CHEMISTRY OF THE SOLUBLE PROTEINS OF FISH MUSCLE

THE CHEMISTRY OF THE SOLUBLE PROTEINS OF FISH MUSCLE; ITS PROBABLE RELATION TO MUSCULAR MOVEMENT AND TO RIGOR MORTIS.

by

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The study of the chemical processes concerned with muscular movement can be followed in two directions. In the first place, we may study the structure of the muscle itself, and the mechanics of its operation. Secondly, we may study the energy supply of the muscle, its source, and the manner in which it is made available.

Our knowledge of the energy supply of the muscle has developed to the extent, that, in all probability, we know more of the reactions taking place in the muscle cells than of those in any other cells of animal tissue. The muscle as a whole lends itself to more varied treatment than do most other tissues or organs. The cells are all similar in character, and results obtained from experiments on muscle in mass can be considered the sum total of the effects of a very large number of individual cells, each identical with its neighbour. As a result, we have a fairly complete picture of the fuel supply of muscle, and of the manner in which this fuel is burned.

A review of the literature of the early work, from which developed the biogen theory, is not of great profit. The first important advance in the chemistry of muscle was made by Fletcher (1898). He showed that resting muscle in an atmosphere of oxygen has a constant output of carbon dioxide. This carbon dioxide increases on stimulation. Fatigue does not occur. He found that a muscle in an atmosphere of nitrogen gives off but slightly more carbon dioxide during contraction than when at rest. Such muscle fatigues rapidly. A muscle, fatigued in an atmosphere of nitrogen, recovers when placed in an atmosphere of oxygen. These experiments show that the muscle respiration is concerned with the recovery of the muscle and not with the contraction.
The role of lactic acid in the contraction of the muscle has been explained in part by Fletcher and Hopkins (1907,1917). By means of a low temperature, they were able to prevent the formation of lactic acid in the muscle during their analytical procedure, and placed the study of lactic acid in muscle on an accurate quantitative basis. They showed the dependence of the muscle on respiration to eliminate the lactic acid produced during the contraction. The experiments of Fletcher, and those of Fletcher and Hopkins showed that the contraction of the muscle is an anaerobic process and that muscle respiration is concerned with the recovery period. The relation between lactic acid and muscle glycogen was demonstrated by Meyerhof,(1919). He showed that, under anaerobic conditions,muscle glycogen is hydrolysed to lactic acid. He found that, during the

recovery

recovery period, the glycogen increases and the lactic acid decreases. Of the total lactic acid, approximately four-fifths only could be accounted for as glycogen. The gas exchange during the recovery period indicated the oxidation of an amount of lactic acid approximately equal to the fraction unaccounted for. Meyerhof concluded that the course of events during a muscular contraction are as follows. The contraction is the result of the very rapid production of lactic acid at the expense of muscle glycogen. The relaxation is the result of the neutralization of the lactic acid. The disappearance of the lactic acid during the recovery period can be accounted for in two ways. Either the lactic acid is all changed to glycogen, the energy required for this reaction being obtained from the combustion of some substance giving an R. Q. of 1, or approximately four-fifths of the lactic acid is reformed to glycogen and the remaining lactic acid is burned. Meyerhof's latest figure for this ratio is 1/4.7. (Meyerhof and Schulz, 1927.)

The production of heat throughout the whole cycle of a contraction of striated muscle has been followed by Hill, (1912). Heat is produced during the contraction period either in the presence or in the absence of oxygen. This heat is, therefore anaerobic. From the

results of the work of Fletcher and Hopkins and that of Meyerhof, one would conclude that this heat is produced, for the most part, by the breakdown of glycogen to lactic acid. There is a delayed anaerobic heat, the role of which is not known. A third heat production occurs after the contraction, but only in the presence of oxygen. The heat production and the lactic acid production have been linked up with the tension of the muscle during the contraction. Hill found that the heat production is proportional to the tension, Meyerhof, that the lactic acid is proportional to the tension, and Hill and Peters, that the lactic acid is proportional to the heat produced. Hill, (1928), found the oxidative heat 2.07 times the anaerobic heat. From this data, the ratio lactic acid oxidized to lactic acid produced was calculated to be 1 to 4.81, a very good agreement with the figure given by Meyerhof and Schulz.

The chemical changes in active muscle are probably far more complex than is indicated by the results cited above. These results are probably sufficiently accurate and complete to give us a correct picture of the general course of events. But they offer no explanation as to the contractile mechanism. This mechanism must be dependent on the structure of the muscles themselves. This structure has been studied from the viewpoints of anatomy and histology. Lack of space will not permit us as complete an account here as can be found in reference works in these fields.

fields. The chemical structure of muscular tissue is more within our field, and the remainder of this thesis will be concerned with this problem, and with an attempt to link up, as far as possible, the chemical structure with the anatomical and histological form of the muscle on one hand, and with the energy supply and the energy consumption on the other. Water and proteins are the most abundant chemical compounds in muscle tissue. They are probably intimately associated with eachother. The chemistry of the proteins of muscle is not well known. It was in the hope , that a study of the chemistry of the proteins of muscle would throw some light on muscle phenomena, that the present work was attempted. The first problem attacked was the isolation and the characterization of these proteins.

The literature in connection with the separation and classification of the proteins of muscle is not large. Important work has been done however, and a fairly thorough review of this work seems essential . The foundation of our knowledge of the proteins of muscle was laid by Kuhne in 1864. He found that the plasma of frog muscle goes almost instantaneously into an insoluble clot at 40°C. This change occurs more slowly at room temperature, and very slowly at 0°C. The fresh plasma was found to have a syrupy consistency and a faintly alkaline reaction. He named the clotted protein, myosin, and the liquid that separated out, muscle serum. Kuhne was unable to obtain plasma from

mammalian

muscle, because, he believed, of the clotting of the plasma before it could be isolated.

Halliburton (1888), made a study of the plasma proteins of mammalian muscle and their tendency to coagulate. He obtained plasma from rabbit muscle in the following manner. The blood was perfused from the hind limbs with physiological salt solution, the muscle was dissected free of connective tissue, frozen and ground and pressed while still frozen. The press juice coagulated in 20 to 30 minutes at 40°C. The coagulation was accompanied by the development of an acid reaction. The clot was found to be soluble in 10% NaCl, or in 0.2% HCl. As the formation of the clot was found to be reversible, the proteins could not have been denatured. Muscle press juice obtained from muscles in rigor did not pass into a clot but it coagulated at 40°C. This coagulum was found insoluble except in strong mineral acids. The failure of the muscle press juice from muscles in rigor to form a clot was thought due to a clotting in the muscles. Therefore Halliburton advanced the theory that rigor is due to a clotting of the proteins in the muscle, and the passing off of rigor, to the reversal of this process.

Various protein fractions were obtained from press juice by means of heat coagulation and by salting out. Five proteins, paramyosinogen, myosinogen, myoglobulin, albumose, and albumin were obtained. Paramyosinogen was found to be soluble in salt solution, and completely

precipitated

precipitated with $MgSO_4$, 50%, or NaCl, 23%. It is insoluble in water. The coagulation temperature is 45 to 50°C. Myosinogen was precipitated with $MgSO_4$, 94%, or NaCl, 36%. It became insoluble after several washings with saturated salt. The coagulation temperature was found to be 56°C. The protein could be coagulated at room temperature by means of a myosin ferment which the author isolated from muscle. Myoglobulin was obtained by the complete saturation of the solution with $MgSO_4$. It coagulated at a temperature of 63°C. It precipitated on dialysis. It was thought to take no part in the formation of the clot. The myoalbumin was found to have the same coagulation temperature as blood albumin. It was isolated from the press juice or extract, after the removal of the myoglobulin, by heating to 73°C and filtering. It was thought to be identical with blood albumin and its presence in the muscle was believed to be due to the incomplete removal of the lymph from the muscle during the preliminary washing. Albumose, a protein that gave a weak biuret and that did not coagulate on heating, was obtained by saturating the above filtrate with ammonium sulphate.

Von Furth, (1895), studied the proteins of the muscle plasma of the dog, the rabbit, the frog, and the fish. The animals were perfused with physiological salt solution, the muscles were ground, and the plasma was expressed with a tincture press. The paramyosinogen was isolated in two ways, by means of dialysis and by salting out. The press juice was dialysed in

parchment

parchment tubes, 12 to 24 hours against running tap water, and the same time against distilled water. It was filtered and washed with distilled water till the filtrate was protein free. Part of the protein was denatured. The undenatured paramyosinogen was separated from the denatured protein by extraction with NH_4Cl, 10 to 15%. Purification of the paramyosinogen by means of salting out was accomplished as follows. Saturated $(NH_4)_2SO4$ was added to the plasma in the ratio of 1½ cc. salt solution to 2 cc. plasma. The plasma was filtered and the precipitate was washed with 23% $(NH_4)_2SO_4$. It was redissolved in NaCl solution and reprecipitated with $(NH_4)_2SO4$. Each stage of the process denatured part of the protein. The coagulum was insoluble in all salt solutions, but was slightly soluble in dilute acids and alkalis. It clouded on heating to 44 to 47°C, and flocked at a temperature of 47 to 50°C. It was completely precipitated with $(NH_4)_2SO_4$ 28%, with NaCl 15%, and with $MgSO_4$ 30%. It precipitated on the addition of dilute acids and dissolved either with an excess of acid or of alkali.

The myosinogen of Halliburton was isolated by dialysing the press juice and filtering off the paramyosinogen. It was isolated by heating the press juice to 52°C., and filtering off the coagulated paramyosinogen, or by the removal of the paramyosinogen with $(NH_4)_2SO_4$, 28%. It was found to be a clear liquid with an amber tinge. It coagulated

at

at a temperature between 55°C and 65°C. It was not precipitated on dialysis. It was precipitated by 40% $(NH_4)_2SO_4$. It was but partially precipitated with saturated NaCl or saturated $MgSO_4$. Halliburton believed that both paramyosinogen and myosinogen participated in the formation of the clot. Von Furth came to a different conclusion. Paramyosinogen, or as he named it 'myosin', was believed to pass directly into the form contained in the clot. Myosinogen, called myogen by Von Furth, was believed to pass through an intermediate stage which he called 'soluble myogen fibrin'. Soluble myogen fibrin coagulated at 40°C and was salted out at the same salt concentration as was myosin. The change, myogen to soluble myogen fibrin was slow, especially at temperatures near the freezing point. From the results of Kuhne, von Furth concluded that soluble myogen fibrin occurs in living frog muscle. Von Furth could find but questionable traces in living mammalian muscle. Salts were found to favour the formation of soluble myogen fibrin. Proteins obtained by dialysis were stable, not clotting after 24 hrs.

Von Furth found no evidence of myoglobulin and concluded that it was simply a fraction of myogen. Myoalbumose could not be demonstrated. Albumin was found in small amounts. A myoproteid was isolated from fish muscle plasma by boiling the plasma for 10 minutes and filtering

off

off the coagulum. The proteid was obtained from the filtrate by salting out with the appropriate concentration of salt.

In summarizing the results of these workers, it is desirable to point out; (1) the variation in the results, (2) the indefiniteness of the coagulation temperatures and the uncertainty of results based on them, (3) the extreme instability of the proteins concerned. This summary is given in Table 1.

The proteins, isolated from muscle by Halliburton and by von Furth, were obtained either from press juice or from extracts made of the muscle with physiological salt solution. Stewart and Sollman (1899), used a much stronger salt solution as extraction medium. The separation of the protein fractions in the extract was accomplished by means of heat coagulation. They found two protein fractions corresponding to von Furth's myosin and myogen. Von Furth found the myosin to myogen ratio about 1 to 4. Stewart and Sollman found this ratio about 2 or 3 to 1. This difference in the results is probably due to the difference in the extraction media used. Evidence for this will be given later. Stewart and Sollman were able to extract the proteins from muscle in rigor in the same relative proportions as from fresh muscle.

TABLE 1.

Proteins of Muscle according to Halliburton and von Furth.

Precipitant.	Halliburton.	von Furth.
	Did not observe soluble myogen fibrin.	Soluble myogen fibrin.
Heat		30-40°C.
Salts		Precipitated by the same salt concentration as myogen.
	Paramyosinogen	Myosin
Heat	47°C.	47-50°C.
$MgSO_4 7H_2O$	37-50% solution or 1.75 molar.	
NaCl.	15-26% or 3.94 molar	
$(NH_4)_2SO_4$		12- 24% or 1.0-1.75 molar.
Dialysis	Precipitated	Precipitated.
Acids	Not precipitated	Precipitated by acetic acid mineral acids and CO_2.
Alcohol		Precipitated, denatured on standing.
	Myosinogen.	Myogen.
Heat	56°C.	55-65°C.
$MgSO_4 7H_2O$	About 2.64 molar	Partial precipitation by saturated solution.
NaCl.	About 5.38 molar	" "
$(NH_4)_2SO_4$		26-40% or 2.00-3.75 molar.
Dialysis	Precipitated	Not precipitated
Acids		Precipitated in the presence of salts.
Alcohol		Precipitated, denatured on standing.

TABLE 1. (Cont.)

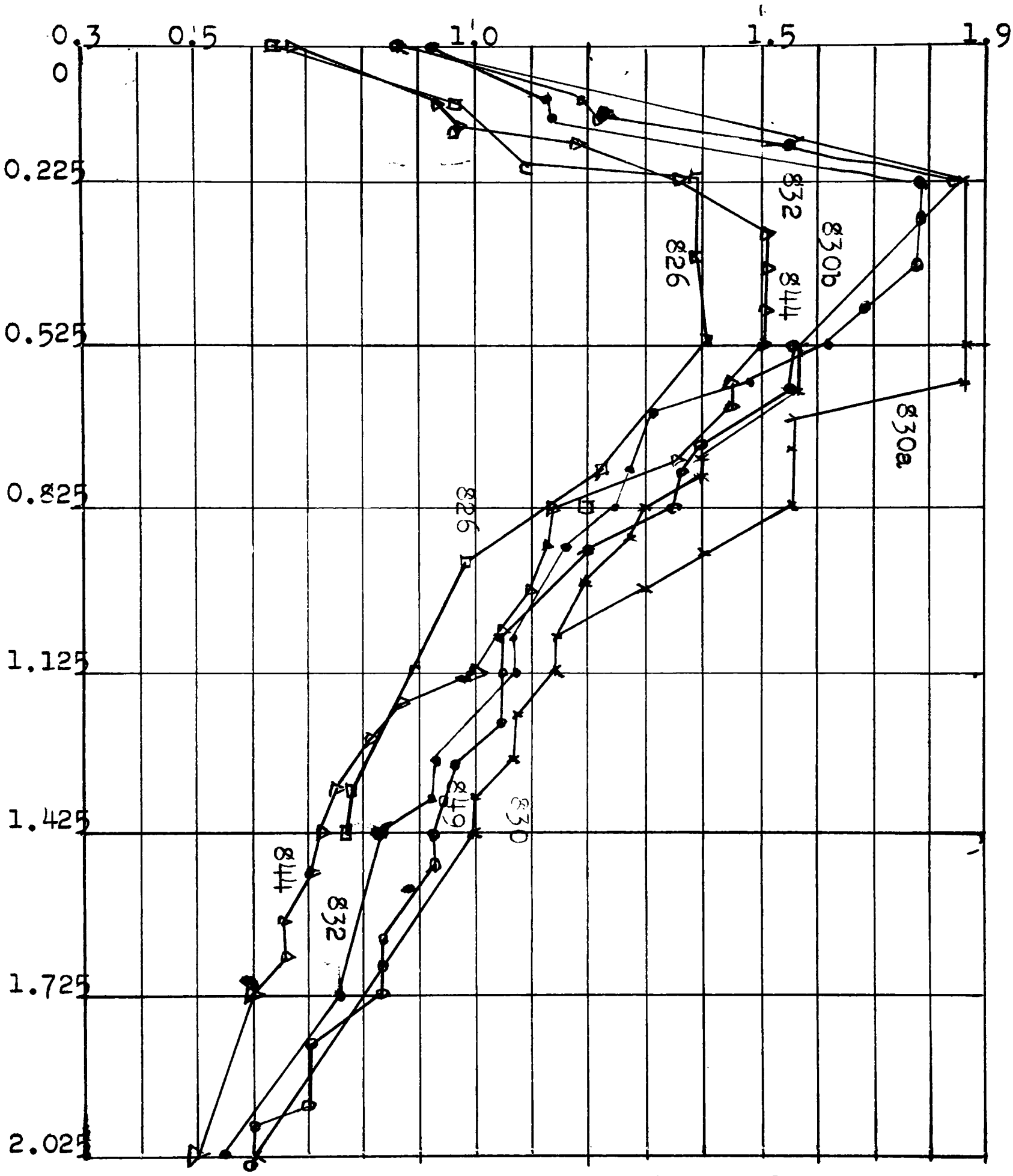
Precipitant.	Halliburton	von Furth.
Heat $MgSO_4 \cdot 7H_2O$ NaCl	Myoglobulin. 63°C. 4.0 molar Saturated.	Could not distinguish myoglobulin from myosin.
	Albumin.	Albumin.
	Considered identical with serum albumin.	Considered identical with serum albumin.
	Albumose.	Could not identify.
Heat $(NH_4)_2SO_4$ Dialysis	Not precipitated. Saturated solution. Precipitated	
		Myoproteid.
Heat $(NH_4)_2SO_4$ NaCl. $MgSO_4 \cdot 7H_2O$ Ethyl Alcohol		Not precipitated. 35%. 36%. 50%. 60%, Not denatured.

Howe,(1924) extracted the muscle of the rabbit and of the cow with monopotassium phosphate and dipotassium phosphate in the ratio of 1 to 2, giving a reaction of pH 7. He found increasing amounts of protein in the extracts as the salt concentration was increased from 0 to 0.225 molar. Increase in the salt concentration above this value produced a diminution in the solubility of the protein, until at about 2.025 molar phosphate, the proteins were almost completely salted out. Howe claims to have found "critical zones" which indicate different proteins, in the curve of decreasing solubilities. These zones occurred at salt concentrations of 0.525, 0.825, 1.125, 1.425, 1.725 and 2.025 molar phosphate. Howe believes the myosinogen of Halliburton and the paramyosinogen of Halliburton to be included in the myosin of von Furth, and the myoalbumin of Halliburton to be identical with the myogen of von Furth. According to Howe's data, myosinogen is salted out at a salt concentration between 1.125 and 1.725 molar, and paramyosinogen is salted out at a salt concentration between 0.225 and 1.125 molar. Each fraction is believed to consist of two proteins. Myoglobulin is supposed to be salted out at salt concentrations between 1.725 and 2.025 molar, and albumin to be the fraction salted out above this salt concentration.

Howe has given quantitative data on three proteins only, paramyosinogen, myosinogen and albumin. In calf muscle he found, paramyosinogen 46%, myosinogen 39%, and albumin 15%. In beef muscle, paramyosinogen was found to be about 52%, myosinogen about 34%, and albumin about 14%. Rabbit muscle yielded values, paramyosinogen about 52%, myosinogen about 38%,and albumin about11%.

The procedure of Howe is of great advantage in that it permits the experimenter to work rapidly. The unstable nature of the proteins, especially of myogen, (myosinogen), enforces working with rapidity and at low temperatures. But the author can see no evidence of critical zones in the data of Howe that seem to indicate different proteins. A copy of Howe's curves is given in Figure 1, page 15. With the exception of curve # 830, the curves are fairly regular, and the breaks in one curve that should indicate differences in the solubility of the proteins do not occur at the same salt concentrations in other curves. It is possible that Howe did get a fairly complete separation of the proteins as he was able to use the considerable experience which he has gained in working with blood proteins,and used heat coagulation as well as solubility measurements to make the separation. It is interesting to note that the values given by Howe for paramyosinogen and myosinogen are in closer agreement with those of Stewart and Sollman than with those of von Furth.

Figure 1.



The effect of increasing concentrations of a mixture of monopotassium and dipotassium phosphates 1:2 on the quantity of protein extracted from muscle. Results are expressed as gms. N_2 per 100 gms. meat. (Howe, Jour. Biol. Chem. 1924, Vol. 61, P.497).

Since the greater part of the experimental work of the auther was done, Ritchie and Hogan, (1929), have reported analyses of the soluble proteins of the muscle of the rabbit. The rabbits were perfused with physiological salt solution, and the muscles were ground and extracted with 10 % NaCl. The proteins of the extract were fractionated by four different procedures; salting out, dialysis,heat coagulation, and irradiation with a quartz mercury light. The nitrogen compounds of the extract were divided into three groups, an albumin, a globulin and a non-protein fraction. Their results are summarized in Table 2, Page 17. They show a ratio of albumin to globulin of about 1 to 2.5.

The present work on the characterization of the soluble proteins of fish muscle was begun at The Atlantic Biological Station, St. Andrews, N.B., by Dr. J. F. Logan, (1930). His work was done entirely on haddock muscle. The solubility of the proteins in sodium chloride solution was found to increase gradually from a concentration of 0% NaCl. to that of about 4% NaCl. Between concentrations of 4% NaCl and 12% NaCl there was little difference in solubility. At a concentration above 12% NaCl there was a gradual lessening in the amount of dissolved protein. The curve throughout was regular. The work of Howe was repeated with phosphate solutions at pH 7, but there was no evidence of breaks in the curve that might indicate different proteins.

TABLE II

The Soluble Proteins of Rabbit Muscle.

Precipitant.	NaCl Sat.		Irradiation.	Dialysis.	Heat Coagulation.
Reaction	pH 6	pH 7	pH 6		
Substance	Percent of the total nitrogen				
Globulin	61.8 59.3 59.4 61.4 51.3	57.8 57.7 50.1 51.3 57.8	54.7 55.7 53.8 42.3 57.3	68.7 56.0 43.5 46.1 52.3	51.9 53.9 55.7 54.9 47.5 48.5 57.2 60.7
Albumin	20.5 23.9 28.3 27.1 23.5	25.1 24.6 30.8 20.1 25.6	17.1 16.9 29.9 21.1 42.5	12.4 21.3 22.0 16.9 20.9	28.3 16.1 25.3 19.6 18.4 22.0 19.6 22.2

A separation of the proteins of haddock muscle was obtained in the following manner. The muscle was ground, inserted into parchment bags and dialysed till the dialysis water gave no test for chloride or phosphate. The muscle was repeatedly extracted with distilled water till no more protein went into solution. It was then extracted with 0.9 molar NaCl.
A second protein fraction went into solution. Coagulation temperatures were so indefinite that they were considered valueless. Determinations of the isoelectric points of these fractions by means of minimum solubility, showed an isoelectric point for the water-soluble portion of pH 6.0 and an isoelectric point for the salt-soluble portion of pH 4.8-5.0.

The study of the soluble proteins of the muscle of the haddock was continued by the author (1926). Partial amino acid analyses of both fractions were made. The tryptophane content of each was done by the method of May and Rose, (1922). The protein, soluble in distilled water, was found to have a tryptophane content of 1.53%, and the protein soluble in salt solution, a tryptophane content of 1.09%. Casein was used as a standard, the tryptophane content of which was taken as 2.00%. The tyrosine content of each was determined by the method of Folin and Looney, (1922). The water-soluble protein was found to have a tyrosine content of 4.02%,and the salt-soluble protein, a tyrosine content of 3.78%. The cystine content was

determined

determined by the method of Folin and Looney, (1922). The cystine content of the water-soluble portion was found to be 1.62%, and that of the salt-soluble portion, 0.99%.

The following methods have been used in the separation of the soluble proteins from muscle; salting out, dialysis, irradiation with ultraviolet light, extraction with distilled water followed with extraction with salt solutions. The results are extremely confusing. Howe reported 6 proteins, Halliburton reported 5, von Furth 4 proteins in mammalian muscle and 5 proteins in fish muscle, Stewart and Sollman 3 proteins, Ritchie and Hogan 2 proteins, and Logan 2 proteins. The methods for the separation of the protein fractions have been similar. Why is there this great variation in the results? The great difference in the procedures is the extraction method used. Halliburton and von Furth used either expressates or extracts made with physiological salt solution. They reported an excess of the albumin-like myogen. Stewart and Sollman, Howe, and Ritchie and Hogan used strong salt solutions, and all obtained more globulin than albumin. Where more than two proteins are reported the interpretation of the results becomes very difficult. In consideration of the unreliability of the methods employed for the separation of the proteins, one can conclude that only two fractions have been isolated with certainty, an albumin-like protein which we shall call

myogen,

myogen, and a globulin-like protein which we shall call myosin. These terms are not used in the exact manner as that of von Furth, but it is thought better to utilize the terms already in use than to introduce new terms before the chemistry of these proteins is well known.

EXPERIMENTAL.

The Isolation of the Soluble Proteins from the Muscle Press Juice of the Haddock . The work of Logan on the soluble proteins of haddock muscle was done entirely on extracts of the ground muscle. It was thought worth while, especially because of the more rapid dialysis, to attempt to isolate the proteins from the muscle press juice. This work was done at The Atlantic Biological Station, St. Andrews, N.B. The fish were obtained by means of trawls set near the station, and were kept till required 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 the blood sugar was almost invariably higher than that of hand-lined fish, although not so high as that of fish fresh from the trawls. The fish were killed by a blow on the head, and the aorta was severed in front of the bulbus arteriosis. The fish bled freely, leaving the muscle a pale yellow colour except for a band of red fibres along the lateral line. The fish were

filleted,

filleted, and the muscle was ground in a meat chopper and pressed in a screw press. The press juice was dialysed in collodion sacs at about 4°C. till salt free. A protein was precipitated. This was filtered off and the filtrate was brought to various concentrations of hydrogen ion by means of dilute acetic acid. Precipitation occurred in the region of the isoelectric point. This precipitate was filtered off and nitrogen determinations were done on aliquots of the filtrates.

The precipitate in the collodion sacs was treated with 0.9N NaCl, that concentration in which Logan found the proteins most soluble. The greater part of the proteins went into solution. This solution was filtered, and the isoelectric point of the protein in the filtrate was found by the determination of the reaction of minimum solubility. The nitrogen determinations were made by digestion with phosphoric-sulphuric acid followed by nesslerization. The nesslerized solutions were read against eachother, the solution giving the greatest reading having the least protein. The results are given in Table 3, page 22. They are very similar to these of Logan. The myosin fraction has an isoelectric point of about pH 5.0, and the myogen fraction, an isoelectric point about pH 6.0. The hydrogen ion determinations were done colorimetrically and are accurate to about ± 0.1. Both myosin and myogen are found in muscle press juice. The use of the press juice affords a rapid and convenient means of their preparation.

TABLE III.

The Isoelectric Points of Myogen and of Myosin from Haddock Muscle Press Juice.

Fish No.	Myogen.		Myosin.	
	pH.	Colorimetric Reading.	pH.	Colorimetric Reading.
1	5.2	Nessler's precipitated due to too much NH_3	4.4	18.6
	5.4	11.6	4.6	19.9
	5.6	16.5	4.8	20.2*
	5.8	18.9	5.0	20.0
	5.9	19.8	5.1	19.4
	6.0	20.0 *	5.2	18.3
	6.1	20.0 *	5.3	17.1
	6.2	19.6	5.4	16.8
	6.3	19.0		
	6.4	18.6		
2			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 Separation of the soluble Proteins of the Hake and of the Cod. The proteins of the hake were isolated from the muscle press juice as in the former experiment. The isoelectric points were determined by the measurement of the hydrogen ion concentration of minimum solubility. The results are given in Table IV, and Table V, page 24.
The myosin of Table V. was precipitated in the isoelectric region and brought into solution again by the addition of dilute NaOH. It was thought, that with a purer solution, the range over which the protein precipitated would be less, and that therefore the isoelectric point could be determined with greater accuracy. The table shows that the precipitation occurs over as wide a range as in the previous experiment. The isoelectric point of the myogen of hake muscle is about pH 6.0, and that of the myosin about pH 4.8.

The experiment was repeated with cod muscle press juice. The results are given in Table VI, P.25.
The isoelectric point of the myogen is about pH 6.0 and that of the myosin about pH 4.6 to 4.8.

The Isolation of the soluble Proteins of the Muscle of the Catfish. The previous studies were made on members of the cod family. A widely different form was chosen for further study. The common catfish, ameiurus, was chosen because of the ease with which it could be kept alive. These fish were secured in the autumn and were kept till required in a large galvanized tank, through which flowed a continuous stream of water from the

city

The Isoelectric Points of Myogen and of Myosin from Hake Muscle Press Juice.

TABLE IV.

Fish #	Myogen.		Myosin.	
	pH.	Colorimetric Reading	pH.	Colorimetric Reading.
1	6.0	25.0	5.3	20.0
	5.9	25.5	5.2	22.1
	5.8	26.3 *	5.1	22.6 *
	5.6	25.4	5.0	22.4
	5.3	23.4	4.9	22.8 *
	5.0	20.0	4.8	22.3
	6.5	16.4		
	6.3	19.8		
	6.1	20.2		
	5.9	20.5*		
	5.7	20.0		
	5.5	19.2		
	4.8	18.0		

TABLE V.

MYOSIN (Reprecipitated).

Fish #	pH.	Colorimetric Reading.
2	6.0	11.5
	5.7	12.2
	5.4	16.0
	5.2	19.2
	5.1	20.0
	5.0	20.8 *
	4.9	21.3 *
	4.8	21.5 *
	4.7	20.6
	4.6	20.5

TABLE VI.

The Isoelectric Points of Myogen and of Myosin from Cod Muscle Press Juice.

Myogen

Fish #.	pH.	Colorimetric Reading.
1	5.9	20.1
	6.0	20.0
	6.1	19.3
	6.3	19.0
	6.4	18.3
2	5.6	20.0
	5.8	20.8
	6.0	20.9 *
	6.3	19.7
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
	Myosin.	
6	5.4	18.2
	5.2	19.6
	5.0	20.0
	4.6	20.5 *
	4.4	19.8

city mains. During the winter the animals became infected with a fungus growth. It is probable that these fish were not normal, although only the active healthy fish were used for experimental purposes. As the fish were small and the supply was limited, extracts were used for the isolation and the characterization of the proteins. The fish were killed and bled as in the experiments on the haddock. When the flow of blood ceased, the head was severed from the body, a small cannula was inserted into the dorsal aorta, and 1.05% NaCl was perfused through the animal. The perfusate became clear after 200 cc. passed through, but a litre of the perfusing solution was always used. The fish were filleted and the fillets were ground with sand in a mortar. The ground muscle was extracted for an hour with distilled water. It was filtered through cotton, and the filtrate was passed through a Whatman #2 filter paper. The second filtrate was dialysed in a collodion sac against distilled water. The muscle, after the removal of the myogen, was extracted with 0.9 N. NaCl. for 1 hour. The extract was filtered, and dialysed against tap water for 24 hours and then against distilled water for 24 hours. An attempt was made to free the protein solutions of electrolytes by means of electrodialysis, bringing them automatically to their isoelectric points. Three types of cell, with combinations of chromgelatin, parchment, and collodion membranes were used. In

every

every case the reaction went rapidly to the acid side, sometimes as far as pH 3. Very thick collodion membranes were placed in front of the negative electrode to slow up the transport of the positive ions. The drift in reaction was somewhat lessened but the dialysis was much slower. Electrodialysis was abandoned and the proteins were treated as in the previous experiments, except for the following changes. The hydrogen ion determinations were made by means of a hydrogen electrode. The nesslerized solutions were read against an ammonium sulphate standard instead of against eachother. The results are given in Table VII, page 28. The isoelectric point of myogen of the catfish muscle is about pH 6.1, practically identical with the results obtained from the determinations of the isoelectric point of myogen from the members of the gadus family.

The determination of the isoelectric point of myosin has been carried out heretofore always in the presence of approximately normal sodium chloride. The possibility of using the method of electrometric titration, (Pattem and Kellems, 1920), was considered. The method is adapted to the use of dilute protein solutions and hence to a minimum of electrolyte. The method was used with both myogen and myosin. In the case of myogen, 25 cc. of a dilute solution was placed in a 250 cc. beaker containing the hydrogen electrode and the salt bridge. The reaction was measured. Small amounts of tenth normal HCl were added, the

reaction

TABLE VII.

The Isoelectric Point of Myogen from Catfish Muscle.

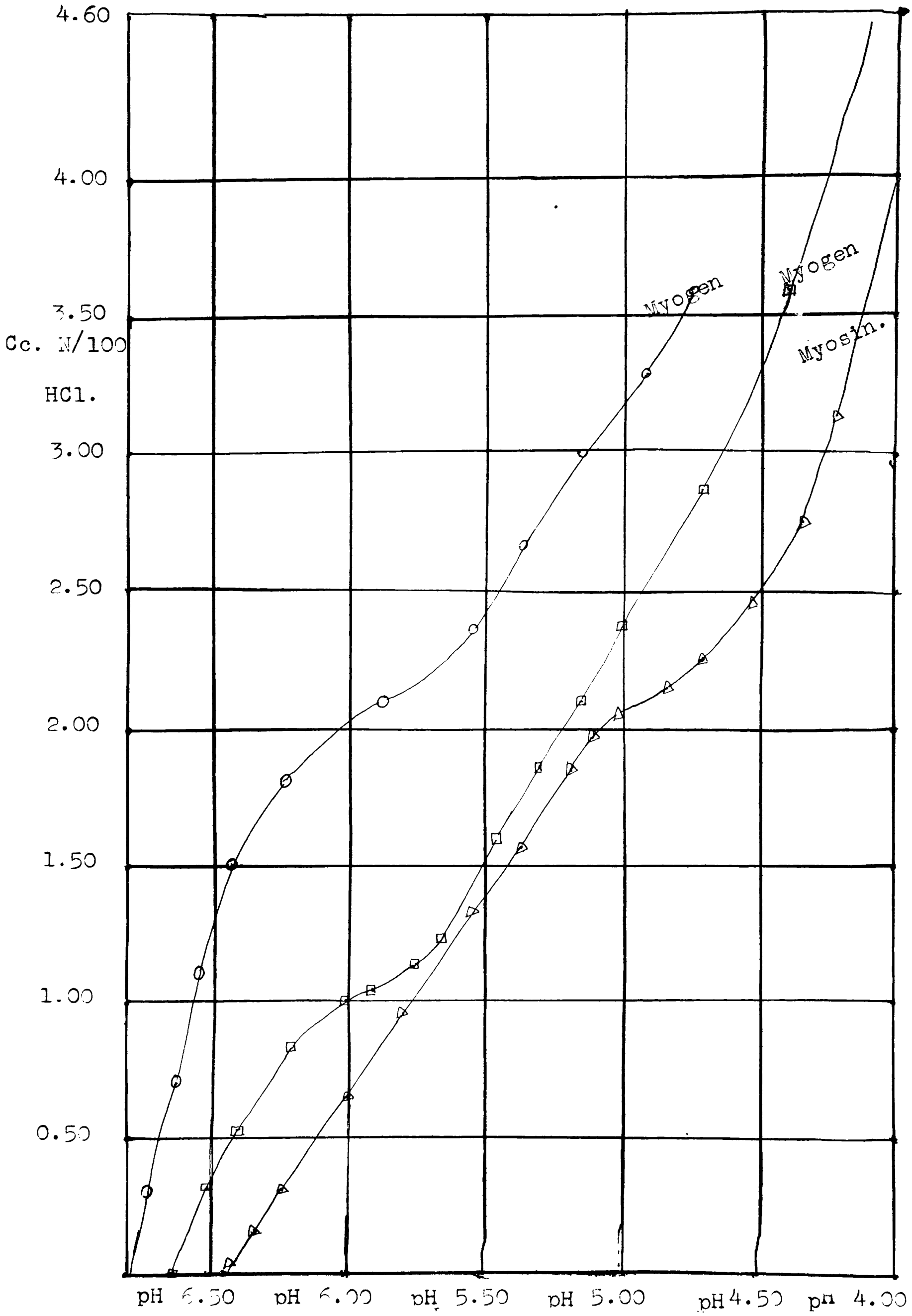
Fish No.	pH	Nitrogen per 100 cc. Extract
1	4.80	14.0 mgs.
	5.23	10.9
	5.47	10.1
	5.81	9.0*
	6.25	9.0*
	6.57	9.6
	6.70	9.7
	7.00	10.6
2	5.85	11.5
	5.70	11.4
	6.08	10.2*
	5.25	10.5
	6.55	11.8
	6.57	11.9

reaction being measured after each addition. The titration curves are given in Figure II, page 30. The break is seen to occur in the region of pH 6.0. The method was used for myosin. As pure a solution as possible was prepared, and dissolved in 1% NaCl. 25 cc. of this solution was titrated as in the previous experiment. This curve is also given in Table II. The isoelectric point of this protein is about pH 4.8.

Results of determinations of the isoelectric points of the soluble proteins of muscle are in fair agreement. Wohlisch, (1925), reported the isoelectric point of myosin pH 5.0. Vles and Coulon, (1924), gave a value more acid than pH 5 for a fraction of mouse muscle proteins. Quaglieriello, (1924), found the minimum swelling point of muscle to be pH 4.5 to pH 5.0. Webar, (1925), found the isoelectric point of the myosin of frog muscle to be pH 5.0, and that of myogen of frog muscle, pH 6.3. Collip, (1922), found the reaction of minimum solubility of frog muscle tp be pH 6.3. This indicates an isoelectric pointtof pH 6.3 for the myogen fraction. These results are in good agreement with those of Logan and the author. Some results not in agreement with the results quoted above have been reported. Wohlisch, (1925), gives a value pH 4.2 for myogen. Vles and Coulon, (1924), found two points for this fraction, one between pH 6 and pH 7, and another point between pH 9 and pH 10. It is seen that the majority of results give an isoelectric

point

FIGURE II.


4.60
4.00
3.50
Cc. N/100
HCl.
3.00
2.50
2.00
1.50
1.00
0.50
Myogen
Myogen
Myosin.
pH 6.50
pH 6.00
pH 5.50
pH 5.00
pH 4.50
pH 4.00

point about pH 5.0 for myosin, and an isoelectric point about pH 6.0 to 6.3 for myogen. These isoelectric points were determined on protein isolated from muscle of rabbit, of frog, and of several different species of fish. It appears probable that these protein fractions are similar in all vertebrate forms.

The Quantitative Relation of Myosin to Myogen in Muscle. The quantitative separation of the protein fractions is very difficult, and the results of such analyses are not in good agreement. Von Furth reported a ratio myosin to myogen of 1 to 4. Stewart and Sollman, and Howe reported an excess of myosin. Ritchie and Hogan gave the ratio myosin to myogen about 2.5 to 1. Logan did not report any quantitative data, but from his curves of the solubility of the muscle proteins in NaCl and in phosphate solutions, the ratio of protein soluble in salt solution to protein soluble in water is greater than 1. The author's attempts to fractionate the proteins quantitatively by means of heat coagulation and by salting out were unsuccessful. The coagulation temperatures were influenced by the concentration of the protein, the age of the extract, and the rate of heating. The salting out of the proteins was influenced to so great an extent by the concentration of protein in solution that wide variation in results could be obtained by dilution of the protein solution. The isoelectric

precipitate

precipitate of myosin, in the author's experiments, was always greater than that of myogen. The results to date fall into two classes, those that give a great excess of myogen, and those that give an excess of myosin. The former results were obtained either from muscle press juice or from extracts of ground muscle with physiological salt solution. The latter have been obtained by extracting the muscle with a much stronger salt solution. This difference in results can be explained by the supposition that all the soluble protein of the muscle is not in solution in the muscle. The addition of a more concentrated salt solution than the muscle press juice brings more of the myosin into solution, resulting in a value for the latter greater than that of myogen. This supposition has been tested, and evidence is advanced demonstrating its correctness. As this is an important point in conclusions drawn later, the attention is specially directed to it. The water content of cod muscle is about 82% by weight. If equilibrium exists throughout the muscle, and the total water of the muscle is available for the solution of the proteins, the maximum amount of proteinthat can possibly be in solution in 100 gms, muscle is that contained in 82 cc. press juice. The determination of the total soluble protein in 100 gms. muscle was made by extraction with 0.9 molar sodium chloride. This work was done in midwinter. Fish were far off, and live fish were hard to procure. Three

cod

cod only were used. They were caught on trawl lines and were in an exhausted condition when procured. The fish were killed and bled. The muscle of one side was removed and frozen in a wax paper bag immersed in ice and salt. The muscle of the other side was ground in a meat chopper and pressed with a screw press. The press juice was frozen in ice and salt. On reaching the laboratory, the press juice was thawed and filtered. The total nitrogen of an aliquot of the filtrate was determined by digestion and nesslerization. The proteins were precipitated with sodium tungstate and sulphuric acid, and the non-protein nitrogen determinations were made. The protein nitrogen was calculated by difference.

The frozen muscle was ground very fine by passing it through a meat grinder several times while still frozen in a room maintained at -20°C. Approximately 5 gms. muscle was accurately weighed and transferred to a flask containing 100 cc. of a 5% sodium chloride solution, giving a final volume of water of 104 cc. This solution was kept below 5°C., and the proteins were extracted for 2 hrs. with frequent stirring. The extract was filtered and analysed in the same manner as the press juice. The results are given in Table VIII, page 34. It is unfortunate that the number of experiments is so small. But the results are conclusive. If all the water in the muscle were available for the solution of the proteins, only approximately two thirds of the total soluble proteins can be in solution.

TABLE VLII.

The Solubility of the Proteins in Muscle Press Juice.

Fish No.	Press Juice. Protein N_2 per 82 cc. (100 gms.muscle).	Extract. Protein N_2 per 100 Gms. muscle.
1	0.78 gms.	1.25 gms.
2	0.70 "	1.11 "
3	0.74 "	1.10 "

The Physical State of the Proteins of Resting Muscle. It is very important that we have an accurate knowledge of the physical condition of the proteins in the muscle under normal resting conditions. As the muscle is affected by almost any manipulation, it is wealnigh impossible to make such study.. Some light has been shed on this matter by an accidental discovery made during the isolation of the proteins from the muscle press juice. It was found that if the muscle of resting fish were ground rapidly and pressed within a very short time after the death of the fish, for a short interval no press juice appeared, even with the greatest pressure that could be applied with a screw press. After a short time, the press juice welled up very readily. As the juice makes its appearance rather suddenly, it was possible to determine the time interval between the maceration of the muscle and the appearance of the press juice, sufficiently accurately to determine some of the factors associated with this phenomenon.

The fish used were taken from aquaria at The Atlantic Biological Station, St. Andrews, N. B. These fish were given sufficient time to recover from the exhaustion incurred during their struggle on the trawl. They were very active when excited, showing none of the sluggishness of trawl-caught fish.

In the first experiment, a haddock was removed from the tank and immediately stunned by a blow on the head. It was rapidly filleted, one fillet was packed in cracked ice, and the other fillet was run through a meat chopper and pressed.

The time between the maceration of the muscle and the appearance of the press juice was noted. The experiment was repeated six times, and in every case the press juice appeared between 5 and 7 minutes after the maceration of the muscle. The fillets placed in cracked ice were macerated and pressed after 25 to 35 minutes. Press juice appeared, in the six cases after an interval of 4 minutes.

Hake muscle appears to be of a more stable character than haddock muscle. Two hake were treated in the same manner as haddock. They were small, about 1 lb. in weight. They were caught on the trawls and kept in the aquaria till needed. The press juice of the first hake appeared 6 minutes after maceration, and that of the second 5 minutes after maceration.

The above fish were all in as nearly resting state as possible. The effect of exhaustion was determined. Haddock were removed from the aquaria, and teased in a small volume of water till they ceased to struggle. They were treated in the same manner as the resting fish. In only one case out of 4 could the pressure be applied to the ground muscle before the press juices welled up readily. The press juice of this fish appeared 3 minutes after the maceration of the muscle.

During the spring of 1930, press juice was obtained from 6 cod which had been taken on trawl lines. They were all dormant and exhausted almost to the point of death.

death. The work was done in a boat at sea, and rapid work was impossible. The pressure was applied about 4 minutes after maceration. The press juice welled up readily in every case.

These experiments show that the water is firmly retained in resting muscle. The loss of water from the proteins, and the breakdown of the gel structure occur after the maceration of resting muscle, and either before or during the maceration of exhausted muscle. This change is associated with and is probably caused by the development of acidity which occurs under the above conditions. The isoelectric points of myosin and of myogen are in the neighbourhood of pH 5.0 and pH 6.0 respectively. Benson, (1928), measured the reaction of the muscles of 'pen fish', fish that were allowed several days to recover from the effects of the trawl, and of trawl-caught fish, both immediately after the death of the fish and at the height of rigor. She obtained a reaction for the fresh muscle of pen haddock of pH 7.30, and for muscle in maximum rigor, pH 6.48. Fresh muscle of trawl-caught haddock had an acidity pH 6.85 to pH 6.54, and the muscle in rigor pH 6.68 to pH 6.53. There was, in the majority of cases, no difference between the reaction of the fresh muscle and that of the muscle in rigor of the exhausted fish. Good agreement is found on comparison of these results with those of Furusawa and Kerridge, (1927), on the reaction of cat muscle, measured with the glass electrode. The resting skeletal muscle had a

reaction

reaction of pH 7.04, fatigued muscle pH 6.26, and muscle in rigor pH 6.02. The reaction of the resting muscle of the cat is almost the same as that of the fish. The reaction of fatigue is considerably greater than that of muscle at rest. The reaction of rigor is only slightly greater than that of fatigue. A comparison of these results with those of the author points to the change in reaction as the factor determining the release of the press juice from the muscle. This hypothesis was tested in the following manner. Codfish were killed, and one fillet was removed as rapidly as possible. It was ground and pressed at once. If the pressure were applied before the press juice welled up freely, the reaction of the first drop was measured with the quinhydrone electrode. The reaction was measured again when the press juice welled up freely. When the fish was in rigor, the second fillet was removed, ground, and pressed. The reaction of the press juice from this muscle was measured. The results are given in Table 9, page 39. The press juice appears simultaneously with the development of the acid reaction. The breakdown of the gel structure is due to the change in reaction towards the isoelectric points of the proteins of the muscle. As Benson, and Furusawa and Kerridge have shown that the reaction of the muscle in fatigue is almost as acid as that of muscle in rigor, it follows that the breakdown of the gel structure probably occurs during fatigue, and that the gel structure is restored when the acidity of fatigue is removed.

TABLE 9.

The Hydrogen Ion Concentration of the Press Juice of Fish Muscle before and during Rigor.

Fish	Press Juice First to appear.	Free welling Press Juice.	Press Juice during Rigor.
Cod 1	pH 7.13	pH 6.72	p H 6.68.
" 2	pH 6.79	pH 6.67	pH 6.68.
" 3	pH 7.03	pH 6.94	pH 6.85.
Sea Raven.	pH 6.92	pH 6.84	pH 6.78

The Concentration of the soluble Proteins in the Press Juice of Muscle from freshly killed Fish and from Fish in maximum Rigor. It has been shown that the proteins of resting muscle take up all the water present in the muscle, forming a gel structure, and that this gel structure is broken down by the postmortem development of acid. It has been shown also that this gel structure is probably destroyed during advanced fatigue. The postmortem acidity and the acidity of advanced fatigue are almost the same as that of rigor. If the reaction of the muscle is the only factor determining the solubility of the proteins, the concentration of protein in the press juice of muscle from freshly killed fish should be practically equal to that of press juice from muscle in rigor. But the concentration of protein in the press juice is greater during rigor than in the pre-rigor period. Experiments proving this fact have been carried on both at the Atlantic Biological Station St. Andrews, N. B. and at the Atlantic Fisheries Experimental Station, Halifax. The experiments done at the former station during the summer of 1928, were performed on haddock from the aquaria. Sufficient time was allowed the fish to recover from the effects of the trawl. Each fish was killed by a blow on the head, and bled by severing the aorta behind the gills. A fillet was removed from one side, ground in a meat chopper, and pressed. The fish was hung by the head and allowed to pass into rigor. Maximum rigor was shown by maximum curvature of the body. During maximum rigor, the second fillet was removed, and press juice was obtained from it. The volume of

press

press juice was usually about one half as large from muscle in rigor as from fresh muscle. The total nitrogen of the press juice was obtained by digestion and nesslerization of an aliquot. The proteins of the press juice were precipitated with sodium tungstate and sulphuric acid, and the non-protein fraction was determined by digestion and nesslerization. The difference between those values was taken as the protein nitrogen.

The work was continued during the spring of 1930 at Halifax. Pen fish were not available, and the scarcity of fish made it impossible to procure them with hand lines. Therefore the only fish available were those obtained from the trawls of the fishermen. As these trawls were usually in the water 8 to 10 hours, the fish were exhausted. They were extremely sluggish. This work was done entirely on cod. They were treated in the same manner as the haddock. The results obtained with both haddock and cod are given in Table 10, page42. In every case, the concentration of protein in the press juice from muscle in rigor is greater than that from muscle in the pre-rigor period. The water binding power of the muscle is intimately related to the solubility of the proteins. The factors that affect this water binding power also affect the solubility of the proteins. The two probable factors influencing the solubility of the proteins are the reaction of the muscle and the concentration of electrolytes contained therein. It has been shown that the destruction of the gel structure of the muscle is associated with and probably

caused

TABLE 10.

A Comparison of the Concentration of Protein in the Press Juice of fresh Muscle with that of the Press Juice from Muscle in Rigor.

Fish.	Condition	Total N_2 per 100 cc. press juice.	Non-protein N_2 per 100 cc. press juice.	Protein N_2 per 100 cc. press juice.
		Gms.	Gms.	Gms.
Haddock				
1	Fresh	1.24	0.55	0.69
	Rigor	1.92	0.53	1.39
2	Fresh	0.56	0.28	0.28
	Rigor	1.43	0.38	1.05
3	Fresh	1.21	0.35	0.86
	Rigor	1.41	0.37	1.04
4	Fresh	0.94	0.32	0.69
	Rigor	1.54	0.33	1.21
Cod.				
1	Fresh	0.94	0.34	0.60
	Rigor	1.01	0.36	0.65
2	Fresh	1.05	0.32	0.73.
	Rigor	1.38	0.31	1.07
3	Fresh	1.14	0.25	0.89
	Rigor	1.38	0.24	1.14
4	Fresh	1.37	0.28	1.09
	Rigor	1.68	0.30	1.38

caused by the increase in acidity. If the solubility of the proteins in the press juice of muscle in rigor can be accounted for by a change in the reaction of the muscle, the reaction of muscle in rigor must be either more acid than pH 5, the isoelectric point of myosin, or must be more basic than the reaction of the pre-rigor period. The acidity of the press juice of 6 codfish, including the last 4 fish of Table 10, was measured both from muscle in the pre-rigor period and from muscle in rigor, by means of the quinhydrone electrode. The results are given in Table 11, page 44. The reaction of the press juice is practically constant throughout the postmortem period. These results are in agreement with those of Furusawa and Kerridge, (1927), and of Benson, (1928). The non-protein nitrogenous constituents of the muscle have no effect. Urea does not cause swelling of fish muscle until the concentration reaches 16%. Creatine causes no swelling in a saturated solution. As the swelling of the muscle is associated with an increase in the solubility of the proteins, neither of these have any effect on the solubility in the concentrations found in muscle.

By the process of elimination, the only known cause for the increase in the solubility of the proteins is an increase in the salt concentration of the muscle. This increase in electrolyte is sufficient to cause an increase in the solubility of the globulin of the muscle. This hypothesis has not been proven. The probable electrolytes concerned and their source can be dealt with best in a later portion of this work.

TABLE 11.

The Reaction of Press Juice from Muscle before and during Rigor.

Fish No.	pH of Press Juice from fresh Muscle.	pH of Press Juice from Muscle in Rigor.
1	6.75	6.60
2	6.62	6.62
3	6.58	6.60
4	6.51	6.55
5	6.50	6.38
6	6.52	6.47.

The Effect of Electrolytes on Fish Muscle. The effect of salts on the muscle proteins is very pronounced. It has been noted that the results of analytical data, in relation to the proportions of myosin to myogen in muscle, vary according to the concentration of salt used to extract the proteins. Throughout the work of the author, unfilterable extracts of jelly-like viscosity were always encountered when removing the myosin from the ground muscle with 0.9 normal sodium chloride. The effect of salt solutions on muscle was studied by Meigs, (1910). He found that frog muscle, when immersed in salt solutions, underwent two kinds of swelling. The first was due to the water entering the walls of the fibrils. This swelling was accompanied by a loss in irritability. The second swelling, the result of the entrance of the salt, was thought due to the loss by the muscle of the ability to keep back the electrolytes. As a consequence, the salts and the water entered the sarcostyles, the latter became congested and the muscle shortened. Chipman, (1930), made a detailed study of the effect of salt solutions on cod muscle. Weighed pieces of muscle were placed in salt solutions of various concentrations. The pieces were removed and weighed at intervals till approximate equilibrium was reached. The results showed a remarkable swelling of the muscle in salt concentrations between 1% and 5% NaCl. The author duplicated these curves for several salts, carrying the effect to equilibrium. All salts that

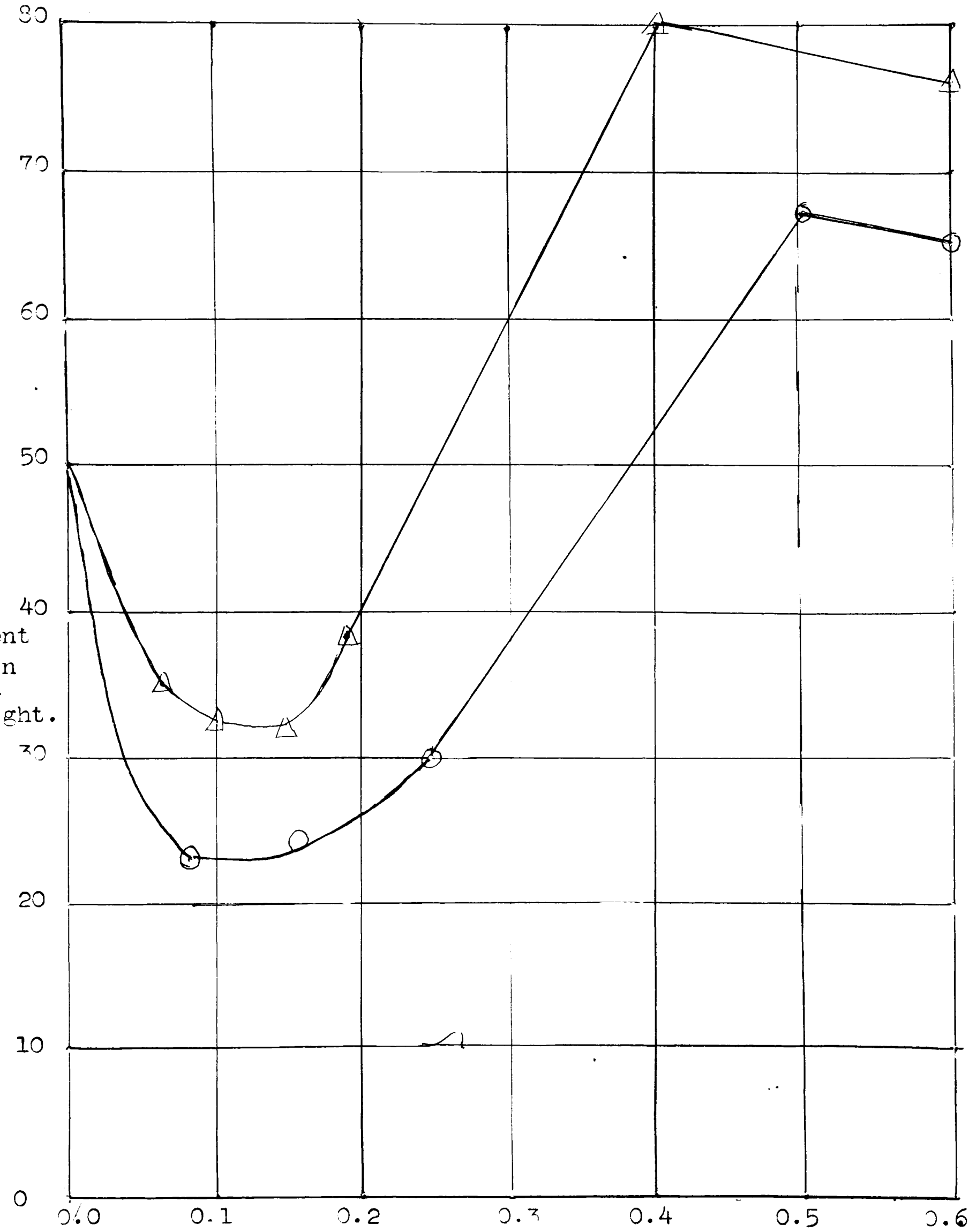
do

do not precipitate the proteins and have a neutral or basic reaction produce the same type of curve. The swelling curves of NaCl and KCl are given in Figure 3, page 47. They are typical curves and are very similar to eachother. There are three divisions to the curve. The first part shows a decrease in swelling between concentrations of 0 and about 0.1 molar. The second part shows a rapid increase in weight between concentrations 0.1 molar and 0.4 to 0.5 molar. In the third region, there is a gradual falling off in weight till at a concentration of 6 molar, the original weight of the fish muscle is reached. The gain in weight in distilled water is probably due entirely to osmosis, the subsequent decrease in weight being due to the approach of the concentration of the salt solution to the salt concentration of the muscle. It is difficult to understand why, when a salt concentration outside the muscle equal to that inside the muscle is reached, the weight of the fish is still greater than its original value. It may be that at the minimum point, the swelling is due to the osmotic pressure of the proteins. The remaining part of the curve resembles the curves obtained by Loeb, (1924), for gelatin. Loeb accounted for the swelling of proteins by means of Donnan membrane equilibrium. Hardy, (1905-6), postulated a union between globulins and salt not unlike the union of acids and alkalis with proteins. Hardy's hypothesis permits of protein ionization and the functioning

of

FIGURE 3.

The Swelling of Cod Muscle in Solutions of KCl. and of NaCl.



of Donnan membrane equilibrium. It is fairly certain at least that the swelling of the muscle in salt solution is due to an increase in the solubility of the muscle proteins. The close relation of swelling to the solubility of the proteins is seen on comparison of the curves in Figure 3, with the solubility curves of Logan.,

These curves have been worked out by Chipman, (unpublished), for a large number of salts. The maximum swelling is produced with salts of mineral acids. The only salts found to give no swelling are lithium lactate and sodium lactate. The swelling is related to the hydrogen ion concentration, but this is not the only factor. Mixtures of sodium carbonate and sodium bicarbonate pH 9.5, produce a greater swelling than does sodium carbonate alone, but it does not produce as great a swelling as does sodium chloride at the reaction of the muscle. This swelling is believed due to the effect of the salt on the myosin fraction. Myosin has the characteristics of a globulin whereas myogen resembles an albumin. This swelling was encountered during the isolation of the myosin from the muscle after the removal of the myogen. Attention is called to the considerable swelling resulting from relatively small increase in salt concentration. This increase is still greater than is shown by the curve. Chipman,(unpublished), has obtained a gain in weight of muscle that had been previously frozen, of 100 % of the original weight. This greater swelling

is

is due to the opening up of the muscle by the ice crystals allowing a more rapid transfer of the salt through the muscle.

The Location of the soluble Proteins in the Muscle Fibrils. At the present time the evidence points entirely to the myosin fraction as the one affected by the addition of salt solutions to the muscle. Advantage of this has been taken in an attempt to determine the location of this protein in the muscle. Teased specimens of cod, haddock,halibut and flounder muscle are all strongly doubly refractive when viewed through a polarizing microscope. The teased muscle was treated on the microscope slide with 10% sodium tungstate and 0.66 N. sulphuric acid. The proteins became white and opaque. The teased layer was thin enough to allow sufficient light through for observation with non-polarized light. When viewed through the crossed prisms the field was a very dark grey due to reflected light. There was no evidence of double refraction. Picric acid, tannic acid, trichloracetic acid, and mercuric chloride produced the same results. The action of ethyl alcohol on the teased muscle was very much slower, but eventually the double refraction was destroyed. These results point to the proteins as the substances causing the double refraction. Teased muscle was thoroughly extracted with distilled water and viewed under the polarizing microscope. It was impossible to measure the degree of double refraction with the above procedure. It was the author's impression that distilled water did lessen the degree of double refraction to a slight extent.

The teased muscle was extracted with sodium chloride solutions both 5% and 10%. The muscle became swollen. The striated appearance of the muscle practically disappeared. The field was dark when viewed through polarized light. The following interpretation appears the most logical. The double refraction in muscle is due to a protein, the molecules of which are in some regular order as are those of many crystals. This regular order is disrupted when the proteins are coagulated. The action of salt on the muscle points to the myosin as the protein concerned with the double refraction. Salts cause this protein to take up water and hence to swell. This swelling may be great enough to break down the regular orientation of the molecules, and the double refraction disappears. While this molecular orientation lasts, the protein seems to be in the form of a gel. An observation of Kuhne demonstrates this. He observed a nematode making its way along a fibril of frog muscle. On reaching the doubly refractive band, it was able to push it aside and to pass on, after which the band settled back into its original position. The presence of this gel structure explains why muscle press juice does not have in solution all the soluble proteins of the muscle. It also explains why the extracts of Stewart and Sollman, of Howe, and of Ritchie and Hogan contained more myosin than myogen, while those of Halliburton and of von Furth contained an excess of myogen. Von Murat and Edsall, (1929), demonstrated double refraction, artificially produced, in a globulin obtained

from

from muscle. They reported the isoelectric point of their protein pH 6.0, the result obtained by the author for myogen of fish muscle. The details of their method of preparation have not been given. As myogen has the common characteristics of an albumin, it is probable that the muscle globulin of von Murat and Edsall is either myosin or a mixture of myosin and myogen. Further work is necessary to discover the reason for this discrepancy.

If myosin is located in the doubly refractive band, where is the myogen located? It will be recalled that the water is entirely bound in resting muscle. For this reason, protein must be present in the isotropic band also. It is unfortunate that the discrepancy is so large in the values given for the relative proportions of myogen and myosin. The extraction curve of Logan probably gives the most accurate value. This value is about 1 to 1. This considerable amount of protein must be located in the light band of the muscle. Striated muscle, therefore, probably consists of alternate layers of myogen and of myosin, the latter protein having some regular molecular orientation.

<u>Summary of Experimental Data.</u> Before attempting to interpret physiological phenomena by means of our experimental findings, it is of advantage to summarize this data and the conclusions drawn from them. They are as follows:-

(1) The soluble proteins of striated fish muscle can be divided into two fractions, a globulin-like protein

protein with an isoelectric point pH 5.0 corresponding closely to the myosin of von Furth, and an albumin-like protein with an isoelectric point pH 6.0 corresponding to the myogen of von Furth.

(2) These proteins are not all in solution in the muscle. The fact that the maximum amount of protein can be extracted with salt solutions, indicates that the protein not all in solution is myosin.

(3) Resting muscle was found to have a gel structure which breaks down on the formation of postmortem acidity, and possibly with the acidity of fatigue. The acidity of advanced fatigue and that of the pre-rigor period are very nearly as high as that of maximum rigor.

(4) The concentration of protein in the press juice of muscle in rigor is much greater than that of muscle in the pre-rigor period.

(5) Salt solutions cause a pronounced swelling of fish muscle. This is believed due to the peptization of myosin.

(6) The muscle is believed to be composed of alternate layers of myosin and myogen, corresponding to the cross-striations of the muscle.

Discussion.

Theories of Rigor and of Muscular Contraction. The data on which to base a theory of muscular contraction are very meagre. The production of lactic acid is the outstanding feature of the known changes that occur during the contraction. The observation of the increase in the diameter of the anisotropic band, coupled with the pronounced tendency of the muscle to swell in solutions of dilute acids, point to an acid swelling mechanism. This view has been advanced by Furth, (1919, 1921). This explanation is plausible on superficial examination. On the development of the lactic acid, the proteins swell, the diameter of the muscle increases and the muscle shortens. The original condition obtains as soon as the acid is neutralized. But this theory fails in the light of recent knowledge of the acidity of the muscle during rest, during contraction, and during rigor. Benson, and Furusawa and Kerridge have shown that the reaction of the resting muscle is in the region pH 7.0 to pH 7.3. They have shown that the contraction of rigor is accompanied by a reaction pH 6.3 to pH 6.9, the reaction being somewhat more acid in mammals than in fish. The reaction of the muscle during a single muscle twitch is probably not as acid as that of maximum rigor. The isoelectric points of the soluble proteins are pH 6.0 and pH 5, 0. To produce an acid swelling, the reaction of the muscle must be on the acid side of the isoelectric points of the soluble proteins, that is more acid than pH 6 and

possibly

possibly more acid than pH 5. Such acidity has not been found after contraction, during fatigue, of during rigor. Therefore an acid swelling theory such as that outlined above cannot explain either the mechanism of the single muscle twitch or that of the contraction of rigor mortis.,

A theory based on the swelling of the muscle as the result of the formation of carbon di-oxide, has been advanced by Wacker, (1916, 1927). The increase in the acidity, caused by the stimulation of the muscle, has a tendency to produce carbon di-oxide gas within the muscle at the expense of the bicarbonates. The reaction of sodium bicarbonate in solution is about pH 8.3. At the reaction of the muscle, there must be an equilibrium between the bicarbonates and dissolved carbon di-oxide. If the solubility of the carbon di-oxide is exceeded, there is also an equilibrium between the gas and the dissolved carbon di-oxide. An increase in the acidity upsets these equilibria and the volume of gas increases. Wacker has shown, by means of rubber models, the possibility of an increase in gas in a system such as muscle causing a shortening of the system. Relaxation is accounted for by the neutralization of the lactic acid.

If such mechanism be the cause of the contraction, the contraction should be accompanied by an increase in the total volume of the muscle. The volume changes are so small during contraction that they are still somewhat in doubt.

The evidence to date tends to show that there is a very slight decrease in the total volume of a muscle during contraction. If gaseous carbon di-oxide is produced at all during the contraction it must be to a very limited extent.

A. V. Hill, (1925), has outlined a scheme to account for the contraction of striated muscle, that takes little for granted, that Hill believes is capable of developing the required force, and that fits well with the few known facts. The reaction of the muscle is on the basic side of the isoelectric points of the proteins. The proteins are therefore negatively charged and are probably surrounded with the corresponding positive ions. Because of the mutual repulsion of like charges, the system is under strain. Lactic acid is produced on stimulation of the muscle. This acid neutralizes the charges on the proteins and the system shortens to its natural length, producing the contraction. The neutralization of the lactic acid results in the restoration of the charges on the proteins, and the original condition is restored. In order to account for the neutralization of the lactic acid by the protein, Meyerhof, (1924), has supposed two kinds of protein present, a 'verkurzungsort' which is charged during the resting state and whose charge is lost on the production of lactic acid, and an 'ermudungsort' which acts as a buffer to neutralize the lactic acid permitting the restoration of the charge on the verkurzungsort. This

theory

theory is probably the most acceptable at the present time. There are no contrary facts and the theory does account for the contraction on the production of the lactic acid, and for the relaxation on the neutralization of the acid. The evidence in favor of it is so small that for the present one must consider it only as one of the possibilities.

Theories based on sufface tension changes in muscle have been eliminated by Hill, (1925). He calculated the amount of lactic acid per gm. frog muscle developing a given force. On calculation of the area of this acid, if spread in a layer 1 molecule thick, he found that the acid should cover about 2% of the surface of the fibrils. He found that the surface tension developed by this amount of acid should have to be far greater than any known substance to account for the tension a muscle is capable of developing. This renders the possibility of an adequate explanation of the mechanism of muscular contraction, based on surface tension, very remote.

A theory of muscular movement, based on the anisotropy of muscle tissue, has been advanced by Garner, (1925). The anisotropic segments of the muscle are believed to consist of long chains of carbon atoms, possibly lipoid, possibly protein. These chains appear to run the longitudinal direction of the muscle. Either the formation of liquid crystals or a molecular rearrangement differing from the

original

original, should result in a shortening of the chains. These changes in the molecular arrangement are brought about by the action of the lactic acid on the surfaces of these regions. Calculations based on data from Hill's work show that the force developed is of about the same order as that developed in muscle.

Clark, (1927), has demonstrated the possibility of a mechanism such as that outlined above. She showed that the contractile force within systems changing from a liquid crystal to a solid crystal state, is of the same order as the values of Hill for the contractile force of muscle. She showed retraction in ammonium oleate as a result of a change from a liquid crystal state to a solid crystal state, caused by a change in the reaction of the system from pH 7.6 to pH 5.6. She found evidence from X-ray diffraction patterns of muscle, that the muscle in the resting state is in the form of liquid crystals, and in ordinary rigor or in rigor induced by chloroform, it consists of solid crystals.

Such reactions as those indicated above may be the cause of the muscular contraction, but they cannot be the cause of rigor. The hypotheses of Hill, Meyerhof, Garner, and Clark, all depend on a change of reaction of the muscle. The change in reaction of the muscle has little if any influence on the development of rigor. The development of lactic acid is often as great in advanced fatigue as it is in rigor. The pre-rigor acidity is usually as great as that

of

rigor. Rigor disappears while the reaction of the muscle is still acid. Hoet and Marks,(1926), have shown that rigor can occur while the muscle is alkaline. Hence some factor other than a change in reaction must be the cause of the contraction of rigor, unless we accept the suggestion of Hill (1926), that acid is necessary to start the process which may continue without the presence of acid. This suggestion is not supported by experimental evidence.

The earliest explanation of the cause of rigor was based on the observation of Kuhne and that of Halliburton, on the tendency of muscle press juice of freshly killed animals to pass into a gel. They found that press juice obtained from muscle in rigor does not pass into this gel form. Halliburton concluded that rigor is due to the clotting of the proteins in the muscle. Folin, (1903), supported this view. The tendency of the muscle proteins to gel was not studied further till 1930. Smith, (1930), froze and ground fresh muscle, then allowed it to thaw at 0^{o}C. and pressed it. The expressate was gelatinous. Contrary to the findings of Halliburton, the proteins did not gel. The addition of a small amount of salt to the expressate caused it to set to a firm gel. The ability of the proteins to gel on the addition of salt was found to decrease with the onset of rigor. The clotting of the expressate of Halliburton was believed due to the contamination with salt from the

salt

salt and ice mixture used to freeze the muscle. But this tendency of the proteins to gel on the addition of salt may be of great importance. It runs parallel with the tendency of the muscle to swell on the addition of salt. The gel structure is similar to the condition of resting muscle. The gel structure of the muscle is destroyed on the development of postmortem acidity. The fact that the expressate from muscle in rigor does not gel points to some change in the muscle proteins hitherto unrecognized. Halliburtonfs view as to the cause of rigor is possibly nearer the truth than any other view held at the present time.

Another possible cause of the rigor contraction must be considered. The postmortem development of lactic acid may change the reaction of the muscle from a hydrogen ion concentration slightly more basic than pH 7 almost to the isoelectric point of the myogen. This results in the isoelectric precipitation ofmpart of the myogen. Collip, (1922), found the minimum solubility of the muscle proteins to be at a hydrogen ion concentration of pH 6.3. As muscle often reaches this reaction during rigor, he suggested that rigor may be due to the isoelectric precipitation of the proteins. This hypothesis does not explain the cause of alkaline rigor. It does not explain the mechanism by which the muscle contracts. As the difference between the hydrogen ion concentration of resting muscle and that of the muscle in the pre-rigor period is often great,

and

and the difference between the hydrogen ion concentration of muscle in the pre-rigor period and that of muscle in rigor is often negligible, it seems improbable that the precipitation should occur over such a narrow range of acidity. Isoelectric precipitation does probably occur, but it is not the cause of the rigor contraction.

Of the theories of muscular contraction that have been reviewed, only the Hill-Meyerhof theory, or those of Garner and of Clark can be considered possible. They are all sadly lacking in experimental proof. They all depend on a change in the acidity of the muscle. Rigor appears to be independent of the acidity of the muscle. Therefore none of these theories throw any light on the rigor contraction.

The Probable Mechanism of the Rigor Contraction. The data of the author pertains entirely to postmortem changes. For this reason, the problem of rigor development will be dealt with before the problem of muscular contraction. Evidence has been advanced indicating that the muscle proteins are in the form of a gel, and that this gel structure is disrupted by the postmortem development of lactic acid. Rigor has been shown to be accompanied by a considerable increase in the concentration of protein in the press juice. The only reasonable hypothesis explaining this increase is an increase in the salt concentration of the muscle, sufficiently large to repeptize part of the myosin. The probable location of the myosin is in the anisotropic segments, and of the myogen in the isotropic segments of the muscle. Smith has shown that

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the press juice from fresh muscle tends to gel on the addition of a small amount of salt. Logan demonstrated the increasing solubility of the muscle proteins in solutions of increasing salt concentration. Chipman demonstrated the appreciable tendency of the muscle to take up water as the concentration of salt is increased. Thus, the tendency of the proteins to gel and to swell are phenomena depending on the increasing solubility of the proteins. From this data, a hypothesis of the mechanism of rigor can be formulated which fits the facts better than any former theory, and which suggests a new attack on the problem of muscular movement. In resting muscle the proteins hold firmly to the available water. An equilibrium exists in the muscle between the proteins with their bound ions and the water, depending on the solution pressure of each protein. The solubility of the myogen depends on the hydrogen ion concentration, while the solubility of myosin depends on both the hydrogen ion concentration and the salt concentration. A pronounced change in the hydrogen ion concentration of the muscle towards the isoelectric points of the proteins causes a release of water from both the isotropic and the anisotropic segments. This condition is thought to exist in fatigue great enough to prevent the muscle from contracting. Fatigue is thought to be due to the loss of the gel structure of the proteins. The proteins have been found to be more soluble after the onset of rigor. This is not due to any inherent change in the solubility of the proteins themselves, as Deuticke, (1930), has shown that the solubility of the proteins, that is the amount of protein that can be extracted from muscle by

salt

salt solutions, is slightly less after rigor than before rigor. Therefore the increased concentration of protein in the press juice of muscle in rigor is due to a change in the solvent and not to a change in the protein. The hydrogen ion concentration of the muscle does not change to an appreciable extent during this period, the usual small change being an increase in the acidity. This should produce a diminution in the solubility of the proteins. The only reasonable explanation of this increased solubility of the protein is an increase in the salt concentration of the water in the muscle cells. The amount of myosin that can be dissolved in a given amount of water depends on the hydrogen ion concentration and on the salt concentration. Hence an increase in the salt concentration of the muscle increases the solubility of the myosin. This protein takes up water both from the interstices of its own micelles and from the isotropic segments. This results in a lessened amount of press juice in the muscle in rigor. The increase in the salt concentration tends to revert the myosin to its original gel form, as in resting muscle. The contraction of rigor can now be explained in two ways. The Hill-Meyerhof explanation or those of Garner or Clark may be applied, supposing however that some factor other than the change in hydrogen ion concentration is responsible for the mechanism. A more plausible explanation can be given by a continuation of the line of reasoning advanced above. Histological examination shows that during the contraction, the anisotropic segment expands laterally only. This is probably because of the

the definite molecular orientation of the constituents of this segment. An increase in the salt concentration above the minimum required to produce the original resting condition in the anisotropic segment, causes this segment to swell. As the swelling is lateral to the fibrils, and the water is taken from the isotropic segments, the result is a shortening of the latter segment and a widening of both. This swelling widens the intermolecular spaces and lessens the degree of double refraction. Steubel and Liang, (1928), demonstrated a decrease in the double refraction of muscle with the onset of rigor.

No theory has adequately explained the passing off of rigor. The data is too scanty at the present time to provide more than a hypothesis for future work. A possible explanation can be given by means of the salt effect. Rigor is possible only if the anisotropic band expands laterally to the fibril. The lateral expansion is probably due to the definite molecular orientation of the constituents of this band. The same molecular orientation probably prevents the diffusion of the proteins from one band into the other. If this molecular orientation is disrupted, the muscle must relax. It has been shown that an excess of salt in the muscle destroys the double refraction. Steubel and Liang demonstrated a lessening of the double refraction of the muscle during the whole course of rigor. It is possible that the anisotropic segments swell to such an extent that

the

the regular molecular orientation is partially disrupted allowing the myosin to expand in all directions. This would result in a falling off in the tension within the muscle cells.

What salt is produced in the muscle during the development of rigor,and what is the source of this salt? The first to suggest themselves are sodium and potassium lactates. Lactic acid is a fairly strong acid, and in the region between pH 6 and pH 7, it can be considered entirely neutralized without appreciable error. The concentration of lactate in fish muscle at the height of rigor is slightly over 0.2%, (Leim, Macleod and Simpson, 1927). The normal salt concentration of fish muscle is isotonic with about 1% NaCl. An increase in the salt concentration of 0.2% should cause a swelling sufficient to account for the rigor contraction. But the salt which peptizes the myosin is not lactate. The acidity of the muscle is practically as high in the pre-rigor period as in the height of rigor. Either all the lactic acid is produced in the pre-rigor period or the muscle is a much better buffer in the region about pH 6.5 than in the region about pH 7.0 To settle this question, the lactate concentration of the press juice of cod muscle was determined both during the pre-rigor period and at the height of rigor. The cod were killed and the press juice was obtained as in the previous experiments. The press juice from one fillet was taken while the muscle was in the pre-rigor period, and that of the other fillet while the muscle was in the height of rigor.

The proteins of the press juice were precipitated with sodium tungstate and sulphuric acid. Lactic acid of aliquots of the filtrates was determined by the method of Friedman and Kendall, (1929). The results are given in Table 12, page 66. They show that there is not sufficient increase in the lactic acid in the later pre-rigor period to change the solubility of the proteins. Since this work was done, Chipman, (Unpublished), has shown that sodium lactate and lithium lactate produce no swelling of fish muscle. Also the results of Hoet and Marks on the production of alkaline rigor cannot be explained on the above basis. Therefore it is concluded that lactates do not produce the rigor contraction.

The only probable salt, other than lactate, that might act as a peptizing agent for the resolution of the myosin is sodium or potassium ortho-phosphate. The relation of the various phosphorus compounds in muscle to muscular physiology is in a state of flux at the present time. The discovery of Fiske and Subbarow, (1927), and of Eggleton and Eggleton, (1927), of creatine phosphoric acid compounds, and that of Lohmann, (1928), of pyrophosphate in muscle, have brought about an entire revision of our ideas of the role of phosphorus in muscle. Previous work must be interpreted very carefully or possibly discarded. The phosphorus cpmpounds now known to be present in muscle are;- orthophosphate, pyrophosphate, phosphagen, hexose monophosphate, adenylic acid, inosinic acid, and in some cases hexose diphosphate. Complete quantitative analyses of

TABLE 12.

The Lactic Acid Concentration of the Muscle Press Juice before and during Rigor.

Fish #	Lactic acid per 100 cc. Press Juice. Before Rigor	During Rigor.
	Mgs.	Mgs.
1	221	228.
2	224.	239.
3	205.	231.
4	218.	220

of these compounds on the same muscle have not been done. The approximate concentration of each can be determined only from the results of different workers on muscle from various sources. The orthophosphate content of resting muscle is very low. Sacks and Davenport, (1928), found 0.015 to 0.020%, by direct analysis. Stella, (1928), found a solution of orthophosphate, 0.008 to 0.010% to be in equilibrium with resting muscle. This very low value increases with fatigue, Stella finding twice the concentration. For muscle in rigor, Stella found a value of 0.100% orthophosphate phosphorus.

Phosphagen is an unstable compound that breaks down in both fatigue and in rigor. The value for resting muscle is about 0.065% phosphagen phosphorus, (Eggleton and Eggleton, 1928). It forms creatine and orthophosphate in both fatigue and in rigor. Eggleton and Eggleton (1928), found that the phosphagen of the muscle of the ray, in an atmosphere of nitrogen or hydrogen, decreases simultaneously with an equimolecular increase in orthophosphate, till all but 5 to 15% of the original phosphagen is gone, before there is a measurable breakdown of other phosphorus compounds.

Pyrophosphate exists in resting muscle in a concentration of about 0.025%. It is more stable than phosphagen and is changed to orthophosphate only after the death of the muscle, (Lohmann, 1928).

Hexose diphosphate is found in muscle in very small amounts, and is probably absent from resting muscle. The concentration of hexose momophosphate in muscle is about 0.08% phosphorus, (Embden and Jost, 1928). Embden and Jost believe hexose monophosphate to be the immediate precursor of lactic acid. They have obtained results that indicate a resynthesis of hexosemonophosphate during the recovery period.

These phosphorus compounds are all potential sources of orthophosphate. The concentration of orthophosphate in resting muscle is at least as low as 0.015% phosphorus, and may be lower. The remaining phosphorus compounds are changed practically quantitatively to orthophosphate by the time the muscle is in rigor. The total phosphate of the protein-free filtrate of haddock muscle is between 0.13 and 0.16% phosphorus. These results were obtained by Leim, Macleod and Simpson, (1927), using the method of Bell and Doisy. They represent mainly orthophosphate and phosphagen phosphorus. As the phosphagen has split to phosphate and creatine by the time the muscle is in rigor, the results may be accepted as very close to the true orthophosphate values for muscle in rigor. Hence, between the resting condition and rigor, there is a rise in the orthophosphate content of haddock muscle of over 0.1% phosphorus. This increase in the salt concentration of the

muscle

muscle is sufficient to cause a considerable swelling. The development of orthophosphate can be advanced tentatively as the probable factor which causes the rigor contraction. Before the view can be considered more than a working hypothesis, a considerable number of questions must be answered, among which are the effect of the orthophosphate precursors on the muscle proteins, and the relationship between the development of the orthophosphate, the increasing solubility of the proteins in the available water of the muscle, and the onset of the rigor contraction.

The phenomenon of alkaline rigor still offers difficulty. If the theory advanced as to the cause of acid rigor be applied, the orthophosphate content of the muscles in alkaline rigor should be high, for the solubility of the myosin must increase sufficiently to withdraw water from the myogen which should still be in the gel state. As the breakdown of the gel structure is due to increased acidity, the muscle in alkaline rigor should yield little or no press juice. An attempt was made to prove this. It was found that fish (cod), could not be put into convulsions with insulin. A cod of 2½ lbs. was given 25 units of insulin intramuscularly. After 10 minutes without results, 25 units were injected into the bulbus arteriosis. This second dose had no effect. A second cod of 3 lbs. was given 50 units, the insulin being injected into the blood stream through the bulbus arteriosis. Both fish were brought to the laboratory and

and were found normal 3 hours after the administration of the insulin. A third cod of 2 lbs. received 200 units injected directly into the blood stream. The animal was living 3 hours after and showed no effect of the insulin. The muscle passed into rigor normally 4 hours after the death of the fish. The expressate of the muscle in rigor had an acidity of pH 6.73. The study of alkaline rigor has been temporarily postponed. Some animal other than fish will be used for this work.

The Mechanism of Muscular Contraction. The presence of alternate segments in the fibrils of striated muscle offers more than one explanation of the mechanism of the muscle twitch. The Hill-Meyerhof theory is supported by this conception, the 'verkurzungsort' corresponding to myosin and the 'ermudungsort' corresponding to myogen. The basis of all theories of the muscle twitch must be the production of lactic acid, as the lactic acid production has been found to be proportional to the strength of the contraction. As the lactic acid production also parallels the change in hydrogen ion concentration, it is possible that, whatever the changes in muscle during contraction may be, they are due to a change in the hydrogen ion concentration of the muscle. The role of phosphates during the contraction has not been explained. Phosphocreatine is thought by Meyerhof to be related to the stimulation of the muscle and not to the contraction. Fiske believes phosphocreatine neutralizes the lactic acid.

Hexose monophosphate is possibly the precursor of lactic acid. At the present time, the physiology of the phosphorus compounds of muscle is so poorly understood that an attempt to fit them into a theory of muscular contraction is useless.

The swelling theories of muscular contraction previously advanced, are untenable because during the contractio the reaction of the muscle approaches the isoelectric points of the muscle proteins. This results in a reduction of the water held by the proteins. A swelling theory as to the mechanism of the contraction of rigor has been advanced by the author which overcomes this difficulty. The muscle twitch is also possibly due to the swelling of the muscle protein. The water of resting muscle is held firmly by the proteins. The amount held by each protein depends on the solubility of the proteins. An equilibrium is maintained between the two proteins as long as the muscle is at rest. Lactic acid is produced on stimulation of the muscle. The lactic acid can be distributed in the fibrils in three ways. It may be produced only in the anisotropic segments, only in the isotropic segments, or in both segments. A swelling theory can account for the contraction in two out of the three possibilities. If the acid is produced only in the isotropic segments in sufficient concentration to change the reaction of the proteins, the amount of water held by the protein is lessened. The equilibrium between the two segments is upset, the anisotropic segment takes up water from the isotropic

segment

segment, and the muscle shortens. The neutralization of the lactic acid restores the original equilibrium. There is no salt effect because the lactate is not capable of producing swelling of the muscle. The process can be repeated until the alkali reserve is depleted and the acid reaches a concentration great enough to allow diffusion into the anisotropic segment, weakening the water binding power of the protein therein. In this state (fatigue?) the muscle cannot contract.

The lactic acid may be produced all along the fibril. In this case the weakening of the water binding powers of the proteins depends on the concentration of the proteins and the reaction of the muscle in relation to the isoelectric points of the proteins. The values given for the ratio myosin to myogen vary from slightly greater than 1 to as high as 2.5. The isoelectric points of the proteins are pH 5.0 and pH 6.0 respectively. Lactic acid affects the solubility of myogen more than myosin because the reaction of the muscle is much closer to the isoelectric point of the former protein. Hence, a limited amount of acid causes a release of water from the isotropic segment without affecting the anisotropic segment. The latter segment takes up the released water, and the muscle shortens. Neutralization of the lactic acid results in the return of the original equilibrium. An excess of acid lessens the solubility of both proteins, and the muscle is rendered incapable of contraction.

The source of the alkali utilized in the neutralization of the lactic acid is not known. The buffer salts of the muscle certainly play some part. Meyerhof believes that most of the acid is neutralized by the proteins . Fiske has shown that the breakdown of phosphocreatine releases sufficient base to neutralize a considerable amount of lactic acid. Bliss, (1928), maintains that the lactic acid is neutralized by the amide groups of the proteins. A knowledge of the true source of the alkali of muscle should be of great assistance in solving the question of muscular contraction.

The theory of rigor, based on the peptizing action of salts on the myosin, has been tested qualitatively. It is admitted that the method is crude and that the number of uncontrolled factors is large. The experiments are reported and the results are interpreted. The reader is left to asses their value. Cunner (Tautogolabrus Adspersus) were killed and were hung by the head. When rigor set in, the bodies were found exactly straight. This shows that the tension of the muscles on the opposite sides of the body was the same. This provides us with a mechanism for measuring the effect of substances injected into the muscle on the rate of the development of rigor. If a substance delays rigor, the curvature is towards the uninjected side, if the substance hastens rigor, the curvature is towards the injected side. The fish for all the following experiments were killed and were kept in water till a direct maximal

electric

electric stimulus of the muscle did not produce a contraction. In this way nervous effects and effects that might be classed as life processes are eliminated. When the fish no longer responded to electric stimulation, rigor had started to develop in the tail region. The injections were made just in front of this region. The fish were hung by the head, and the effect of the injected substances was noted by the curvature of the body.

In the first experiment, distilled water was injected into the muscle of one side of the body. The results are given in Table 13, page 76. They show that the curvature is always towards the side injected with distilled water. In Experiment II, the fish were injected with distilled water in one side and with NaCl solution, 25% in the other. The results are given in Table 14, page 77. They show that salt in concentration much greater than 1% of the muscle causes a relaxation, and in concentration of about 1% it causes a contraction. The effect of more dilute salt solution was studied in Experiment III. See Table 15, page 78. This experiment shows that even with the unequal distribution of saline obtained with injection by means of a syringe, an amount of salt less than 1% of the weight of the muscle in excess of the salt already in the muscle is capable of producing a contraction.

TABLE 13.

The Effect of Injected Distilled Water on Rigor.

Fish No.	Weight. Gms.	Injected		Curvature.
		Left Side.	Right Side.	
1	95	1 cc.	0	Immediately towards left side
2	125	0	1 cc.	Immediately towards right side.
3	105	2 cc.	0	Immediately towards left side.
4	75	0	5 cc.	Immediately towards right side.
5	89	0	10 cc.	Immediately towards right side.

TABLE 14.

The Effect of Injected Sodium Chloride Solution 25%, on Rigor.

Fish No.	Weight Gms.	Injected		Curvature.
		Left Side.	Right Side.	
6	100	5 cc. NaCl	5 cc. Water	Right.
7	124	10 cc. Saline	10 cc. Water	Right
8	103	5 cc. Saline	5 cc. Water	Right.
9	62	5 cc. Water	5 cc. Saline	Left.
10	138	5 cc. Water.	5 cc. Saline.	Left
11	108	1 cc. Saline	1 cc. Water.	Left
12	59	0.4 cc. Saline	0.4 cc. Water	Left
13	71	0.5 cc. Saline.	0.5 cc. Water.	Left
14	64	0.3 cc. Saline.	0.3 cc. Water.	Left
15	110	0.6 cc. Saline .	0.6 cc. Water.	Left.
16	56	0.3 cc. Saline.	0.3 cc. Water.	Left

TABLE 15.

The Effect of Small Amounts of Sodium Chloride Solutions on the Rigor Contraction.

Fish No.	Weight. Gms.	Concentration of NaCl.	Injected		Curvature.
			Left Side	Right Side.	
17	69	12½%	0.5 cc. Saline.	0.5 cc. Water.	Left (Slight).
18	70	"	0.5 cc. Saline.	0.5 cc. Water.	None.
19	208	"	1.2 cc. Saline.	1.2 cc. Water.	Left (Slight.).
20	-	"	0.6 cc. Saline.	0.6 cc. Water.	Left (Slight).
21	210	"	0.6 cc. Saline.	0.6 cc. Water.	Left. (Slight.)
22	60	"	0.4 cc., Saline.	0.4 cc. Water.	Left.
23*	70	"	1 cc. Saline.	1 cc. Water.	Left. Definitely*
24*	156	"	1 cc. Saline	1.cc. Water.	Left Slight.
25*	90	"	0.5 cc. Saline.	0.5 cc. Water.	Left Slight.
26*	165	"	2 cc. Saline.	2 cc. Water.	Left Definitely.

* These fish were injected along the whole muscle while all other fish were injected in the region of the external anal orifice.

Swelling of globulin, under the action of salt, is at a minimum at the isoelectric point of the protein. If the muscle be brought to a hydrogen ion concentration of pH 5.0, either rigor should not develop at all or the contraction should be much weaker than that occurring at pH 6.3 to pH 6.8, the reaction usually found in fish muscle in rigor. An attempt to demonstrate this was made by injecting various amounts of acid in the muscle of one side of cunner, and an equal volume of 0.9% NaCl in the other side. The results are given in Table 16, page 80. The minimum tension seems to be developed on the addition of 0.5 to 1 cc. of normal acid. In every case the curvature was towards the side injected with the acid.

In Experiment 5, Table 17, page 81, the effect of base on the muscle was determined. In some cases acid was injected in the opposite side to equalize the pressure, and in other cases saline was used. The body was found to curve towards the side injected with base. When acid was used, the body curved first towards the side injected with base and then curved towards the side injected with acid.

In all these experiments there must have been a considerable gradient between the centre of the injected region and the periphery. This makes the results difficult to interpret. The results are as follows. An increase in the salt concentration of the muscle by NaCl of about 1%

produces

TABLE 16.

The Effect of Injected Acid on Rigor.

Fish No.	Weight Gms.	Injected		Curvature.
		Left Side.	Right Side.	
27	160	3 cc. N/14 HCl.	3 cc. 0.9% NaCl.	Left.
28	152	1 cc. N. HCl.	1 cc. 0.9% NaCl.	Very slight to Left.
29	50	1 cc. N. HCl.	1.cc. 0.9% NaCl.	Definitely to left
30	168	2 cc. N. HCl.	2 cc. 0.9% NaCl.	Definitely to left.
31	125	1 cc. 2 N. HCl.	1 cc. 0.9% NaCl.	Definitely to left.
32	104	2 cc. 2 N. HCl.	2 cc. 0.9% NaCl.	Definitely to left.
33	117	0.5 cc. N HCl.	0.5 cc. 0.9% NaCl.	Very slight to left.
34	124	0.5 cc. N. HCl.	0.5 cc. 0.9% NaCl.	Very slight to left.
35	144	0.5 cc. N. HCl.	0.5 cc. 0.9% NaCl.	More to left than # 34.

TABLE 17.

The Effect of Injected Alkali on Rigor.

Fish No.	Weight Gms.	Injected		Curvature.
		Left Side.	Right Side.	
36	103	1 cc. N NaOH.	1. cc. 0.9%. NaCl.	Slight to left.
37	108	1 cc. N. NaOH.	1 cc. 0.9% NaCl.	" " "
38	125	1 cc. 2 N. NaOH.	1 cc. 0.9% NaCl.	Slight to right.
39	160	3 cc. N/14 HCl.	3 cc. N/14 NaOH.	Immediate towards right side. After 10 minutes towards left side.
40	98	1 cc. N. NaOH.	1 cc. N. HCl.	First to left, then to right.
41	198	1 cc. acid	1 cc. base	First to right, then to left.
42	185	0.5 cc. acid	0.5 cc. base.	First slight to right, then decidedly to left.

produees a contraction. An increase greater than 1% inhibits the contraction. It was because of this result that the author concluded that rigor passes off because of an excess of salt in the fibril. An examination of the tissue invaded by an excess of salt shows the characteristics of tissue dipped in salt solution. The inhibition of the contraction is due to the disruption of the fibres by the swelling caused by the salt. The experiments on the effect of acid are disappointing. There seems to be a concentration of acid which produces a minimum effect. This minimum effect is still greater than the rigor in the side which received no acid. The failure to bring the proteins to their isoelectric points, and so to inhibit the rigor contraction, is possibly due to diffusion gradient from the centre of the injected region outward. The concentration of acid was so large that in some cases the muscle must have contracted on the acid side of the isoelectric points. In fish numbers 28,34, and 35, the isoelectric precipitation of the protein was probably greatest. The unexpected results of Table 17 offer a possible explanation. The theory advanced as to the cause of the rigor contraction depends on the gelation of the myosin. This gelation is favoured by an alkaline reaction and by an increase in the salt concentration. The fish in all the experiments above were on the verge of rigor. A small injection of alkali into the muscle of one side causes a swelling of the myosin of that side. This results in a contraction of the muscle of this side. The breakdown of the phosphorus compounds

in

in muscle is favoured by an acid reaction. Therefore the orthophosphate production is greater on the side injected with acid than on the side injected with base. The acid and alkali both tend to become neutralized by their diffusion through the muscle. In a short time the difference in reaction between the two sides becomes negligible. But the phosphate production has proceeded faster on the side injected with acid. The result is a reversal of the curvature of the fish. If this explanation be true, the effect of acid in rigor formation is the hastening of the production of orthophosphate. The view that acid is not of primary importance in the development of the rigor cpntraction is in keeping with all previous data.

SUMMARY.

Two protein fractions have been isolated from the striated muscle of the cod, the haddock, the hake, and the catfish. One is a globulin resembling the myosin of von Furth, while the other is an albumin similar to the myogen of von Furth. The isoelectric point of the former is about pH 5.0 and that of the latter is about pH 6.0 .

Evidence has been given indicating the proteins of resting muscle to be in the form of a gel. This gel structure is destroyed by postmortem acidity.

The protein content of the muscle press juice is much higher from muscle in rigor than from muscle in the pre-rigor period. This is believed to be the result of

of an increase of salt, probably orthophosphate, in the muscle.

The swelling effect of salts on the muscle has been studied. It was found that in salt solutions greater than 1% NaCl. the muscle swells pronouncedly.

The effect of salt solutions on teased muscle indicates the presence of myosin in the anisotropic segments of the muscle.

With the above data, a theory of muscular contraction has been advanced, based on the release of water from the isotropic segment, and its uptake by the isotropic segment, as a result of the increase of the acidity of the muscle.

A theory of rigor is suggested. This theory is based on the salt effect on the myosin of muscle. The presence of salt, probably orthophosphate, increases the s solubility of the myosin in the anisotropic segments. The myosin withdraws water from the isotropic segments, resulting in a shortening of the muscle. Experimentss on artificial rigor demonstrate the possibility of this theory of rigor.

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