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The Chemistry of Fish Muscle Proteins.

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The Chemistry of Fish Muscle Proteins

by

S. A. Beatty, M. A.

The work included in the following report is a continuation of that carried on for the last few years at The Atlantic Biological Station, St. Andrews, N.B., on the proteins of fish muscle plasma. This work was done during the summer of 1928. Four phases of the problem were attacked; (1) the earliest post-mortem changes in the character of the muscle, (2) a comparison of the protein content of fresh plasma and of plasma from fish in maximum rigor, (3) a comparison of the proteins of the plasma of the haddock, the cod, the hake and the skate, and (4) the source of the ammonia produced during the autolysis of the plasma.

The fish used in these experiments were obtained by means of trawls set near the Station and were kept in the basement of the building in cement tanks through which circulated a continuous stream of sea water. The fish seemed perfectly normal except that they almost invariably ran a blood sugar higher than normal.

The Earliest Postmortem Changes in the Character of the Plasma.

During previous work it was noted that the plasma cannot be pressed out of very fresh muscle. A short study of this phase was made to determine the conditions which cause the release of the plasma and to determine the time interval between the maceration of the muscle and the appearance of the juice. It was hoped that the process might be slow enough in order to allow extraction of the proteins and their isolation before they underwent this change. As the change in the muscle that enables it to give up its plasma under pressure is a sudden one, a pretty definite idea of the time of this change can be formed.

Experiment 1: Haddock were removed from the tanks and stunned by a blow on the head as soon as taken from the water. They were rapidly filleted, one fillet packed in cracked ice, and the other run through the meat chopper and placed in the press. The period between the killing of the fish and the first appearance of juice, and the period between the mincing of the muscle and the appearance of the plasma were noted. After a considerable period the second fillet was removed from the ice, ground and subjected to pressure. The results of experiments with six fish are so nearly alike that the reporting of more than one is needless repitition.

Haddock 2, taken from tank and killed without struggle. Rapidly filleted and one fillet placed in cracked ice. Second fillet minced and pressed. Gave slight amount of juice 9 1/2 min. after the death of the fish and 5 1/2 min. after the maceration of the muscle, and a copious flow 6 1/2 min. after macer-

5½ - 6 ↙

ation. The fillet placed on ice was macerated and pressed after 25 min. It yielded its plasma readily 4 min. after the application of pressure. The muscle was still reactive at the time of maceration.

Hake muscle appears to be of a more stable character than that of the haddock. For that reason the experiment was repeated with hake, in the hope that the proteins could be isolated before the change occurred. The fish were small averaging slightly less than 1 lb. each. They were caught on trawls and kept till required in the basement tanks. A typical experiment is as follows. Hake #1 was killed at 10.50 A.M. fillets macerated at 10.56, and pressed at once. Press juice appeared at 11.02. Second hake killed at 10.45, and placed at once in cracked ice, filleted at 11.20, macerated at 11.30 and pressed at once. Press juice appeared at 11.35.

6 + 6

10 + 5

It will be seen that, contrary to expectation, there is little if any difference between the behavior of the haddock and that of the hake. In both cases the main factor in the production of the change is the maceration and the pressing. Whatever the character of this change may be, it is hastened by exhaustion and asphyxia. Haddock exhausted in a small volume of water yielded up their plasma so rapidly that in only 1 case out of 4 could the muscle be macerated and pressed before the ready separation of the plasma from the ground muscle. In this case the plasma appeared 3 minutes after the maceration of the muscle.

The muscle of these fish resembles that of the fillets that were allowed to stand in cracked ice, although the plasma appears even faster than in the latter. This is probably because the rate of reaction is slowed in the latter by the lower temperature. One would expect a greater concentration of lactic acid in the muscles of the former.

At the present time no explanation of this phenomenon can be given. The experiments show that it is useless to attempt to isolate the proteins before they have undergone this very early post-mortem change. It is hoped that the study of this problem can be continued this year.

The Protein Content of fresh Fish Plasma and Plasma of Fish at maximum Rigor.

The cause of the contraction of the muscles after death has received a great deal of attention from scientists, and yet the problem is unsolved. The first definite step forward was made by Kuhne and Chittenden (1) who isolated myosin from frog muscle and made a study of the muscle clot. Halliburton (2) and von Furth (3) isolated several protein fractions from muscle plasma and from muscle extracts. Although not agreeing completely, their results in the main, confirm each other. They isolated at least two protein fractions, which the former called paramyosinogen and myosinogen. They showed the tendency of these proteins, especially of the latter, to pass into an insoluble clot. Von Furth came to the conclusion that rigor mortis consists in the coagulation of these proteins. The studies of the post-mortem chemical changes of muscle, especially of the development of lactic acid (4) has strengthened the general belief in this theory. (5).

A different explanation of the cause of rigor-mortis was given by Meigs (6). He found that frog muscle, when immersed in distilled water, underwent two kinds of swelling. The former was thought due to the entrance of water into the fibrils and the swelling of the contractile elements. This was accompanied by loss in irritability. The second swelling was considered due to the breakdown of the walls of the fibrils, and the loss of their ability to hold back the salts. As a consequence, water and salts both entered the sarcostyles, which became congested and the muscle shortened. It was shown that the sarcostyles swell in dilute acid. The accumulated lactic acid provides a suitable reaction for the swelling of these fibrils. Rigor mortis was considered due to the swelling of the contractile elements of the sarcostyles, caused first by the entrance of water, and, after the disruption of the walls, by the entry of both salts and water. This theory has been supported by von Furth (7) who thought the passing off of rigor due to the coagulation of the proteins.

Wacker (8) has advanced yet another theory to explain both contraction in living muscles and rigor mortis. This theory is also based on the swelling of the sarcostyles, due to the development of carbon-di-oxide. Wacker believes he has shown that the development of acid is too slight to cause either swelling or coagulation of the proteins. He believes that rigor can occur in muscle which has not changed in reaction, the lactic acid being neutralized as fast as it is produced, and some being burned with the development of CO_2.

Wacker has not given very convincing evidence for the above theory. At the present time the theory does not seem nearly so probable as the former two. Therefore we shall consider the evidence available in support or in opposition to the former theories.

The only protein part of the muscle that is thought to be in solution is the sarcoplasm. If rigor is caused by the coagulation of these proteins, because of the development of acid, the maximum coagulation should be expected to occur at the isoelectric point of these proteins. The work of Logan (8) has shown the muscle plasma of the haddock to consist of at least two protein fractions, one with an isoelectric point pH 6.0 and the other with an isoelectric point pH 4.8-5.0. Benson (9) has shown fresh muscle of trawl-caught (exhausted) haddock to have a reaction of pH 6.85 to 6.59, and of pen fish, a reaction of pH about 7.3. At maximum rigor haddock muscle from 6 fish had a reaction ranging from pH 6.68 to 6.49. Therefore rigor mortis in fish muscle is not due to isoelectric precipitation of the proteins.

If the proteins are coagulated during rigor, and there is no absorption of water, the concentration of the muscle press juice in proteins should be lessened. However if water is imbibed by the contractile elements of the sarcostyles, the sarcoplasm should be concentrated in proteins. The following experiments throw some light on this matter.

The haddock for this experiment were obtained from the basement tanks. They were killed and the fillet was removed from one side. The fillet was minced and the press juice pressed out. The fish was hung by the head and maximum rigor was shown by maximum curvature of the fish caused by the contraction of the muscle. During maximum rigor the second fillet was removed and the press juice obtained from it. Obviously not all the plasma of the muscle was obtained, The volume of the plasma of the muscle in rigor was usually about half or somewhat more than half that obtained from the fresh muscle. The total nitrogen and the non protein nitrogen were obtained, the difference between them giving the value of the protein nitrogen. The nitrogen determinations were made by digestion in 25-200 mm. test tubes with concentrated H_2SO_4 and H_2O_2 , and nesslerization. The proteins were precipitated with Na_2WO_4, 10% and H_2SO_4 0.667 N as in the preparation of a protein free filtrate of blood. The results are summarized in the following table.

Fish No.	Period of Rigor	Total N_2 per 100 cc. plasma	Non Protein N_2 per 100 cc. plasma	Protein N_2 per 100 cc.
1	Before	1.24 gms.	0.55 gms.	0.69 gms.
	During	1.92 "	0.53 "	1.39 "
2	Before	0.56 "	0.28 "	0.28 "
	During	1.43 "	0.38 "	1.05 "
3	Before	1.21 "	0.35 "	0.86 "
	During	1.41 "	0.37 "	1.04 "
4	Before	1.75 "	0.29 "	1.46 "
	During	2.15 "	0.34 "	1.81 "
5	Before	0.99 "	0.30 "	0.69 "
	During	1.54 "	0.33 "	1.21 "

It will be noted that the rise in non protein nitrogen is, in all but one instance, negligible. The rise in concentration of protein can be accounted for by either an increased solubility of protein or by a reduction in the amount of water in the press juice. This work is being continued with cat fish and results seem to indicate that there is no increase in the solubility of the proteins that could account for the difference in the concentration found above. These facts lend support to the theory that rigor is accompanied by the imbibition of water into the muscle elements. If water and the non protein fraction are imbibed at approximately equal rates the above results are explained. The details of the work done on cat fish will be given in a later paper.

The Isolation of the Proteins of the Haddock from the Muscle Press Juice.

The work done by Logan on the proteins of the haddock was carried out exclusively by means of extraction. The muscle was ground and dialysed to free it of its natural salts. The rate of dialysis was exceedingly slow. It was thought that the proteins might be more easily obtained from muscle press juice which should be more rapidly dialysed free of its salts. The plasma was obtained as in the previous experiments and dialysed in collodion sacs till the dialysate was salt free. A protein precipitate formed in the sac. This was filtered off and the filtrate brought to various concentrations of hydrogen ion. In some cases the protein was precipitated. The precipitate was filtered off and N_2 determinations were made of aliquots of the filtrate. As comparative values had as great significance as actual values, the digests were nesslerized and read against each other, the greatest colorimetric reading indicating the lowest protein content and therefore the maximum precipitation.

The precipitate that formed in the collodion sac was treated with 0.9N NaCl. The greater part went into solution. It was filtered and the isoelectric point found in the same manner as that of the protein soluble in water.

The procedure is very similar to that of Logan, the main difference being the use of the micro-Kjeldahl and Nessler's solution instead of the macro-Kjeldahl for the determination of the protein nitrogen. The results are as follows.

Fish #1

Water Soluble Protein

pH	Colorimetric Reading.
5.2	Nesslers pptated. due to excess NH$_3$
5.4	11.6
5.6	16.5
5.8	18.9
5.9	19.8
6.0	20.0
6.1	20.0
6.2	19.6
6.3	19.0
6.4	18.6

Salt Soluble Protein

4.4	18.6
4.6	19.9
4.8	20.2
5.0	20.0
5.1	19.4
5.2	18.3
5.37	17.1
5.37	17.6
5.4	16.8

Fish #2

Salt Soluble Protein.

4.4	18.0
4.6	19.3
4.8	20.0
5.0	20.0
5.2	18.5
5.4	17.1

The results are so similar to those of Logan that they need no comment. For small amounts of material the most rapid method of preparation is as follows. The muscle press juice is dialysed till free of salts and filtered. The filtrate is brought to pH 6.0, and the water-soluble protein is precipitated. The precipitate from the dialysis is dissolved in 0.9M NaCl, filtered to free it from a portion that has become denatured, brought to pH 4.8 and filtered. There is a considerable loss in this process as the greater part of the proteins cannot be pressed out of the muscle.

The Isolation of Protein from the Hake, the Cod and the Skate.

The proteins of the above fish were obtained from the muscle press juice by means of dialysis. They were dialysed in collodion sacs till a heavy precipitate appeared in the sac and the dialysis water was salt free. The contents of the sac were filtered, the filtrate brought to definite pH values as in previous experiments, and the amount of protein remaining in solution determined by digestion and nesslerization. Minimum protein values are shown by maximum colorimetric readings.

The precipitate was dissolved in 0.9 N. NaCl and filtered. It was brought to its isoelectric point in the same manner.

Proteins of the Hake 1.

Water-soluble Portion

pH	Col. Reading
6.0	20.0
5.9	25.5
5.8	26.3*
5.6	25.4
5.3	23.4
5.0	20.0

Isoelectric point as indicated by point of minimum solubility is about 5.8.

Salt-soluble portion.

pH	Col. Reading.
5.3	20.0
5.2	22.1
5.1	25.6
5.0	25.4
4.9	25.8
4.8	25.3

The isoelectric point lies between pH 5.1 and 4.8.

Hake 2.

Water-soluble Portion.

pH	Colorimetric Reading.
6.5	16.4
6.3	19.8
6.1	22.0
5.9	24.5*
5.7	23.1
5.5	22.7
4.8	20.0

Salt -soluble Portion. In view of the indefinite results of the former experiment, the proteins were brought to pH 5.0 and centrifuged. The precipitate was dissolved in dilute salt solution to which was added enough alkali to render it faint pink to phenolphthalein. Most of the protein went into solution. It was filtered and the filtrate precipitated at various concentrations of hydrogen ion as before.

pH	Col. Reading.
6.0	8.1
5.7	10.2
5.4	11.5
5.2	12.2
5.1	16.0
5.0	20.0
4.9	19.2
4.9	23.9
4.8	25.4*
4.7	24.8
4.6	24.5

In the second experiment the isoelectric point of the water-soluble portion is about pH 5.9 and that of the salt-soluble protein about pH4.8. The reading 19.2 for pH 4.9 is believed to be an error in analysis. A duplicate analysis gave the reading 23.9.

Unfortunately no more material was available.

Conclusion. The proteins of the hake are very similar to those of the haddock; the isoelectric point of the water-soluble protein being about pH 5.9, and that of the salt-soluble protein about pH 4.8. Determinations of hydrogen ion were done colorimetrically and they are not thought to be correct to more than 0.2.

Proteins of the Cod Muscle Plasma. The above experiments were repeated with cod muscle press juice. The dialysed proteins were precipitated and redissolved at pH 9 in order to get them in a more pure form. The results are as follows.

Water Sol. Proteins.

	pH	Col. Reading.
#1	5.9	20.1*
	6.0	20.0
	6.1	18.3
	6.3	18.0
	6.4	17.3
#2	5.6	20.0
	5.8	21.8*
	6.0	20.9
	6.3	17.7
#3	6.2	20.0
	6.0?	20.7
	5.9	21.3*
	5.8	20.9
#4	5.8	20.6
	5.6	20.0
	5.6	19.4
	5.4	18.1
#5	6.2	18.1
	6.0	19.5
	5.8	20.0*
	5.7?	19.2
	5.4	18.6
	5.2	17.4

Salt Soluble Protein

5.4	18.2
5.2	19.6
5.0	20.0
4.6	20.5

Proteins of the Skate.

Skate muscle juice was dialysed 12 hrs. and centrifuged. The water-soluble portion was treated as in previous experiments. The residue in the centrifuge tubes was shaken up with 0.9 N. NaCl and recentrifuged, the part going into solution being treated as the salt-soluble proteins in the previous experiments.

Water-soluble portion.

pH	Col. Reading.
4.5	20.0
4.7	24.2
4.9	25.6
5.0	26.6*
5.2	24.4
5.6	17.6

Salt-soluble portion.

5.2	20.0
5.0	21.7
4.8	22.3
4.7	22.9*
4.6	22.8*
4.2	21.3
4.1	20.1

Conclusion; The related fish, the haddock, the cod, and the hake have plasma proteins of a very similar nature. Two proteins were found in each fish, and the isoelectric point of the proteins were very similar. In the skate muscle were found two proteins, one soluble in water and the other soluble in dilute saline as in the other forms, but with isoelectric points of about pH 5.0 and 4.7 respectively. The proteins of the catfish are under examination at the present time.

The Source of Ammonia in Autolysing Fish Muscle; As a supplement to the work of Dr. G. B. Reed and co-workers on the autolysis of fish muscle, some work was done on the source of the ammonia produced during the autolysis of fish muscle. Bliss (11) has shown that ammonia can be carried through the body in the form of protein amide nitrogen. He obtained an enzyme from kidney that released ammonia from blood protein and from casien. It was thought possible that ammonia produced during autolysis is derived from the breakdown of the amide groups of the proteins. To determine the correctness of this supposition analyses were made of amide nitrogen and ammonia nitrogen of muscle plasma before and after autolysis. If the decrease in amide nitrogen is equal to the increase in ammonia nitrogen the ammonia must all be derived from this source.

Method; Determination of Ammonia. Two to five c.c. plasma were placed in 25-200 mm. test tubes, 2 cc. of 20% Na_2CO_3 and 1 drop of caprylic alcohol were added and the ammonia aerated into dilute HCl, and nesslerized in the usual manner. Duplicate analyses checked within 2% and added ammonia was recovered within the same error by 1 hr. aeration. All analyses were aerated 1 1/2 hours to ensure complete recovery.

Determination of Amide Nitrogen. One to two cc. plasma were placed in a centrifuge tube and mixed well with Almen's reagent.*

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*Almen's reagent consists of 4 gms. tannic acid in 8 cc. glacial acetic acid and 190 cc. of 50% alcohol.

This was the only precipitant that gave an easily centrifuged precipitate with the autolysed plasma, 8 cc. giving a protein free filtrate with both the fresh and the autolysed plasma. The precipitate was centrifuged and the supernatant liquid discarded. The precipitate was washed twice with the reagent, 4 c.c. of 4 N. HCl were added and the whole was hydrolysed for 4 hours in a boiling water bath. The hydrolysate was washed into a large test tube, made basic with NaOH, and the ammonia aerated into dilute HCl and nesslerized.

The muscle plasma was obtained from fish from the basement tanks. Preliminary determinations of amide nitrogen and of ammonia nitrogen were made. Plasma was set aside, usually 1 cc. in a centrifuge tube for amide nitrogen and 2 cc. in a large test tube for ammonia nitrogen. A buffer pH 6.2 in proportion to the amount of plasma was added to each. Toluol was thoroughly mixed with the plasma in both tubes. Toluol does not produce complete sterility but does not allow an increase in the bacterial content of the medium if it is thoroughly mixed with it. (Dr. G. B. Reed, Personal communication). The tubes were set aside for varying lengths of time to allow the development of the ammonia. After this incubation period analyses of the amide nitrogen and of the ammonia nitrogen were made. The results are included in the following tables.

Experiment No.	N_2 per 100 cc. plasma	Amide Mgs.	Ammonia Mgs.	Incubation Temperature	Incubation Time Days.	Amide Decrease Mgs.	NH_3 Increase Mgs.
1	Preliminary	36.4	11.4	Room			
	Final	31.1	16.5	(18°)	2	<u>5.3</u>	<u>5.1</u>
2	Preliminary	43.1	10.0				
		43.5	9.9	Room	2		
	Average	43.3	10.0				
	Final	36.6	15.4	"	2		
		38.2	15.0				
	Average	37.4	15.2			<u>5.9</u>	<u>5.2</u>
3	Preliminary	53.0	8.0				
		52.0					
	Average	52.5					
	Final	41.5					
		42.0					
	Average	41.7	18.6	"	5	<u>10.8</u>	<u>10.6</u>
4	Preliminary	34.6	11.8				
		34.4					
	Average	34.5					
	Final	21.6	25.2				
		20.7	25.6				
	Average	21.1	25.4	"	5	<u>13.4</u>	<u>13.6</u>
5	Preliminary	34.7	9.2				
		35.6	9.4				
	Average	35.1	9.3				
	Final	20.9	26.9				
			25.9				
	Average		26.4	"	5	<u>14.2</u>	<u>17.1</u>
6	Preliminary	47.4	11.6				
	Final	32.8	25.6	"	5	<u>14.6</u>	<u>14.0</u>
7	Preliminary	47.6	8.1				
		48.5	8.3				
	Average	48.1	8.2				
	Final	38.8	16.5				
		37.4	16.7				
	Average	38.1	16.6	"	5	10.0	8.4

The results show that the greater part, if not all, the ammonia formed in fish muscle plasma is derived from the amide groups of the proteins. As amide groups are present in the proteins of the formed elements of the muscle and as Bliss has shown that desmidases release amide nitrogen from proteins in general, it is extremely probable that all ammonia formed in autolysing fish muscle comes from that source.

Summary: The early postmortem changes in muscle has been studied. It was found that these changes are so rapid that it is impossible to isolate any protein fraction, with the methods at present available, before these proteins have undergone a change in character. The nature of these changes is not known.

It has been found that the concentration of the plasma in protein increases considerably during rigor. It is thought that this observation will lead to proof that during rigor water passes from the plasma to the formed elements of the muscle.

A comparison has been made of the proteins of the haddock, the hake, the cod and the skate. A striking similarity has been found in the proteins of the first three.

It is believed proven that the greater part, if not all the ammonia produced during the autolysis of fish muscle plasma is derived from the amide groups of the proteins.

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