THE DECOMPOSITION OF FISH FLESH UNDER NATURAL CONDITIONS TOGETHER WITH THE QUANTITATIVE ESTIMATION OF SOME OF THE PRODUCTS FORMED

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The Decomposition of Fish Flesh under Natural Conditions together with the Quantitative Estimation of some of the Products Formed

By James Arnold Dauphinee

The experiments reported in the following pages were done at the Fisheries Experimental Station (Atlantic) during the months of August and September of the year 1926. They represent a small amount of the work of this nature carried on by the various workers under the direction of Dr. A.G. Huntsman at the Biological Station at St. Andrews and the Fisheries Experimental Station at Halifax.

The ultimate object of the work on decomposition is to find a quick, simple, and effective means to gauge the stage to which the processes of decomposition have gone on in individual fishes under various conditions of storing and curing. Having established a definite criterion by which to gauge the extent of these decomposition processes, then constructive and result-bringing work could be done upon the mechanisms which hinder or speed up this decomposition and thus enable the industry to be shown how best to treat their product in order to insure a good article being offered to the public both at home and in outside markets.

By the term decomposition as used in this paper is meant the sum total of all the destructive processes which commence to operate the instant that the fish dies whether in or out of the water. These processes can be divided into two great classes, autolytic and bacterial.

A. Autolysis

It is well known that in all the organs and muscles of an animal there are certain autolytic enzymes. During life these enzymes quite likely play a very important part in synthesizing from the contents of the blood stream the more complete proteins, fats, and carbohydrates of which the particular organ is composed. After death, however, the conditions are so altered that the major evident outcome of the activity of these autolytic enzymes is directed towards the hydrolytic aspect. As a consequence of their activity, proteins are broken down, to a greater or less extent, to more simple nitrogenous compounds by "proteases" which have been classified by Dernby (1) into three main groups,-namely, ereptases, tryptases, and peptases,-according to whether they resemble the erepsin, trypsin, or pepsin concerned in the ordinary digestion of proteins; glycogen is broken down into glucose by "glycogenases" and possibly by other enzymes into lactic acid and other hydrolytic products; fats may be broken down by intracellular lipases into their constituent fatty acids and glycerol; and all the other enzymes present are working in the hydrolytic direction.

The word autolysis has frequently been used chiefly in the restricted sense of the action of the proteolytic enzymes in the tissues. In this connection all the tissues that have been examined (with the exception of connective tissue) will autolyse to a greater or less extent (2) The epithelial tissues such as liver, mammary, salivary, thyroid, thymus, kidney and other glandular tissues hydrolyse most rapidly and most thoroughly. Muscle tissue autolyses to some extent but much less than gland tissue.

As we are chiefly interested in the edible portion of fishes the organs that have to be studied in this connection are the muscles. In this connection, it is interesting to note that in the case of cats, Vernon (3) compares the content of muscle erepsin with that of other organs and obtains the following results.

Table I

Tissue	Activity(Average)
Kidney	14.3
Pancreas	6.4
Spleen	7.6
Liver	5.0
Cardiac Muscle	1.59
Skeletal Muscle	0.77
Brain	1.24
Gastric Mucosa	3.9
Duodenal Mucosa	27.7
Jejunal Mucosa	18.2
Ileal Mucosa	14.4
Large Intestine Mucosa	5.8

So that it is seen in the case of mammals the "ereptase" (and very likely all the other proteolytic enzymes) is present in much less concentration in the muscles than in any of the other tissues.

The progress of autolysis has been shown to be speeded up by the addition of acids or substances, which hydrolyse to form acids, to the tissue brei (4) (5); it has been shown to be hindered or definitely prevented by the addition of sodium bicarbonate, sodium hydroxide, calcium carbonate, zinc oxide, magnesium oxide, and other basic compounds which make the reaction alkaline or keep it neutral. (4) It is therefore seen that the rate at which autolysis can go on is determined to a great extent by the reaction of the tissue. In fact Bradley (4(a)) goes so far as to state "As long as this (pH 7.4±) is maintained, there is no evidence that autolysis can go on either in vitro or in vivo." As it might be expected that the tissue would become acid due to post mortem changes, this increase in acidity would increase the rate of auto-digestion. The reactions which account for this acidity are the formation of lactic acid from glycogen and glucose, the formation of fatty acids from fats and the development of free phosphoric acid. In connection with this development of acidity Sevringhaus (6) has demonstrated that of the acids liberated in an autolysing liver, the fatty acids account for 15.8%, the carbon dioxide for 19.2%, the lactic acid for 4.6%, and the phosphoric acid for 60.4%. He found that within 24 hours the phosphoric acid (calculated as mgs. phosphorus) of autolysing liver rose from 94 mgs. per 100 gms. of tissue to 175 mgs. and in 48 hours the value had risen to 217 mgs. Mr. Simpson, working with Dr. MacLeod, has also found a corresponding liberation of phosphoric acid from autolysing fish muscle and although the increase amounted in this case to only 20 or so mgs. of phosphorus per 100 gms. of tissue. Dernby (1) found in an organ ~~free~~ brei that soon after death the pH was 7, that the acidity developed rapidly, and that within 24 hours the hydrogen ion concentration had increased to pH 6.5. For the next 10 days the reaction drifted back slowly to the neutral point as the autolysis proceeded usually reaching a level of pH 6.6 or 6.8. These changes occurred in ground up tissue that was kept sterile. In such cases the post mortem changes would increase the rate at which autolysis normally takes place. In fish muscle the experiments of the writer were somewhat confusing in that certain experiments showed a decided drop in the pH, during a short period immediately after the death of the animal, which soon returned to approximately neutrality. In other cases the pH remained throughout at approximately the same point as observed at the beginning until it became decidedly alkaline due to bacterial action. The cause of this discrepancy is not at all clear, though (providing that the exper -imental details are correct) it is quite possible that, since the development of acidity depends upon the initial presence of glycogen and fat and phosphoric acid yielding substance, the absence of these in some fishes would prevent the development of sufficient acid to overcome to any noticeable degree the buffering material of the tissues.

Oya, Sumi, and Shimata(8) have investigated the influence of various salts on the autolysis of fish muscle at the Imperial Japanese Fishery Institute. They found that sodium chloride and potassium chloride both accelerated the autolysis of fish muscle in low concentrations but whereas the sodium chloride accelerated the digestion even in concentrations as high as 0.2916 mol, the potassium chloride retarded the digestion in concentrations of 0.125 mol or above barium chloride and calcium chloride retarded the digestion even in concentrations as low as 0.0002 mol, and at 0.2916 mol almost completely stopped the digestion. Magnesium chloride accelerated the hydrolysis at about 0.0042 molbof above and caused complete stoppage at a concentration 0.2916 mol. The acidity of the mixture was increased by the addition of the salt which, would, as Bradley (4) has shown, then cause an increase in the rate of digestion. They found that copper and zinc sulphates aluminium chloride and cupric hydroxide retarded the autolysis whereas aluminium hydroxide gave some acceleration. They also found that the digestion of mackerel muscle was not stopped in the presence of large amounts of sodium chloride and went on slowly even in a saturated solution of this salt.

Oya and Shimada (8) found that the autolysis of mackerel muscle is accelerated by acid and retarded by alkali and that the optimum pH for this autolysis was between 4 and 5.

Chen and Bradley (9) have compared the rates of autolysis of various tissues and muscles from different types of animals and conclude (page 162)--" In general we find that warm blooded muscle tissue autolyses under much the same conditions as glandular tissue. This difference is due we believe to the greater connective tissue content of muscle and also to the presence within the muscle cells of structural proteins which are not affected by cell proteases under any condition. Only a small fraction of the total muscle proteins is susceptible to atrophic hydrolysis ..... In the muscles of the higher warm blooded animals we find what seems to be a relation between activity, pigmentation, and autolysis. The more active muscles contain more protein susceptible of autolysis. In some of the very active fishes muscle autolysis is more extensive than in the warm blooded animals and much more so than in the sluggish fishes.....

Thus they found that the muscle of the perch and the carp showed very little autolysis in their control experiments whereas mackerel and menhaden muscle underwent considerable autolysis. However the conditions under which their experiments were carried on were quite different from those on a fish dealers table. In the experiments of Chen and Bradley and practically every one who has worked on autolysis the muscle tissue has been ground up very fine, a definite quantity of water added preserved with a liberal quantity of toluene and placed in a water bath at 37°C. These conditions tend to promote autolysis to its fullest extent and are necessary for the study of this phenomenon. But it is impossible to leave whole fishes, unpreserved in any way for even a day at 37°C. without getting distinct evidence of bacterial contamination whereas the experiments of Chen and Bradley on autolysis were conducted over periods of from 10 to 20 days.

The point that the writer is trying to make is this--that in whole muscle tissue, unpreserved in any way (and these conditions are essentially those of a fish in a dealers hands) the concentration of autolysing enzymes is so small, the conditions are so far from the optimum, and the contamination by bacteria is so inevitable, that the processes of autolysis play a very small part in the decomposition of fish flesh. They hardly get started before they are ruled out and overshadowed by an increasingly active bacterial decomposition. That autolysis can be extensive when measures are taken to eliminate all other factors is certainly true but has little bearing on the question in hand. Factors such as lowering the temperature to stop the bacterial action will also stop the action of the autolytic enzymes and so also most influences which tend to increase the action of the autolytic processes are going to speed up the growth of putrefactive bacteria.

The experimental findings have borne out the above argument and little success has attended the development of a test based upon the autolysis of the proteins of the fish muscle; and as the first few experiments indicated this very little work was done on this phase of the problem.

The process of the autolysis of the flesh was measured in two different ways, first by following the increase in non-protein nitrogen of the fish muscle, and secondly by following the content of free amino-nitrogen of the muscle. The action of the autolytic enzymes should increase both these values.

The fish were obtained from the fishing grounds as fresh as possible, immediately cleaned, and packed in crushed ice. They were then brought to the laboratory and stored in boxes at the required temperature. One difficulty experienced was that one had frequently to take the fisherman's word for the time that the fish had been out of the water. The chief disadvantage of the Halifax Station for this kind of work is the difficulty one has of procuring fresh material when desired. Time and time again has the writer gone to the near by fishing grounds to find the fishermen either not catching anything or not fishing at all, and our own efforts rarely met with success. Any amount of fishes could be had from the National Fish Company, but the previous history of such fishes is quite unknown. However, much of the work has of necessity been done on these and is, therefore, in view of the work of Dr. MacLeod and Mr. Simpson, and of Dr. Huntsman and Dr. Leim on the necessity of controlling the conditions under which the fish have been caught, imperfect on this account.

Samples of the fish flesh were taken at intervals as follows. The skin of a portion of the back was removed and a portion of flesh weighing about 10-15 grams was removed. The portion of muscle was carefully weighed to the nearest decigram, thoroughly ground up with sand in a mortar and extracted on a boiling water bath for four successive periods of fifteen minutes each with 50 cc. 0.05% acetic acid. This coagulated all the coagulable protein and extracted from the coagulum all the soluble constituents. The extracts were all filtered through the same filter paper. The combined filtrate was cooled, treated with 6 grams of trichloracetic acid and made up to a final volume of 200 cc. with distilled water, thus making the final concentration of the acetic acid 3%. A slight amount of flocculent precipitate of non-coagulable protein was thrown down and was filtered off through a folded filter. For the estimation of the quantity of total non-protein nitrogen, 50 cc. of this filtrate were macro-kjeldahled and the ammonia distilled over the usual way. A 75 cc. portion of the remainder of the filtrate was titrated by the Sorēnsen formol method for the estimation of the free amino-nitrogen. This formol titration is not very satisfactory and the estimations would no doubt be far more accurate had they been done by the Van Slyke technique which, however, involves the use of an expensive piece of apparatus.

EXPERIMENT I

One haddock was obtained from the National Fish Company. It was of unknown age but was in good condition as far as could be told from the odor, appearance and firmness of the flesh.

Two portions of the flesh were weighed out.

Sample A. weighed 13.12 gms.
Sample B. weighed 13.49 gms.

Sample A. was ground up thoroughly in a glass mortar with sand, and made up to 100cc. with distilled water. It was allowed to stand for 15 minutes and was then precipitated with 60cc. 10% trichloracetic acid, made up to 200 cc. and was filtered.

Sample B. was ground up thoroughly in a mortar, transferred to a beaker with 50 cc. 0.05% acetic acid, heated on a water bath for 10 minutes, and decanted through a filter paper. This was repeated 4 times--passing through the same filter paper. The volume of filtrate was 150 cc. Added 6 gms. of trichloracetic acid, made up to 200 cc., and allowed the sample to stand for an hour, after which the flocculent precipitate was filtered off.

Two 50 cc. portions of each filtrate were kjeldahled. Sample A. gave a value of 356 mgs. of non-protein nitrogen per 100 gms. fish muscle. Whereas sample B. gave a value of 416 mgs. It is seen then that in order to be throughly extracted the thoroughly ground muscle should be heated with several portions of acidulated water. This is essentially the same technique as given by Van Slyke (10) and adopted by Cathcart (11). The free amino nitrogen was determined by the formol titration method of Sorensen on 50 cc. portions of the filtrates which were boiled to remove the trichloracetic acid. Sample A gave 30.4 mgs. free amino N. per 100 gms. of fresh fish muscle and Sample B. gave 33.5 mgs. free amino N. per 100 gms.

This fish was allowed to remain at room temperature (20° - 25°C) for three days. During this time marked changes took place in the appearance of the fish. The gills became grayish in color, the eyeballs became sunken and grayish, the scales were much less firmly held in their sockets, the flesh had lost a good deal of its elasticity and the fish gave off a very marked putrid odor. In this case as well as in others the external portions,--i.e skin and body cavity lining, gave off a much more intense smell than did the flesh itself. However, the flesh too gave off a very stale and nauseating odor.

13.09 grams of the flesh were ground up with sand in a mortar and extracted 4 times on the water bath with 50 cc. 0.05% acetic acid. It was precipitated in the usual way with trichloracetic acid, made up to 200 cc. with distilled water and filtered. Non-protein nitrogen and free amino nitrogen were determined as above. The N.P.N. was found to be 564 mgs. N. per 100 gms. of fish and the free amino N. (which must have included a portion of ammonia) was found to be 90 mgs. nitrogen per 100 gms. fish muscle.

EXPERIMENT 2

Two Haddock were obtained from fishermen who said the fish had been caught half an hour previously. One of these was immediately surrounded with chopped ice. The other was allowed to remain (protected by netting from flies) at room temperature. Samples were taken from both fish at intervals of six, thirty, and fifty four hours, after the death of the fish and analysed for non protein nitrogen and free amino nitrogen in the manner of sample B. of the last experiment.

The results of this experiment are given in the following table:

Table 2

	Fish on Ice		Fish at Room Temperature	
Time	Non Protein nitrogen	Free Amino Nitrogen	Non Protein nitrogen	Free Amino nitrogen
Hrs.	mgs.%	mgs.%	mgs.%	mgs.%
6	427	31.9	444	31.5
30	426	35.1	471	37.5
54	426	38.7	512	48.8

The initial values for the free amino nitrogen of muscle here found are similar to that found by Denis (12) (32Mgs.%) for the cunner but are lower than those reported by Buglia and Constantino (13) who give the following values: Gobio fluviatilis 74mgs., Labrus turdus 60 mgs., Crenilabrus pavo 65 mgs., and Conger niger 99 mgs.

The values given in the table are, with perhaps the exception of those in the third column, showing a slight increase in the free amino N. in the muscle of a fish kept on ice, quite reasonable. The content of free amino nitrogen and of the non protein nitrogen of the fish kept at room temperature show increase but the increase is comparatively small.

EXPERIMENT 3

However when the samples used for analysis were taken from different fish as they were in the next experiment the results are not nearly so clear cut. Six Haddock were obtained fresh from the trawler where they had been kept on ice. Three of these were kept well surrounded with cracked ice, the other three were kept at a temperature of 19-20°C.

Each smaple was taken from a separate fish. Results in Table III.

Table III

Free Amino Nitrogen Mgs.%

	1 Day	2 Days	3 Days
Fish on Ice	21.8	27.8	27.6
Fish at 20°C.	42	38.5	44

The results given in Table III are very irregular and show the futility of experimenting with fish obtained from such a source. However, when the samples are taken from different individuals it is possible that the individual variations are sufficiently large to account for such results as these. Those fish kept at the higher temperature do show, however, a markedly higher concentration of free amino nitrogen.

DISCUSSION

By the end of the first day period at temperatures 19-22°C. the fish are certainly undergoing bacterial decomposition so that any results obtained after this period must be caused by the activities of the processes of autolysis and bacterial decomposition, with the latter very quickly predominating.

As Dreyer (14) says, the free amino nitrogen may rise 50% or more within a few days, but in comparison to the total amount of protein present this represents only a very small amount of hydrolysis. For instance in the first experiment in which the free amino nitrogen of the muscle rose from 33.5 mgs. to 90 mgs. per 100 gms. of fish in three days, although the initial content of free amino nitrogen is almost trebled it represents, from a rough calculation, only an increase from 2.4% hydrolysis of the total protein to 6.43% hydrolysis. (calculated from an assumption that there are 1400 mgs. possible free amino nitrogen in 100 gms. of fresh fish muscle.) Almy (15) comes to the same conclusion about the flesh of the herring and stated (page 2142) " The autolysis of the muscle tissue plays a comparatively insignificant part in the breakdown of muscle proteins......."

B. Bacterial Decomposition

The fish, soon after being caught (unless kept on ice) exhibit evidence of bacterial contamination. Whether these bacteria are present as inert micro-organisms in the living flesh, whether they come from the sea water, or whether they come from their surroundings, makes little difference. The fish do not remain sterile These bacteria are of many kinds and their products of metabolism are in many cases toxic. Most of the toxic products produced by the bacteria from muscle contain nitrogen., i.e. they are produced from the amino acids of the proteins and from the lecithin which is contained in all cells.

The amino acids formed by the breaking down of muscle protein either by autolytic enzymes or by proteolytic enzymes secreted by the bacteria themselves, are broken down by bacteria in two distinct ways (16).

(1) An amino group may be broken off (deamination).
(2) A carboxyl group may be broken off (decarboxylation).

This second type of reaction may also be brought about in two ways:

(1) By a simple removal of CO_2

$$\begin{matrix} R \\ CH\text{-}COOH \\ NH_2 \end{matrix} \longrightarrow \begin{matrix} R \\ CH\text{-}NH_2 \\ H \end{matrix} + CO_2$$

(2) Or reduction may take place during which the carbon dioxide is removed as formic acid.

$$\begin{matrix} R \\ CH\text{-}NH_2 \\ COOH \end{matrix} + H_2 \longrightarrow \begin{matrix} R \\ CH_2\text{-}NH_2 \end{matrix} + H.COOH$$

The importance of this last reaction is that reduction is brought about and that it takes place under anaerobic conditions, By Decarboxylation of the amino acids the following list of amines may be formed:

Methylamine -- from glycine-- however most likely from the methylamine which is formed from choline.

Iso butylamine -- from d.l. valine.

Iso amylamine -- from leucine.

Putrescine -- from ornithine.

Cadaverine -- from lysine.

Agmatine -- from arginine.

Phenyl ethylamine -- from phenylalanine.

p-hydroxyphenyl ethylamine -- from tyrssine.

Indole ethylamine -- from tryptophane.

B-iminazolyl ethylamine (hi~~ts~~stamine) -- from histidine.

By Deamination of the amino acids the following are obtained:

Ammonia

Corresponding hydroxy acid.

By a combination of these two processes of deamination and decarboxylation by bacteria, the following substances among others have been produced:

Paracresol and Phenol -- from tyrosine.

Indole)
Skatole) -- from tryptophane.

The action of the bacteria can also break down the sulphur containing amino acid cystine with the liberation of hydrogen sulphide. This reaction has been shown by Reed (17) to be the cause of the discolouration of canned lobster.

Most of these products are produced by anaerobic bacteria requiring no atmospheric oxygen. The deaminated products are comparatively inert whereas the amines produced by decarboxylation are physiologically very toxic.

Any one of these might be measured as an index of the amount of growth of the particular organism which produced them. The chief difficulty in the determination of any of these is the separation of them from the rest of the material, and they must be freed from interfering substances before they can be measured with any degree of exactitude.

Indol gives several very sensitive color reactions with various substances. These have been fully reviewed and discussed by Clough. (18) and by Fellers and Clough (19) who have been able to detect by their technique indole in a concentration of one in twenty five million.

The method which they have described for the quantitative detection of indole in fish muscle is as follows: 200 gms. of thoroughly ground and mixed fish muscle are transferred to a liter round bottomed flask with a long neck using about 200 cc. water. A current of live steam is then passed through the mixture in the flask until 500 cc. distillate are collected. A gallon oil can having a long safety tube placed in the opening on top and a rubber tube attached to the spout makes a very satisfactory steam generator. The steam is passed through the fish by means of a glass tube reaching to the bottom of the flask. (flask is inclined at an angle of 45°) and so bent near the end to give a rotatory motion to the contents as the steam issues from it. The flask is kept boiling hot by being placed in a boiling saturated salt solution. The steam from the flask is received in a vertical worm condenser the end of which projects below the surface of a small amount of water in the receiver (600 cc. beaker) Some difficulty is encountered due to foaming but careful watching at the beginning of the distillation will prevent this from becoming troublesome.

About 500 cc. of distillate are collected, transferred to a liter separating funnel, acidified with 100 cc. C.P. concentrated hydrochloric acid and extracted with 120 cc. ethyl ether (U.S.P.) with repeated vigorous shaking. After the ether has separated, the ether layer is transferred to a 250 cc. separatory funnel and washed first with 25 cc. 2.5% NaOH and then with 25 cc. dilute hydrochloric acid. (10% C. P. conc. HCl - 200 cc. water) The first washing is to remove compounds which might interfere with the color test and the second is to neutralize any alkali left in the ether. The ether is then transferred to a small flask containing 10 cc. water and evaporated on a steam bath (passing the vaporized ether through a condenser -- (Take care of Fire!) taking care that while the last of the ether is being driven off the water layer is not heated above the boiling point of the ether, since, the indol might easily be lost by volatilization at this stage. A five cc. portion of the water solution left is then tested by the technique described by the authors. This is carried out as follows:

Reagents required:

(a) para dimethyl amino benzaldehyde, 2 gms. in 100 cc. 95% alcohol.

(b) HCl -- 600 cc. conc. plus 200 cc. water.

(c) Chloroform U.S.P.

Method: to 5 cc. of the water test solution add 0.5 cc. reagent (a) and 1 cc reagent (b): place in a boiling water bath for about 20 seconds shaking vigorously, then place in ice water about ½ minute and extract with reagent (c). The color developed is compared with a series of standard tubes containing 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0. Micromilligrams of indol in 5 cc. water and treated exactly in the same way as the unknown.

The standard color solutions were made up using calculated volumes of a solution of indol containing 4 mgs. of indol per liter plus water to 5 cc. These standard solutions have to be made up fresh every day for they deteriorate rapidly. The solution of indol containing 4 mgs. indole per liter is also made up fresh every day by pipetting 5 cc. of an alcoholic solution of indol containing 80 mgs. indol in 100 cc. into a liter flask and making up to the mark. The concentrated alcoholic solution of indol, if tightly stoppered will keep for months.

Such a test was carried out on a series of haddock which were caught at 8 O'clock one morning. One half of the number were placed on ice and kept well surrounded by chopped ice during the duration of the experiment. The other half were left at room temperature well protected from flies and insects by netting. Samples of the flesh (generally a whole side) were taken at various times and tested by the above technique for the presence of indol. The values found are given in the following table:

Table 4

Time	Fish on Ice		Fish at Room Temp. (65°F	
Hrs.	Remarks	Indole	Remarks	Indole
		Mmgs.%		Mmgs.%
8	good	<0.2	good	0.4
30	good	<0.2	very slightly stale	1.78
50	good	0.2	definitely though not strongly stale	4.0
56	--	--	Stale	
80	good	0.2	Undoubtedly bad	227.
83	--	--	Undoubtedly very bad	327.

A second series of indol determinations on haddock was done with the results given in Table 5.

Table 5

Time	Fish on Ice.		Fish at Room Temp. (58-62°F	
Hrs.	Remarks	Indole	Remarks	Indole
6	good	<0.2	good	0.2
24	good	<0.2	Flesh still good strong fishy odor. Gills are grey.	0.4
48	good	<0.2	Fish is slightly stale	0.4 (?)
72	good	<0.2	Fish is obvious-ly bad. Flesh smells quite stale	5.0
96	good	<0.2	Bad	55

There appears to be some discrepancy between these two series of results. Of course the temperature is slightly lower in the second series but the difference in temperature is hardly sufficient to account for the difference in the rate of production of indole.

It was thought that this test could be modified somewhat so as to include the estimation of the volatile N. (Ammonia and volatile nitrogenous bases). To this end some phenolphthalein was added to the ground up mixture of fish in the flask along with sufficient magnesium oxide to make the mass assume a pink color. An extra 200 cc. water was added to render the mass fluid as the alkali tends to make it gelatinous. The distillate was caught in a 500 cc. volumetric flask containing a sufficient quantity of hydrochloric acid to entrap the ammonia and volatile bases being driven off. The distillation was continued until the flask was filled to the mark. A 50 cc. portion was then titrated with standard alkali to determine the excess acid and the remainder was treated as before to detect the presence of indole. Experiments showed that this modification had little effect upon the quantity of indole found. The titration could also be adapted to the differential estimation of ammonia and trimethylamine. The total bases are titrated using methyl red and methyl green as indicators, Phenalphthalein is then added, the mixture neutralized to this indicator, and the formol titraction is carried out giving the amount of ammonia present. By substracting this from the total bases being estimated a value for the other volatile bases -- chiefly trimethylamine -- is obtained.

This was done on the same series of haddock given in Table 4 and the values so obtained are given in the following table in terms of 100 gms. of fresh fish muscle.

Table 6

Fish at Room Temperature.

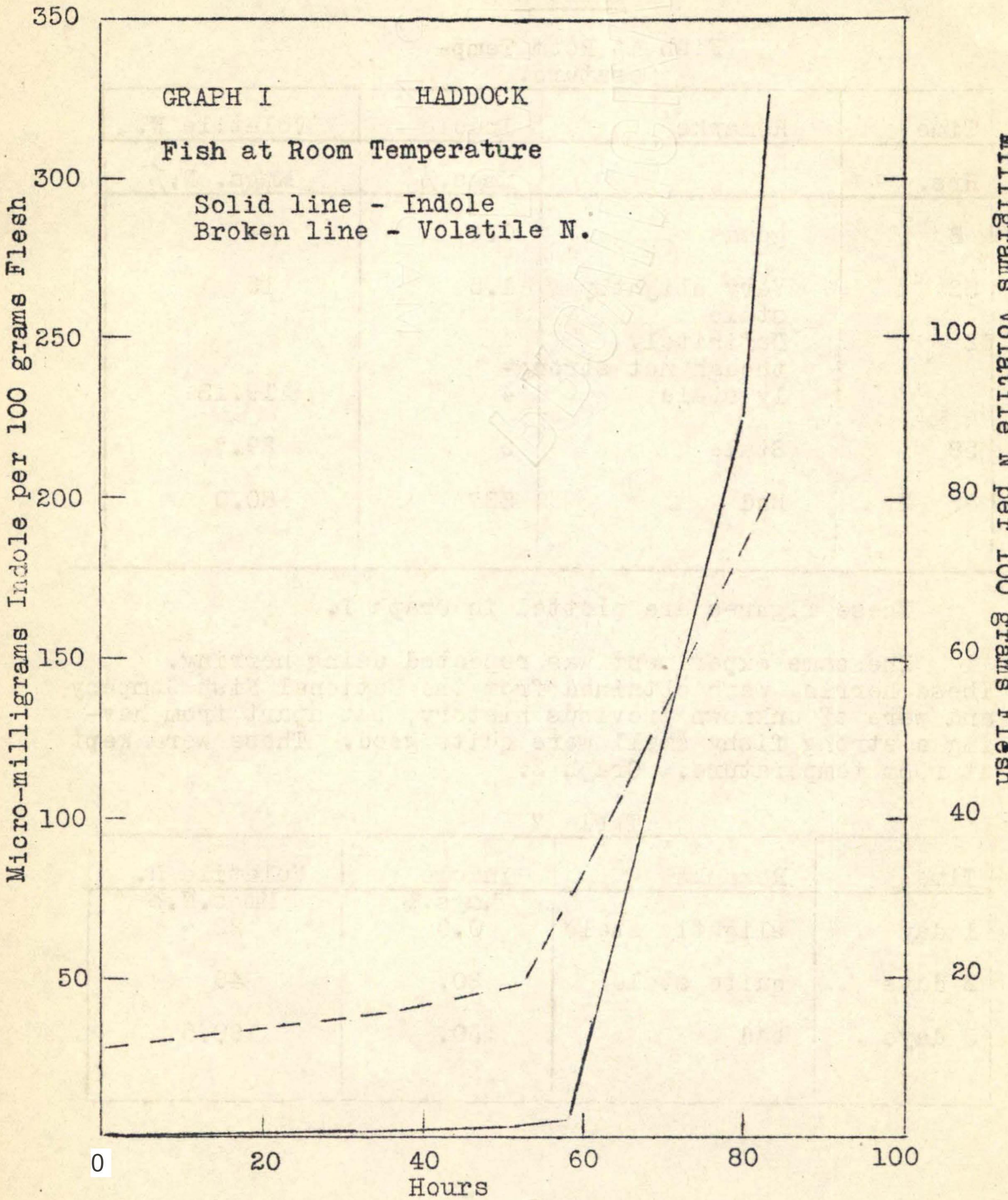
Time	Remarks	Indole	Volatile N.
Hrs.		Mmgs.%	Mmgs. N.%
8	good	0.4	--
32	Very slightly stale	1.5	15
52	Definitely though not strongly stale	4	19.13
58	Stale	5	29.7
83	Bad	327	80.0

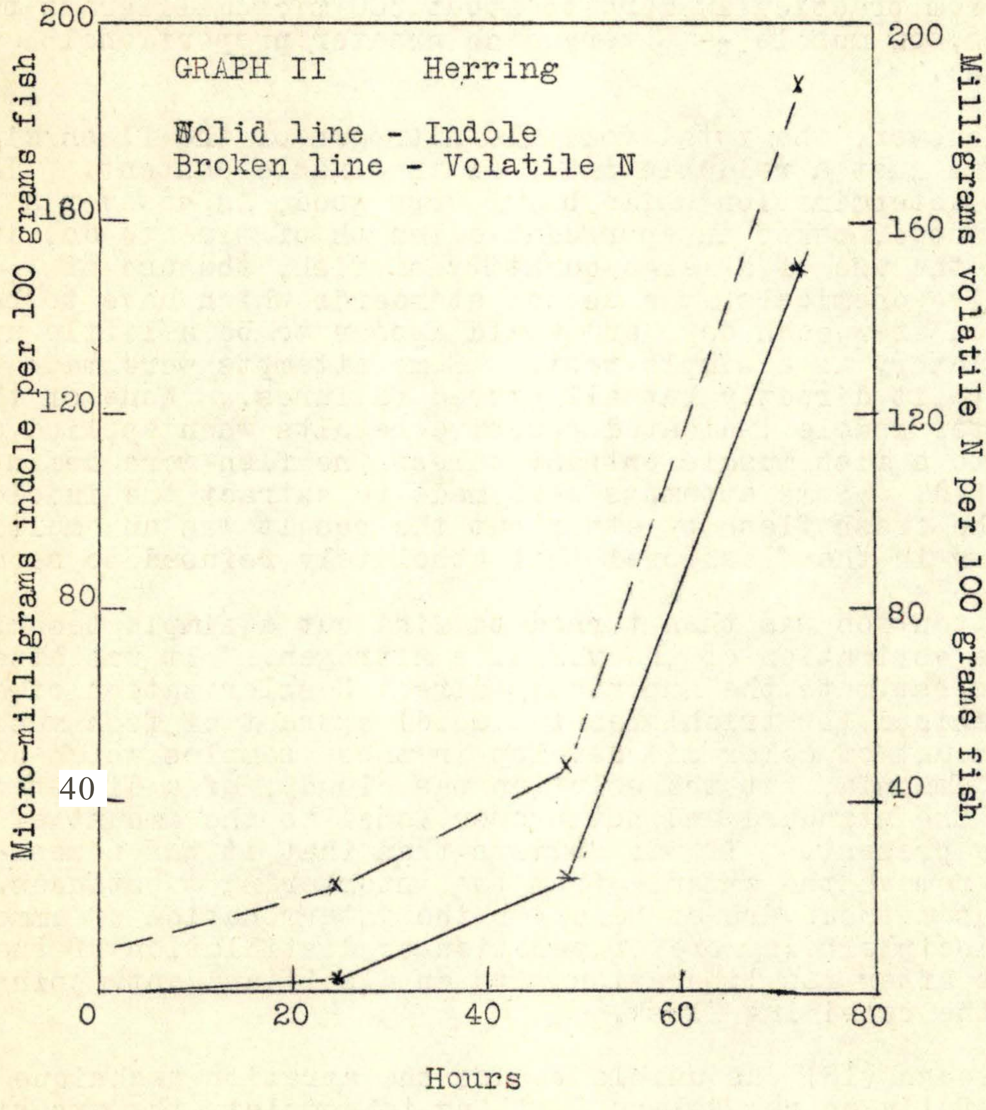
These figures are plotted in Graph I.

The same experiment was repeated using herring. These herring were obtained from the National Fish Company and were of unknown previous history, but apart from having a strong fishy smell were quite good. These were kept at room temperature. Graph 2.

Table 7

Time	Remarks	Indole Mmgs.%	Volatile N. Mmgs.N.%
1 day	slightly stale	0.8	22
2 days	quite stale	20.	48
3 days	bad	150.	188.5


GRAPH I HADDOCK
Fish at Room Temperature
Solid line - Indole
Broken line - Volatile N.
Micro-milligrams Indole per 100 grams Flesh
Milligrams Volatile N per 100 grams Flesh
350
300
250
200
150
100
50
100
80
60
40
20
0
20
40
60
80
100
Hours


GRAPH II Herring
Solid line - Indole
Broken line - Volatile N
Micro-milligrams indole per 100 grams fish
Milligrams volatile N per 100 grams fish
200
160
120
80
40
0
20
40
60
80
Hours

It is quite obvious that the type of curve that one obtains by estimating the volatile nitrogen is similar in nature to that which one obtains with indole: with this difference however, that whereas the total organic volatile bases increases from a normal in fresh fish muscle of about 10 mgs. N per cent. (see below) to about 80 mgs. N per cent in three days at room temperature in haddock, the value for indole goes from practically zero to about 300 micromilligrams per 100 gms. of muscle -- a very much greater proportional increase.

However, the total volatile nitrogen of the flesh might serve as just a reliable index as the indole content. The indole determination while being very good, is however a bit complicated, takes an appreciable length of time to do, involves the use of a large quantity of fish, the use of expensive chemicals, the use of standards which have to be a prepared anew each day, and would appear to be a little unsatisfactory as a simple test. Some attempts were made to estimate it directly but all proved failures. None of the tests for indole indicated positive results when applied directly to a fish muscle extract unless the fish were definitely bad. Some attempts were made to extract the indole from the fresh flesh by ether but the result was an emulsion of ether in the flesh brei that absolutely refused to separate.

Attention was then turned to find out a simple technique for the estimation of the volatile nitrogen. It was attempted to estimate the ammonia by direct Nesslerization of a deproteinized (by trichloracetic acid) extract of fish muscle. Some amount of color did develop in those samples which contained ammonia, but the solution was cloudy, of a different hue to the standard and not proportional to the amount of ammonia present. It was obvious then that it was necessary to remove the ammonia from the interfering substances. Numerous methods are at hand for the determination of ammonia, the principle being one of aeration or distillation of the ammonia after its liberation with an alkali and entrapping it in the receiving flask.

Clough (18) was unable to use the aeration technique successfully on raw salmon, failing to complete the experiment in all but one occasion on account of foaming. He tried paraffin oil, kerosene, alcohol, and other materials as well as various kinds of baffle plates but frothing continued.

However, a technique has been developed by the writer which enables one to estimate the volatile base content of fish muscle quickly, easily, and quite accurately. The method as finally adopted is as follows: 10 gms. of fish muscle are accurately weighed out, ground up thoroughly with sand in a mortar, and transferred with 20 cc. water to a 500 cc. Kjeldahl flask. 80 cc. of ammonia free alcohol are added and two teaspoonsful of magnesium oxide. The flask is connected to a worm condenser the end of which dips into a small Erlenmeyer containing 5 cc. of accurately measured 0.1 N hydrochloric acid. 50 cc. of the alcohol are distilled over and the excess acid is titrated by 0.02 N sodium hydroxide solution, using sodium alizarin sulphonate as the indicator. A simple calculation gives the amount of volatile N per hundred gms. of fish tissue.

The use of the alcohol has the following advantages:

(1) Effectually prevents frothing.
(2) Prevents bumping.
(3) Increases markedly the speed of distillation-- can be completed in 4-5 minutes.
(4) Keeps the temperature down thus diminishing any small tendency the magnesium oxide would have to break off ammonia from the proteins.

The whole test can be carried out in little more than ten minutes.

It was first attempted to estimate the ammonia thus distilled over by Nesslerization of distillate: but it was found that the addition of as little as 1 cc. alcohol to 10 cc. of a dilute solution of ammonium chloride caused a marked difference in the amount of color developed on the addition of Nessler's solution.

The titration by such a weak alkali in such a high concentration of alcohol offers some difficulty. It was found impossible to use methyl red as the indicator. Various indicators were then tried -- bromthymol-blue, brom cresol purple, phenol red, methyl orange, etc. but of a large number the most satisfactory was found to be sodium alizarin sulphonate. The end point was taken as the change from the true pure yellow of the indicator in acid solution to the reddish yellow tint that was the first visible change in the color. The use of a second solution containing the same concentration of indicator in a weakly acid solution greatly facillitated by comparison the determination of the end point.

This technique was used to determine the volatile nitrogen concentration in the muscle tissue of a number of samples of fish. These are described below. The blank for the reagents used was found to be 0.15 mgs. Nitrogen.

Experiment A.

10 gms. of codfish (judged good by smell) were ground up with sand and transferred with 20 cc. water and 80 cc. alcohol to a 500 cc. Erlenmeyer flask. Added 2 teaspoonsful of magnesium oxide. Distilled till the mixture began to foam, into 5.00 cc. 0.1 N hydrochloric acid. Titrated excess acid with 0.02 N NaOH. Found 0.9 mgs. N. Therefore the volatile N of 100 gms. of fresh fish muscle is 9.0 mgs.

Experiment B.

A further 80 cc. of alcohol were added to the flask and distillation was repeated. Found an additional 0.06 mgs. which, being practically fully accounted for by the blank, shows that the first distillation carried over all the volatile N present in the fish. Also shows that the presence of the weak alkali has no destructive action on the proteins present.

Experiment C.

Experiment was repeated on the muscle of the same fish. Found 8.8 mgs. volatile N per 100 gms. fresh fish tissue which is in fair agreement with the value 9.0 mgs.% previously found.

Experiment D.

Same specimen as was used in the last experiment but had been kept at approximately 60°F. for a further 24 hours. The flesh still smelled good. Found in two determinations 11.0 and 11.7 mgs. volatile N per 100 gms. tissue.

Experiment E.

Same specimen as in last experiment but had now been kept for 48 hours at approximately 60°F. The skin and body cavity of this fish had a definite putrid odor. The flesh when cleaned of skin did not smell at all bad but did have a definitely stale odor. The volatile N of this sample was found to be 34.52 mgs. per 100 mgs. of moist tissue.

Experiment F.

Same cod fish as above which had now been kept for 3 days at room temperature. This fish was very bad--putrid. It contained 79.5 mgs. volatile N per 100 gms. tissue.

Experiment G.

Haddock. The haddock muscle used in this experiment was taken from a fish which on the whole smelled quite stale. The flesh on rubbing on one's hand and being held to one's nose seemed perfectly good, having a marked fishy smell similar to that obtained in frozen fish and noted during the process of cooking fish. It could hardly be called stale but nevertheless it had a definite fishy and slightly unpleasant odor. The volatile N content was found to be 14.30 mgs. per 100 gms. moist tissue.

Experiment H.

The same specimen as had been used in experiment G which had now been kept for 24 hours at approximately 60°F. This fish was bad, quite stale, but not putrid. The volatile base content was 45.2 mgs. N per 100 gms. tissue.

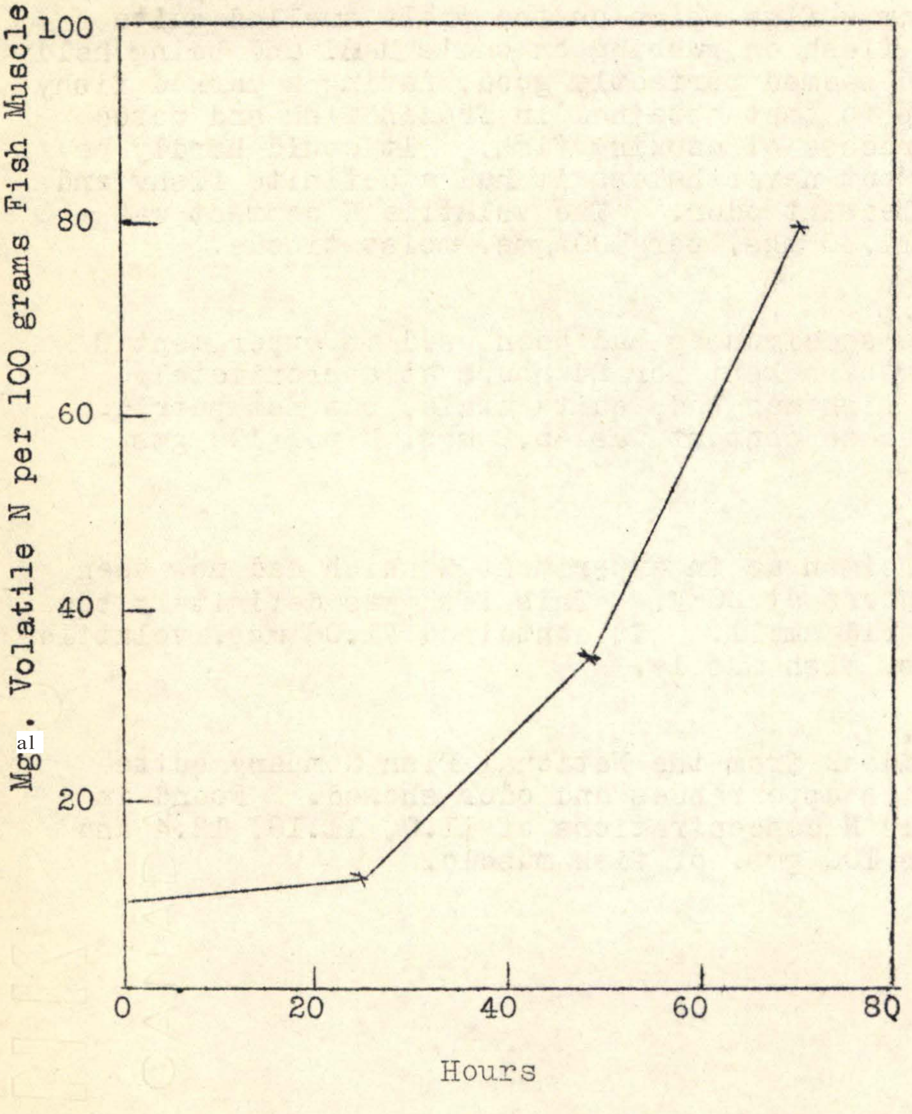
Experiment I.

Same specimen as in experiment G which had now been kept for 48 hours at 60°F. This fish was definitely bad and had a putrid smell. It contained 71.08 mgs. volatile N per 100 gms. fish muscle.

Experiment J.

Four haddock from the National Fish Company quite fresh as far as appearances and odor showed. Found in these volatile N concentrations of 11.0, 11.18, 12.4 and 10.8 mgs. per 100 gms. of fish muscle.

GRAPH III. Showing development of volatile N in codfish kept at 60-65°F.



The writer at this time made many attempts to obtain some absolutely fresh fish but none could be obtained. On this account the following experiment on the development of volatile nitrogen was done on 4 haddock that were obtained from the National Fish Company where they had been kept on ice. They were quickly brought to the laboratory where two of them were surrounded with chopped ice and two were placed in a cool part of the building along with a recording thermometer. At intervals of 24 hours 10 gm. samples were taken from each of the 4 fishes and analysed by the above method for the volatile nitrogen content of their muscle tissue.

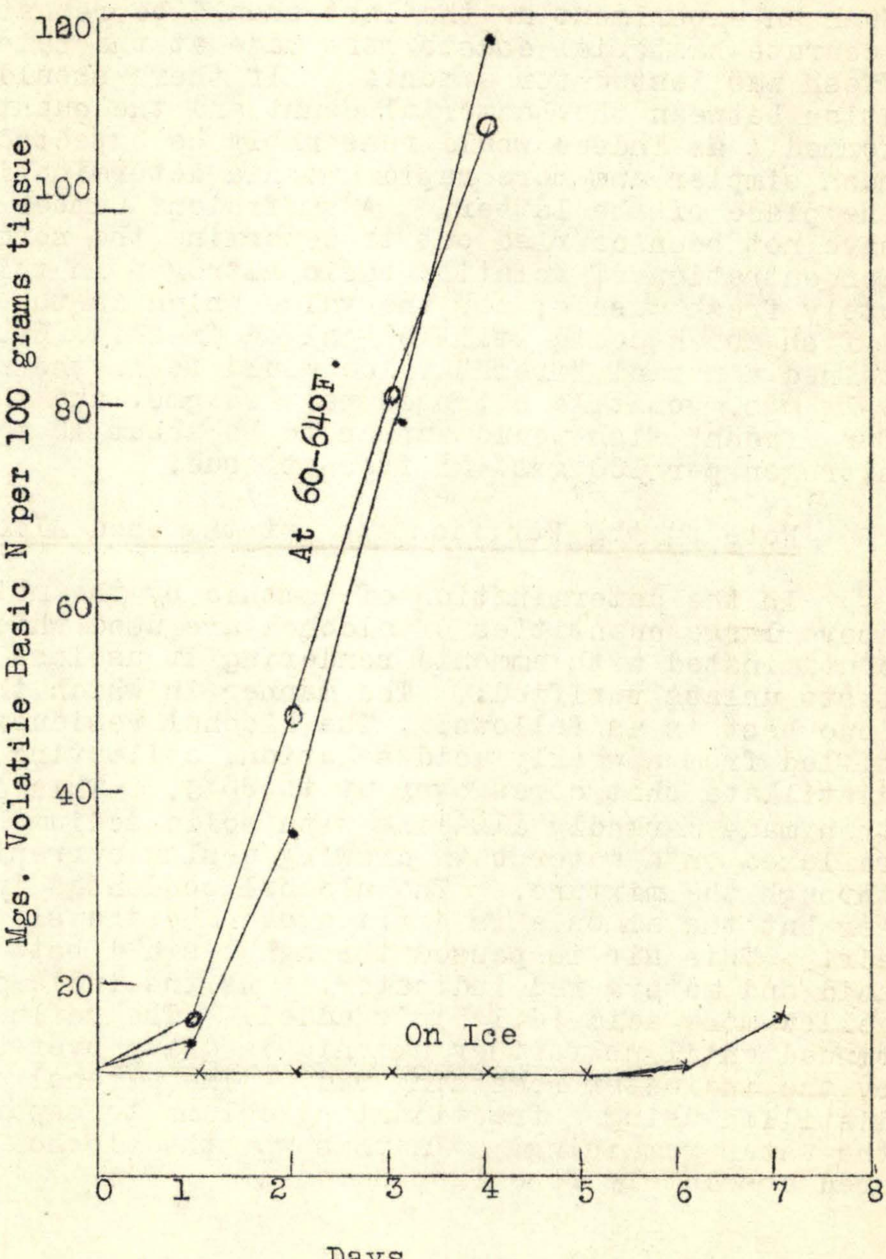
The following table and graph record the results obtained from this experiment. All the 4 fish were initially in excellent condition: the surface and body cavity did not give off any offensive odor and the flesh possessed practically no smell at all.

Table 8

Fish on Ice			
Days Standing	No.	Remarks	Volatile Basic Nitrogen Mmgs.%
0	a b	Good	9.9 11.2
1	a b	Good	10.7 10.8
2	a b	Good	11.0 10.2
3	a b	Good	10.4 10.2
4	a b	Good	10.9 10.6
5	a b	As good as ever. No perceptible disagreeable odor from skin or body cavity.	10.6 10.2
6	a b	Perfectly good	12.8 10.4
7	a b	Very faintly stale. partially exposed from ice.	17.5 15.3

Fish at Room Temperature				
Days Standing	No.	Remarks	Av. Temp. F.	Vol. Basic Nitrogen Mmgs.%
0	c d	Good	--	11.0 11.2
1	c d	Slightly stale. Skin has a markedly stale odor	60.4	13.9 16.3
2	c d	Flesh is definitely stale	60.7	36.00 47.96
3	c d	Bad	64	79.00 81.2
4	c d	Putrid (thrown away)	60.0	116.2 108.7

GRAPH IV. Showing increase in volatile basic N in fishes at various temperatures.



From these results it would appear that such a test for the concentration of volatile basic nitrogen in fish muscle might give some indication of the extent of bacterial decomposition. This would be so, providing that the majority of the bacteria which contaminate the fish produce ammonia. Such an experiment as the last should be repeated in which accurate bacterial counts were made at the same time as the flesh was tested for ammonia. If there should be a correlation between the bacterial count and the quantity of ammonia formed (as indeed would reasonably be expected) then the much simpler and more rapid ammonia determination could take the place of the latter. A sufficient number of experiments have not been carried out to determine the normal value of the concentration of volatile basic nitrogen in this way in absolutely fresh muscle; nor the value which is the upper limit in a fish which could still be called fresh. From the data obtained a normal "fresh" value would be in the neighborhood of 9-11 mgs. volatile nitrogen per 100 gms. and the upper limit for "fresh" fish would appear to be about 15 mgs. of volatile nitrogen per 100 gms. of fresh tissue.

Note on the Purification of the Used Alcohol

In the determination of ammonia by the method described above large quantities of alcohol are used which become contaminated with ammonia rendering it useless for further tests unless purified. The manner in which this can be done best is as follows: The alcohol residues are redistilled from a weakly acid solution, collecting all the distillate that comes over up to 80°C. This distillate is then made markedly alkaline with solid sodium hydroxide and refluxed on a water bath drawing a slow current of air through the mixture. The alcohol condenses in the condenser but the ammonia is carried over by the slow current of air. This air is passed through a small bottle containing acid and methyl red indicator. As the indicator turns yellow more acid (0.1 N) is added. The refluxing is continued until no further ammonia is drawn over as indicated by the indicator remaining red. The alcohol is then again distilled using a fractionating column to separate it from the water remaining. In this way the alcohol can be recovered absolutely free from ammonia.

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