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The Denaturing of Fish Muscle during Storage

By W. W. Johnston

Introduction

Fish frozen and preserved at low temperatures retain, at least approximately, the appearance, flavour and nutritive qualities of fresh fish for some time, but after varying periods generally from 6 to 10 months they begin to lose their flavour and become tough and fibrous. Weld(1) has observed from microscopical examinations of fish muscle that when freshly frozen and thawed it regains its original appearance but when frozen and stored for some time then thawed the tissue fails to recover and appears to be dehydrated thus demonstrating that some irreversible change has taken place in the muscle fibres due to prolonged storage. Moran (2) has shown that eggs frozen below -6°C suffer some irreversible change on thawing, and Callow (3) has also demonstrated that frogs sartorious if frozen at a temperature below -2.4°C behaves quite differently from fresh unfrozen muscle in regard to its ability to absorb water. It was thought possible that the above phenomena of toughening and irreversibility might be due in part at least to the denaturing of a portion of the soluble protein of the muscle by freezing and subsequent storage at low temperatures, hence the present investigation was undertaken.

Methods

Extraction medium

In his work on frog muscle Deuticke (4) recommended and used as a means of extraction of the soluble proteins from cold blooded animals a solution prepared by mixing a solution containing 4.56% mono potassium phosphate with a solution containing 4.96% di sodium phosphate. By using these solutions in the proportion of about 1 of the former to 4 of the latter a solution with a pH of approximately 7.2 was

obtained and was used as his extraction medium. Solutions prepared in the same manner and adjusted to the same hydrogen ion concentration were used in the work to be reported below for the extraction of the soluble fraction of the protein from fish muscle the pH being measured either by the quinhydrone electrode or by using brom thymol blue in the Gillespie and Walters series of indicators.

smith criticism of Sentiche

Method of Extraction

Three hundred cubic centimetres of the above extraction solution were measured into a 500 cc. Erlenmeyer flask by means of a 250 cc. volumetric flask and a 50 cc. pipette. The flasks were then weighed and removed to a room held at a temperature of about 0°C. until they had reached the same temperature when about 10 gm of the minced muscle was added to each and the flask left for extraction for a period of from 15 to 20 hours, each flask being shaken several times during the interval.

Preparation of the muscle for extraction

Since it was considered that the outer edges of a frozen fillet might have dehydrated to a greater extent than the central portion these were first removed and discarded and the remainder then ground to a fine white powder by passing it several times (usually 4) through a meat chopper. The grinding was done in the cold storage room thus allowing the muscle no opportunity of thawing before being placed in the extraction medium.

Experimental Technique

In the preliminary experiments after allowing the extraction to proceed from 15 - 20 hours the Erlenmeyers and contents were re-weighed in the cold room and the muscle filtered and 10 cc. of the filtrate added in duplicate sample to the digestion tubes for total nitrogen determinations. However as later work showed that no precipitation took place for some time after allowing the flasks to come to room temperature this precaution was subsequently abandoned and the flasks were taken to the laboratory and allowed to warm up before weighing, filtering and sampling. In order to determine the non-protein nitrogen the protein was precipitated

from the filtered extract by means of Schenk's reagent, the technique being as follows:- To a 50 cc. volumetric flask containing 10 cc. of 4% hydrochloric acid and 10 cc. of 5% mercuric chloride solution was added 25 cc. of the above extract and the flask then filled to the mark with distilled water. This was allowed to stand for 3 hours when it was filtered and nitrogen determinations made in duplicate on 15 cc. samples of the filtrate.

Nitrogen determinations

The digestion mixture used for the conversion of not more than 0.125gm. of protein nitrogen to ammonia consisted of 1 cc. of concentrated sulphuric acid, 1 cc. of a saturated solution of sodium sulphate and 4 drops of a 10% solution of copper sulphate. The above digestion mixture and the sample containing the nitrogen were heated together in pyrex test tubes until the digestion was completed, when the copper, and in the case of the non-protein nitrogens the mercury, was precipitated with a solution of 8% sodium thiosulphate and the ammonia then determined by a steam distillation into $\frac{N}{70}$ hydrochloric acid using Pregl's modified nitrogen apparatus.

Moisture determinations

The moisture determinations were carried out by drying a 10gm. sample of the minced muscle to constant weight at a temperature of 110°C.

Fat determinations

These determinations were done by Miss A. M. Wilson of the staff of the Fisheries Experimental Station.

Preliminary Experiments

Probable error in the results obtained

In the results to be reported below the individual nitrogen values were always determined in duplicate samples and the total nitrogen values checked to within an accuracy of 1%, the non-protein nitrogens to within an accuracy of 2%. In order to determine the probable error in the method of extraction, extracts were made in triplicate on a mackerel fillet and 4 extracts made on a haddock fillet with the results shown in the following tables. In actual practice each fillet was

generally extracted in duplicate only, the nitrogen content of the different extracts agreeing for the most part to within 3% with occasional differences as great as 6%.

Table I

Mackerel Extracts

Fillet Extract	Total Nit. per Gram Fish	N.P.N. per Gram Fish	Prot. Nit. per Gram Fish
1	0.0118	0.00390	0.00790
2	0.0119	0.00396	0.00794
3	0.0118	0.00390	0.00790

Table II

Haddock Extracts

Fillet Extract	Total Nit. per Gram Fish	N.P.N. per Gram Fish
1	0.00988	Not done
2	0.01020	"
3	0.01005	"
4	0.01026	"

Variation in nitrogen content of extracts from different fillets of the same age.

In order to determine what variation in soluble protein content was to be expected in the case of different fillets held in storage for the same period 3 different haddock fillets which had been frozen for 14½ months were extracted in the usual manner and nitrogen determinations done with the results shown in the following table.

Table III

Fillet	Tot. Nit.	N.P.N.	Prot. Nit.
1	0.00985gm.	0.00407gm.	0.00578gm.
2	0.01035gm.	0.00434gm.	0.00602gm.
3	0.00998gm.	0.00404gm.	0.00594gm.

The above table shows the variation in nitrogen content usually met with in extracts of different fillets of the same age and with the exception of two cases the variation shown is as great as any found during the course of this work. The greatest difference occurring in one of the cases mentioned, amounted to 10% but a variation of this magnitude could almost certainly be attributed to an experimental error.

Effect of temperature on the muscle extract

As it was known that muscle juice obtained by grinding fish muscle and subjecting it to pressure, rapidly becomes cloudy when brought into a warm room it was thought that the muscle extract might behave similarly under the same conditions, hence the following experiment was carried out. An extract of cod fish was made in the usual manner, the flask weighed, a portion of the extract filtered and samples taken for analyses before removing the flask from the cold room, then the flask with the remainder of its contents was taken to the laboratory and allowed to reach room temperature when a second portion of the extract was filtered and samples of the filtrate again taken for total nitrogen determinations. The amount of nitrogen per gram of muscle found in the former case was 0.01020gm that in the latter 0.01013gm showing that no appreciable amount of protein had been precipitated by allowing the extract to stand at room temperature for a short period.

The Effect of time of extraction

The amount of nitrogen found in an extract of cod muscle extracted with the usual salts and in the usual manner after 20 hours was 0.01007gm per gram of fish while after a period of 60 hours extraction the same figure was found, thus demonstrating that the muscle is completely extracted in a period somewhat less than 20 hours.

Effect of freezing on the amount of soluble protein

In order to determine whether the process of freezing itself exerted any influence on the amount of protein that could be extracted in the manner employed, two cod fish were caught and brought to the station in a barrel of water. They were then immediately killed and filleted and a fillet of each ground at once and samples placed in the extraction medium. The remaining fillet of each was then frozen in a brine tank at -14°C. , ground to a powder and also extracted in the usual manner, the results of both extractions being shown in the following table.

Table IV

Fillet	Condition	Tot. Nit.	N.P.N.	Prot. Nit.
1	unfrozen	0.00997gm.	0.00398	0.00599gm.
1	frozen	0.01035gm.	0.00431	0.00604gm.
2	unfrozen	0.00983gm.	0.00395	0.00587gm.
2	frozen	0.00997gm.	0.00417	0.00580gm.

The above table shows no difference in the amount of nitrogen extracted greater than the probable experimental error between the frozen and unfrozen samples of cod fish and hence it must be concluded that the process of freezing itself exerts no influence on the amount of protein soluble in the salt solutions employed.

Experimental

Denaturing of protein observed with mackerel

In the table to be given below the results obtained by the extraction of mackerel fillets in the manner previously described are listed.

Table V

Age of Fillet	No. of Fillets Extracted	Average% of fat in fillet	Average% Residue at 110°C.	Average Tot. Nit. per gm.Fish	Average N.P.N. per gm. fish	Average Prot. Nit. per gm.Fish.	Corrected Average Prot. Nit. per gm. Fish.
0 mos.	2	10.2	29.6	0.0129g.	0.00388g.	0.00902gm.	0.00885gm.
1 "	2	7.7	27.3	0.0132g.	0.00394g.	0.00928gm.	0.00900gm.
8 "	3	20.1	37.1	0.0118g.	0.00392g.	0.00788gm.	0.00884gm.

In column 1 is given the period that the fish had been held in storage, column 2 the number of the fillets that were extracted while column 4 shows the average % residue after drying samples of the fillets to constant weight at 110°C. Column 3 shows the average % of fat in the different fillets used as determined by Miss A. M. Wilson of the staff of the Fisheries Experimental Station. Columns 5,6, and 7 are self explanatory while the last column was obtained by calculating the amount of soluble protein in the different fillets based on a total protein content of 19%.

Employing column 8 as a basis for comparison we find that there appears to be no denaturing of protein over a period of 8 months and since no samples of mackerel that had been stored for longer than this period could be procured no information is available on mackerel fillets over this age.

Observations on haddock muscle

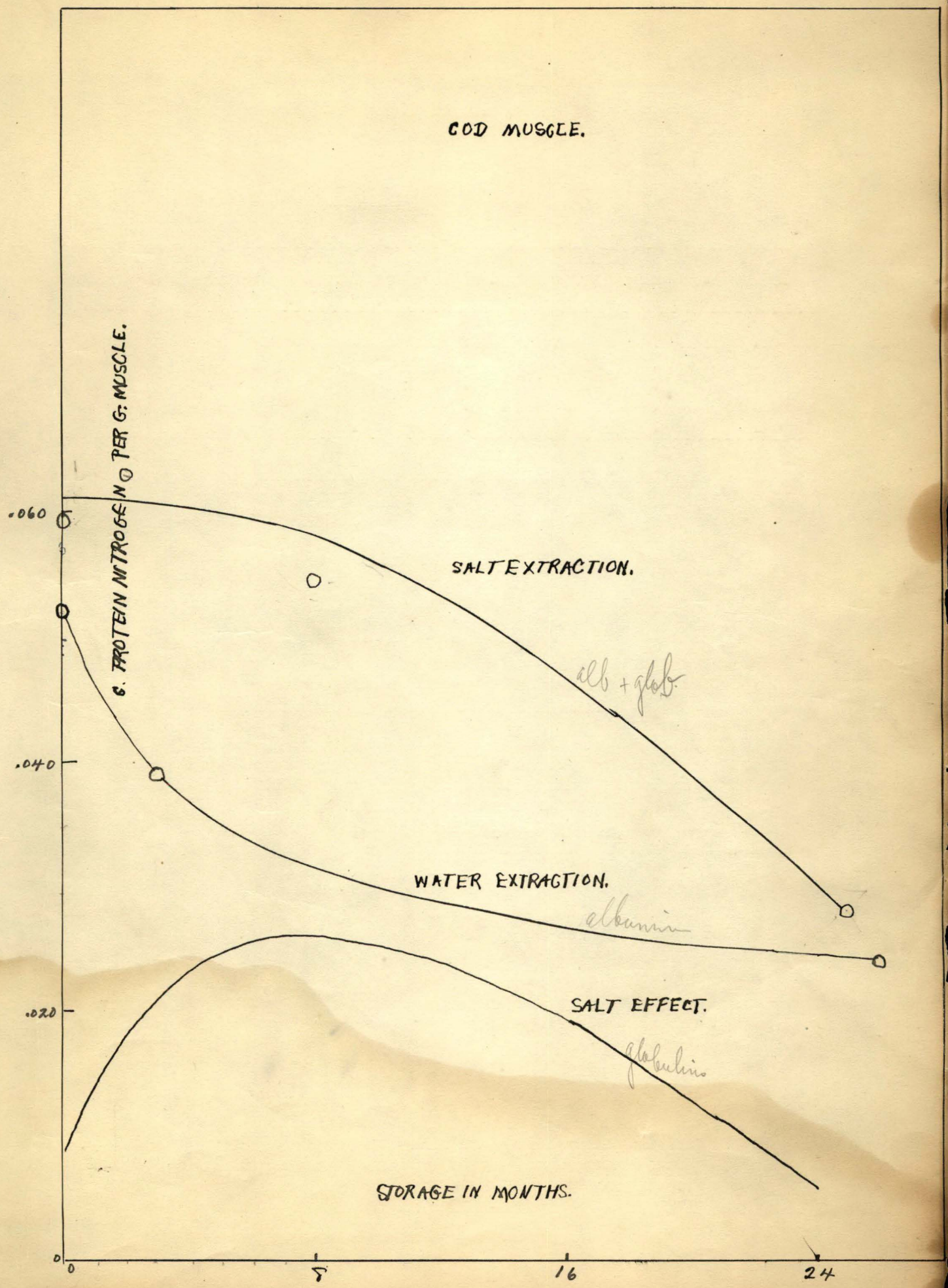
The results obtained on the denaturing of the protein in haddock muscle are summarized in the following table.

Table VI

Age of Muscle.	No. of Fillets Extracted.	Average % Residue at 110°C.	Average Tot. Nit. per gm. Fish	Average N.P.N. per gm. Fish.	Prot. Nit. per Gm. Fish.	Prot. Nit Calculated on Basis of 19% Residue.
0 mos.	4	18.2%	0.0102gm.	0.00324gm.	0.00696 gm.	0.00727gm.
9 "	3	19.4%	0.0110gm.	0.00378gm.	0.00722 gm.	0.00707gm.
15 "	3	not done	0.0100gm.	0.00413gm.	0.00597 gm.	0.00597gm.
17 "	3	" "	0.00940gm.	0.00368gm.	0.00572 gm.	0.00572gm.
25 "	3	19.2%	0.00777gm.	0.00345gm.	0.00432 gm.	0.00425gm.

In connection with the table given above it must be mentioned that the samples of haddock labelled 0 months were purchased from the National Fish Company and nothing of their history is known previous to freezing. In connection with the samples of haddock which were 25 months old it should be remembered that they had not been frozen in the usual fashion but by immersion in a brine bath. In comparing the above figures the fact must also be taken into consideration that although all samples had been stored at the same temperature yet those which were 25 months old had not been wrapped in individual waxed papers as the remainder were and had been packed loosely in a large wooden box, while those which were 9 months old had been wrapped in the usual manner but were packed in a closed tin pail. The remaining samples were preserved in the usual commercial containers, that is they were wrapped in a waxed paper and stored in a cardboard box.

By comparison of the figures recorded in the last column of the above table or of the accompanying graph with the denaturing curve to be shown for cod fish a striking similarity is observed, neither curves indicating any appreciable denaturing until after a period of about 10 months. Both of these curves appear to be an auto-catalytic type the significance of which might possibly be attributed to an accumu-


COD MUSCLE.
G. PROTEIN NITROGEN PER G. MUSCLE.
.060
.040
.020
0
SALT EXTRACTION.
alb + glob.
WATER EXTRACTION.
albumin
SALT EFFECT.
globulins
STORAGE IN MONTHS.
0
8
16
24

Temp. of storage
reason for slowing up!! W.D.

lation of hydrogen ions which would progressively increase the rate of denaturation but since the pH. of the samples at different ages was not taken and moreover, since the water extraction curve as will be shown later is not of a similar type as would be expected if the accumulation of hydrogen ions was correlated with the rate of denaturation of the protein this suggestion appears to be very doubtful. A more probable explanation of this phenomenon lies in the fact that at least in the early stages, the albumen seems to bear the main burden of the denaturing processes and that the first step in the denaturation of the albumen is its passage into a pseudo globulin fraction which is still dissolved by the phosphate solutions so that no apparent denaturation takes place until after this "converted" albumen becomes more insoluble and will no longer dissolve in the salt solutions. This explanation will become clearer after a perusal of the water extraction curve, given in connection with cod fish muscle.

Denaturing of cod fish muscle

In the following table are given the results obtained by extracting cod fish muscle with the usual salt solutions.

Table VII

Age of Muscle.	No. of Fillets Extracted.	Average % Residue at 110°C.	Average Total Nit. per gm. Fish.	Average N.P.N. per gm. Fish
0 mos.	4	19.1%	0.01002gm.	0.00411gm.
2 "	2	17.4%	0.00924gm.	0.00342gm.
8 "	3	not done	0.00956gm.	0.00410gm.
25 "	4	18.4%	0.00627gm.	0.00353gm.

Average Prot. Nit. per gm. Fish	Average Prot. Nit. per gm. Fish Calculated on Basis of 19% Residue.
0.00591 gm.	0.00591 gm.
0.00582 gm.	0.00629 gm.
0.00546 gm.	0.00546 gm.
0.00274 gm.	0.00282 gm.

In connection with the above table the fillets listed as 0 months were from cod fish which were brought living to the Fisheries Experimental Station, in a barrel of water and were killed and filleted just prior to freezing and extracting. Another point which must be remembered when considering the figures given above is that the fish which were 25 months old had been stored at a temperature of -8°F. while all remaining samples had been held at a temperature of -1°F. From a review of the above table or graph accompanying it one may observe that up to a period of approximately 10 months there appears to be little denaturing of protein but after this time the rate increases rapidly, this curve resembling to a marked degree the curve shown in the case of haddock muscle.

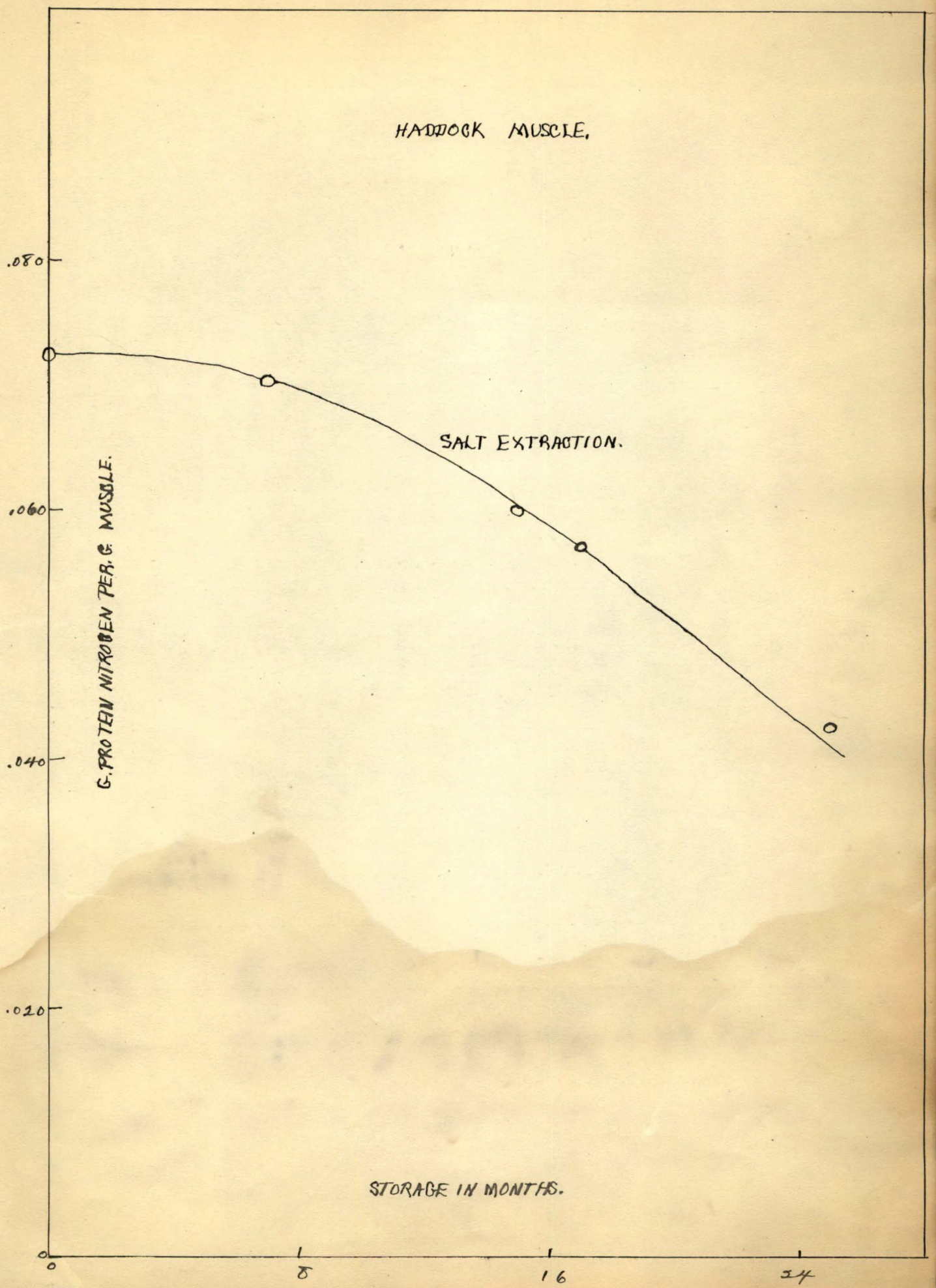
The aqueous extraction of cod fish muscle

As the phosphate solutions employed in the previous work extract both the albumens and globulins, the curves show the denaturing effect of these combined proteins and in order to obtain the albumen curve alone and thus differentiate between the denaturing of the two proteins a water extract on cod muscle was made. The technique employed in this work was the same as that used in the extraction with the salt solutions and the results obtained in this way are listed in the table below.

Table VIII

Age of Fillet.	No. of Fillets Extracted.	Tot. Nit. per gm. Fish.	N.P.N. per gm. Fish	Prot. Nit. per gm. Fish
0 Mos.	1	0.00856 gm.	0.00338 gm.	0.00518 gm.
3½ "	2	0.00711 gm.	0.00324 gm.	0.00387 gm.
26 "	2	0.00565 gm.	0.00331 gm.	0.00234 gm.

From an observation of the curve accompanying this table we may observe that the form of this curve is quite different from that of the phosphate extraction curve. By plotting the differences between this and that of the phosphate extraction curve we are able to derive a curve which should repre-


HADDOCK MUSCLE.
.080
.060
.040
.020
0
G. PROTEIN NITROGEN PER G. MUSCLE.
SALT EXTRACTION.
STORAGE IN MONTHS.
0
8
16
24

sent the course of the globulin denaturation but as can be observed this curve is "impossible."

An explanation of this apparent anomaly may lie, however, as previously explained in the fact that the denaturing of the albumen during the early periods of storage may proceed to just such an extent that while it no longer remains soluble in distilled water may still retain the property of dissolving in phosphate solutions and thus made to appear as a globulin, with the result that there appears to be no denaturing of protein until after this pseudo globulin becomes still more insoluble and finally refuses to be extracted by the phosphate solution.

General conclusions and summary

(1) As determined by extraction of the muscle with a phosphate buffer mixture there appears to be little denaturation of protein until after a period of 10 months when the rate is accelerated considerably.

(2) The rate of denaturing of albumen as shown by the water extraction curve is quite rapid during the first 6 months of storage after which it becomes considerably reduced. This curve follows more closely than the phosphate extraction curve that which would be expected from a knowledge of the rate at which stored fish loses its palatability and becomes tough and fibrous.

(3) From a consideration of tables V, VI, and VII we find as a side observation that the amount of soluble protein in the muscle of mackerel is considerably higher than that of haddock which is in turn somewhat greater than that occurring in cod.

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