the blood of the squirrel hake. The duplicate plate was sterile.

(7) The agar plates found to contain molds, (included in (4)), were plates from the 1/10,000 and 1/100,000 dilution mixture of the blood of silver hake No. 1. The duplicate plates were found to be sterile in each case.

From the foregoing it can be seen that in no case have bacteria been found, in agar plates and broth tubes made

from fish blood material, in such a manner as to suggest the possibility that they were present in the fish blood.

THE DECOMPOSITION OF FISH MUSCLE DUE TO BACTERIAL AND ENZYME ACTION

Enzyme Decomposition of Fish Muscle at 0° C.

According to the values found for amino nitrogen and carboxyl groups, enzyme hydrolysis of haddock muscle reaches a maximum, when the fish is held at 0° C., in about 48 hours. The decrease in amino acids is continuous from this time up to the end of the 120 hour period.

Bacterial Decomposition of Fish Muscle at 0° C.

Bacterial action, as shown by biochemical tests for amino acids, is very slight, if at all, in haddock muscle held at 0° C., until the 72 hour period has passed. Up until this time the values for amino acids in infected muscle follow those for amino acids in sterile muscle. After the 72 hour period the amino acid values for the infected muscle increase continuously.

Enzyme Decomposition of Fish Muscle at + 5° C.

The values for amino nitrogen and carboxyl groups indicate that there is an increase in the amino acid content of sterile haddock muscle up to the end of the 72 hour period. Ammonium nitrogen also increased up to this time. The values for amino acids thereafter decrease progressively and there is also a very slight decrease in ammonium nitrogen. The decrease in ammonium nitrogen is probably due to the loss of volatile basic products.

Bacterial Decomposition of Fish Muscle at + 5° C.

The values for amino acids in infected haddock muscle at + 5° C., show some discrepancies. In one fish amino acid values followed

those found in the sterile muscle up to the 72 hour period. In the other fish incubated at + 5° C., bacterial action made itself manifest, as shown by siginficant increase in amino acids, after 48 hours. Ammonium nitrogen values indicated that there is no appreciable increase in bacterial action until the third day has passed.

Enzyme Decomposition of Fish Muscle at + 10° C.

Enzyme action in sterile haddock muscle incubated at + 10° C. causes an increase in amino acids up to the end of the second day. The decreased in amino acids is thereafter noticeable to the 120 hour period. The increase in amino acids during the first 24 hours is much greater than that during the next 24 hour period. Likewise the decrease in amino acids during the 48 to 96 hour period is more pronounced than that of the next 24 hours. Ammonium nitrogen shows an increase until the 48 hour period has passed, there is thereafter a slight decrease in ammonium nitrogen.

Bacterial Decomposition of Fish Muscle at + 10° C.

In both fish incubated at + 10° C., the increase in amino acids during the first 24 hours was greater than that shown by the sterile muscle. The decrease in amino acids took place after this time. Bacterial action, as indicated by a significant increase in amino acids and ammonium nitrogen, was apparent after the 48 hour period.

Enzyme Decomposition of Fish Muscle at + 15 to 17° C.

Both ammonium nitrogen and amino acids increase for 24 hours in

sterile haddock muscle incubated at + 15 to 17° C. The decrease in amino acids, noted at lower temperatures, takes place after this period.

Bacterial Decomposition of Fish Muscle at + 15 to 17° C. There is no appreciable amount of bacterial action in fish muscle held at + 15 to 17° C., until the 48 hour period has passed. Ammonium nitrogen increases enormously after this period.

DISCUSSION OF THE RELATIVE VALUES OF CERTAIN BIOCHEMICAL TESTS USED ON FISH MUSCLE

The Van Slyke Method. The Van Slyke method seems to give correct values for amino acids. However, due to the fact that there is a decrease in amino acids in haddock muscle, during certain stages of the incubation, this method would be of little use to determine freshness in the haddock. In the sterile muscle the values are often lower in the final stages of the incubation than they were at the start. Some trouble has been experienced in obtaining blanks below 0.300 cc. of gas at the start of each days work. This seems to be due to an accumulation of gas in some part of the apparatus, probably in the alkaline permanganate solution, which can be removed only by running the apparatus for some time.

The Electrometric Titration Method. This test is of doubtful value in following the proteolysis of haddock muscle. The B value, which is supposed to measure bacterial decomposition and according to Lemon and Stansby, should never exceed 8 unless some bacterial decomposition has taken place, has been found to vary from 6.11 to 14.55 in haddock muscle which was sterile. Nor do these values agree with any of the other tests used to follow decomposition, as the incubation continues. The A value, or measure of enzyme decomposition, has been found to follow proteolysis no better than does the B value, being often much lower than the conditions warrant.

The reason for the irregular results of the electrometric titration test would seem to be due to the fact that the method is founded on two contrasting principles. The smaller the amount of decomposition of any kind, the more acid used, due to the fact that the protein molecule acts as a buffer. On the other hand, the greater the amount of decomposition of any kind, the more acid used, due to the fact that basic products are formed which neutralize acid.

The Harris Modification of the Formol Titration. The Harris modification of the formol titration has been found to be very good as a method by which to determine amino acids. However, as in the case of the Van Slyke method, it is of doubtful value to determine freshness in the haddock where a decrease in amino acids is likely to take place.

The Ammonium Nitrogen Test. The ammonium nitrogen test is an excellent method by which to follow decomposition in haddock muscle. The values for ammonium nitrogen in infected muscle increase progressively and serve well to indicate the amount of decomposition. In the sterile muscle there is a slight decrease in ammonium nitrogen values in the later stages of the incubation, nevertheless, the decrease is not sufficient to nullify the results obtained.

General. Observations show that sterile muscle has no bad odor after it has been held for 96 hours at temperatures as high as 17° C. A slight fishy odor is given off by sterile muscle during certain stages of the incubation at higher temper-

atures.

There is a marked difference in the odor of the haddock itself and that of the muscle removed from the haddock. Whereas the fish itself has a putrid odor in the later stages of the incubation at higher temperatures; (15 to 17°C) the muscle, removed to a beaker, never has more than a slightly stale odor at the end of the 96 hour incubation period.

The fact that no other worker in this field has reported a decrease in amino acids in the later stages of the incubation of sterile haddock muscle, would seem to designate that toluene does not sufficiently inhibit bacterial action when used as a disinfectant in a fish muscle mash.

It is probable that the method of grinding fish muscle and placing it in flasks does not present the true picture of bacterial action as it takes place in the fish. The surface area of fish muscle is greatly increased by grinding, hence bacterial action and proliferation is facilitated. The fact that the values, found by other workers for ammonium nitrogen, and pH, are much higher than those found during this research, suggests that decomposition of ground fish is much more rapid than is the decomposition of the whole fish.

Different fish were used as the temperature was varied, for the series of proteolytic determinations. The results show that there are some few discrepancies in the values found. This is to be expected since it is probable that the enzyme concentration is not identical in different fish of the same species.

In the same manner there is doubtlessly a difference in the number of bacteria found on the gills and in the slime of different haddock.

CHANGE IN pH

In sterile haddock muscle the pH first increased and then decreases slightly in the later stages of the incubation.

In the infected muscle the pH increased progressively until the end of the incubation period was reached.

The pH of sterile muscle was never found to be less than 6.55

The pH of the infected muscle never reached a value of more than 6.95

Reed, Rice and Sinclair, (48), found that the pH of ground, infected haddock muscle, incubated at about 20° C., reached a value of 7.0 in 24 hours. These findings do not agree with those obtained during the research here reported. It is probable that this discrepancy in the results of the two investigations is due to a difference in the methods used in each case. In the former research the infected fish muscle was ground and placed in flasks, while during the present research the infected muscle was taken from the incubated fish itself.

The Relative Values of Certain Kinds of Commercial Ice as Refrigerants. The results obtained in the cooling experiments indicate that there is some advantage in using "flake ice" rather than ordinary broken ice as a refrigerant to cool fish. The cooling curves,(page 90-95),show that it takes from 25 to 30 minutes longer to cool a fish from 20-25° C., to 0° C., with ordinary ice than it does to do the same thing with "flake ice".

It is to be expected that it should take longer to cool a substance with ordinary broken ice than it does with "flake ice". Ordinary broken ice varies in size, having many large pieces as well as small pieces with the consequence that it does not pack together well and there are many air spaces, some of which are between the surface of the fish and the ice. Flake ice, on the other hand, is of a relatively small size, and what is more important, all of the pieces of flake ice are of the same shape the result is that it packs together so as to leave only a few small air spaces. Moreover, flake ice is flat and has a greater surface area per unit weight than ordinary ice. For these reasons flake ice makes better contact with the surface of a fish than does ordinary crushed ice.

The rate at which heat is carried away by conduction is dependent upon the ability of the surrounding medium to conduct heat away. This is a very important factor in cooling since different substances vary greatly in their ability to conduct heat.

Air is a very poor conductor of heat while ice is a very good one. The thermal conductivity of air at 0° C., is 0.0000568 calories per cm. per degree per second, while that of ice is 0.00568 calories per cm, per degree per second. The amount of surface contact which the ice makes with the substance to be cooled is, therefore, vastly important since good contact means that heat is mostly being carried away through the ice, while poor contact means that the heat must first traverse an air space before it can be carried away by the ice.

Toluene as a Disinfectant. From the experiments carried out, it appears that toluene is not sufficiently bactericidal to be used as a disinfectant in a fish muscle mash. In fish flesh which is fairly heavily contaminated at the start there is a large increase in the number of bacteria even in 48 hours after the toluene is added. Large amounts of toluene, (20 per cent by volume) do not inhibit bacterial growth altogether since after 72 hours the bacteria have increased their number by more than 100 per cent.

It would appear that when large amounts of toluene are added to mashes of fish muscle, there is first a decrease followed by a progressive increase in the number of bacteria present. It is probable that only certain types of bacteria are able to proliferate in the presence of toluene since the colonies on the agar plates, made from the toluene muscle mixture, were mostly of two types after the 48 hour period had passed.

A POSSIBLE EXPLANATION OF THE DECREASE IN AMINO ACIDS WHICH TAKES PLACE DURING THE LATER STAGES OF THE INCUBATION IN STERILE MUSCLE

In the present investigation it has been shown, that during the later stages of incubation of sterile muscle, amino nitrogen and carboxyl groups decrease while the values for ammonium nitrogen remains virtually constant. It has also been possible to demonstrate something which clearly resembles a plastein formation in the incubated sterile muscle. Percentage curves, (page 80 to page 83), show that in some cases the decrease in amino nitrogen is greater than 28 per cent during certain stages of the incubation. The decrease in amino nitrogen and carboxyl groups varies somewhat with the temperature at which the muscle is incubated.

These results suggest that an enzyme synthesis takes place in fish muscle after enzyme action has first carried out an hydrolysis of proteins.

Rona and Chrometzka, (52), found that the ammonium nitrogen increases along with the decrease in amino nitrogen during plastein formation from casein digests. They believe that the decrease in amino nitrogen is due to a deaminization of the amino acids, since the carboxyl groups do not decrease. These findings are contrary to those of other workers.

The values found for ammonium nitrogen in this research show a slight decrease, rather than an increase, in the final stages of the incubation of sterile muscle. It cannot be

said, then, that the decrease in amino nitrogen is due to the deaminization of the amino group. The slight decrease in ammonia in the later stages of the incubation is probably due to the loss of volatile basic products. It has been found that there is always a decrease in carboxyl groups of amino acids during the later stages of the incubation of the sterile muscle.

There is a remote possibility that rigor mortis is connected, in some manner, with plastein formation, or an enzyme synthesis of protein in the muscle. It was once believed that rigor mortis is due to the formation of lactic acid in the muscle. It has since been shown by Claude Bernard,(5), and by Hoet and Marks,(34), that rigor mortis can take place without any production of lactic acid, although the quantity present will determine the amount of shortening and the tension developed by the muscle when rigor appears. Hoet and Marks suggest that there is needed a rapid disappearance of hexose-phosphate from the muscle, through the failure of resynthesis, in rigor mortis. The resynthesis normally goes on for some time after the death of the animal.

The failure of resynthesis may be due to the adsorption of the hexose-phosphate enzyme upon the plastein formed in the muscle, if plastein formation really takes place at this time.

Smith,(56), showed that the gelation of muscle plasma is accompanied by no constant change in acidity. He was of the opinion that the process of gelation is identical with the stiffening of the muscle in rigor mortis. Smith later,(57),

found that the coagulation of muscle plasma is due to the precipitation of myosin from a sol not originally present in muscle but formed by the addition of salt to the muscle before the muscle is expressed. He concludes that not more than 10 per cent of the total myosin in rabbit muscle is present in the form of a sol so that gelation of myosin probably has little to do with rigor mortis.

It is clear that little is known about rigor mortis or the manner in which rigor mortis takes place. Two facts would tend to eliminate the possibility of plastein formation during rigor mortis: (1) Macpherson, (40), Macleod and Simpson, (39), and Leim, Macleod and Simpson, (37), state that rigor mortis takes place in the fish in from 1 to 3 hours after the fish is caught. This would seem to be too soon to allow a sufficient accumulation of amino acids to support an enzyme synthesis of protein. (2) Hewer and Jiaram, (33) have shown that there is an increase in diamino acids, rather than a decrease, during rigor mortis. This is opposed to the findings during enzyme syntheses of protein.

CONCLUSIONS

1. Six of the 158 haddock muscle samples examined showed the presence of bacteria. Considering the fact that the methods used to isolate the muscle did not rule out the possibility of accidental contamination, the low percentage of positive samples supports the probability that the living tissues of normal haddock are sterile.

2. The blood of one squirrel hake, (Urophycis chuss), and two silver hake, (Merluccius bilinearis), has been found to be sterile.

3. At temperatures between 0°C., and + 17° C., enzyme hydrolysis of proteins in haddock muscle takes place for from 48 to 96 hours hours after the start of the incubation period.

4. At temperatures between 0° C., and + 17° C., there is no significant invasion of bacteria into the muscle of the haddock, as shown by bio-chemical tests, until 48 hours after the start of the incubation period.

5. The pH of infected haddock muscle increases only very slowly. Haddock muscle which is definitely stale has a pH of not more than 6.95. The pH of the sterile muscle shows a slight decrease in the later stages of the incubation.

6. In sterile haddock muscle the values for amino acids, Van Slyke amino nitrogen, and formol titration values,

have been found always to decrease in the later stages of the incubation period.

7. Both the Van Slyke method for determining primary aliphatic amino nitrogen and the Harris modification of the formol titration appear to give satisfactory values for amino acids.

8. The electrometric titration method has been found to give irregular results in following the proteolysis of haddock muscle.

9. The ammonium nitrogen test is very satisfactor as a method by which to follow proteolysis of haddock muscle.

10. Flake ice is of more value than ordinary broken ice as a refrigerant to lower the temperature of fish.

11. Toluene does not inhibit bacterial action sufficiently to warrant its use as a disinfectant in fish muscle which is incubated over a period of more than forty-eight hours.

12. Low temperatures, (0° F.), for a period of 21 days, have little or no destructive effect on the enzyme trypsin.

SUGGESTIONS FOR FUTURE INVESTIGATIONS

In order to determine whether or not an enzyme synthesis takes place in fish muscle it is necessary that determinations be made for amino and carboxyl groups and for total nitrogen before and after the plastein formation. An investigation of this kind would be of great value and would show definitely the course of the enzyme synthesis.

Rigor Mortis and its Relation to Enzyme Synthesis. An investigation to determine the hourly change in amino and carbóxyl groups before and during rigor mortis in fish would be of definite scientific value. At present little is known of the course and cause of rigor mortis, the formation of lactic acid and the disappearance of glycogen have been shown to be incidental to the process of rigor mortis. Something might be learned of the relation of rigor mortis to enzyme synthesis if such a research were carried out. It should be emphasized that an investigation of this kind would virtually necessitate the use of a floating laboratory.

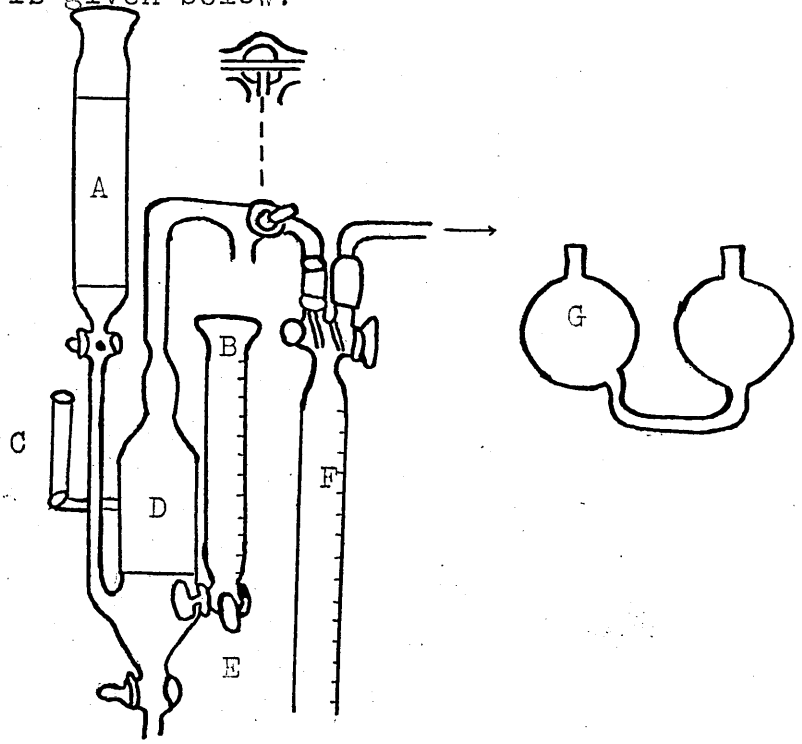
Decomposition as it Takes Place in Fresh Fish and in Fish Which Have Been Frozen. It is very probable that there is some difference in the speed with which decomposition takes place in a fresh fish and in a fish which has previously been frozen. Something might be learned of the best manner in which to prevent decomposition in fish which have been frozen from an investigation of this kind.

Changes That Take Place in Frozen Fish During Cold Storage. Certain changes take place in fish muscle during storage at low temperatures. The change at very low temperatures is less than that which takes place at temperatures below but near 0° C. Certain investigators claim that the change is due to a denaturation of protein in the fish muscle which takes place in a relatively short period. Other workers are of the opinion that such changes take place only after long periods of storgae. An investigation of proteolytic changes which take place during storage would throw some light on this problem in that it would determine whether or not these changes are due to enzyme action.

The problem of quick freezing is also involved in this question. Is the difference between products which are quickly frozen and those which are frozen more slowly, due to the fact that large crystals, which injure cells, are formed during slow freezing; or are other factors involved? A research of this kind would possibly contribute something both to science and to commerce.

APPENDIX

Description of the Van Slyke Apparatus. A diagram of the Van Slyke apparatus which was used to determine amino acids in fish muscle is given below:



A, reagent burette.
B, sample burette.
C, Capryl alcohol burette.
D, deaminizing bulb.
E, stopcock connecting B to the waste and B to D.
F, gas measuring burette.
G, Hempel pipette.

The reagents, glacial acetic acid and sodium nitrate solution, are measured into the deaminizing bulb through A. The deaminizing bulb is then shaken until the reacting solution is forced back to a calibration on D. The stopcock connecting A and D is then closed, thus assuring a constant amount of react-

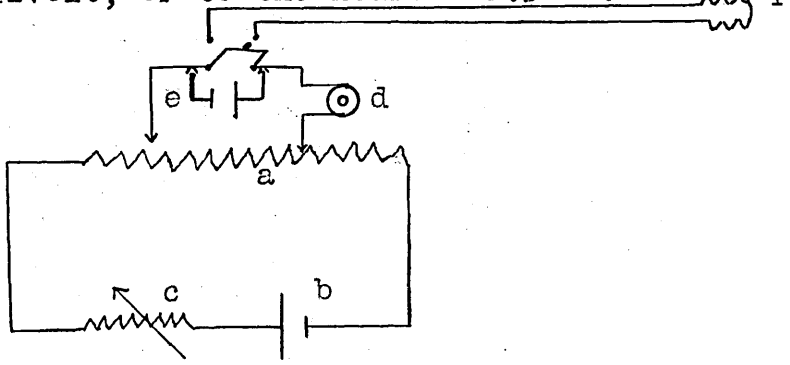
ing solution.

The unknown solution is then measured into D through B after which the deaminizing bulb is shaken for three minutes. Capryl alcohol is run into the deaminizing bulb before the bulb is shaken in order to prevent foaming.

After deaminization the gas produced is forced into the hemple pipette where the nitric oxide is absorbed in an alkaline permanganate solution. The Hemple pipette is shaken during the process.

After the nitric oxide has been absorbed the nitrogen gas, which remains, is forced into the gas measuring burette where it is measured.

The Thermocouple Setup. The following is a diagram of the thermocouple setup used to determine the temperature of fish which were being cooled by ice. The potentiometer is first set at the e.m.f. of the standard cell and the rheostat is adjusted until the galvanometer shows no deflection, the potentiometer then reads directly in millivolts. The thermocouple was first standardized by keeping the hot junction at 100° C., and varying the temperature of the cold junction from 0° C., to 30° C.; the readings obtained at the different temperatures were tabulated and a graph was made from the readings obtained. The e.m.f. could be determined potentiometrically to the nearest 0.1 millivolt, or to the nearest 0.5° C.



a, rheostat
b, dry cell
c, potentiometer
d, galvanometer
e, standard cell
f, thermocouple

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