ACTA CHEMICA SCANDINAVICA 12 (1958) 1851—1868

Electrophoretic and Ultracentrifugal Study of Plaice Myogen

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The protein composition of water extracts and myogen of plaice muscle has been investigated by electrophoresis and ultracentrifugation. Plaice myogen has been fractionated with neutral ammonium sulphate in the whole range of saturation and the different fractions analysed by both methods. The salting-out range of the various electrophoretic and ultracentrifugal gradients has been determined. Usually the components precipitating at a higher salt concentration sediment more slowly and migrate more rapidly in the electrical field. Some properties of two crystalline albumins have been described. The isolation of a third component by preparative electrophoresis has allowed to show that the protein of abnormal amino acid composition discovered in carp myogen also occurs in plaice. Although the muscle extracts of low ionic strength present a high specificity from the electrophoretic point of view, their more detailed study will make the common properties of their protein constituents more conspicuous.

Fish muscle extracts of low ionic strength have been investigated electrophoretically in several laboratories. Carp [1–7] and cod [8,9] have been examined fairly extensively while general surveys of about 25 species including *Gadidae*, *Pleuronectidae*, *Selachii* and fresh-water fishes have been carried out by *Connell* [10] and by Nikkilä and Linko [11]. These surveys have, however, been limited to the electrophoretic analysis of whole extracts. A fractionation scheme with ethanol at low temperature has been described [11] but applied only on a small scale and to pike muscle extracts, the fractions obtained being characterized by paper electrophoresis. The separation of the albumins and globulins of the whole extracts by dialysis against water and the electrophoretic analysis of the myogens isolated in this way have been carried out on cod [8], carp [1–4] and plaice [12]. The fractionation of the carp myogen with neutral ammonium sulphate has been investigated in Liège by Henrotte [5–7] who crystallized two carp albumins and described some of their properties (*cf.* also Hamoir [13]). Preliminary experiments have also been done on plaice myogen resulting in the crystallization of another muscle albumin [12,14].

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A systematic study of the fractionation of fish myogens is thus still lacking. We have thought it therefore of interest to investigate more completely the electrophoretic and ultracentrifugal behaviour of plaice muscle extracts of low ionic strength, of plaice myogen and of fractions isolated from it with neutral ammonium sulphate in the whole range of saturation. A preliminary report of a part of this work has already been published [15].

METHODS

Plaice *(Pleuronectes platessa* L.) was caught on the Belgian coast, immediately eviscerated and kept in ice for about 20 h before filleting. The muscles are cut into small blocks and stored at −20°C for 1 to 30 days in closed containers in order to avoid evaporation. All operations are carried out at 0°C. Muscles fragments are cut in slices 40 μ thick with an automatic freezing microtome [16]. The muscle pulp is mixed with one volume of water and gently stirred for one hour. The extract is sometimes dialyzed for 48 h against 2 l of a phosphate buffer of μ 0.05 and pH 7.5 renewed once (0.0156 M Na_2HPO_4 + 0.0035 M KH_2PO_4). The precipitate formed is spun down and the supernatant examined by electrophoresis or ultracentrifugation. Usually the dialysis is carried out against the same volume of distilled water renewed several times for three days in order to remove completely the globulins. The myogen is then fractionated with neutral ammonium sulphate or dialyzed against the usual buffer of μ 0.05 and pH 7.5 before electrophoretic or ultracentrifugal analysis.

For fractionation, a mixture of solid ammonium sulphate and disodium phosphate (0.71 g $Na_2HPO_4 \cdot 12\ H_2O$/100 g ammonium sulphate) is added to the rapidly stirred solution. The precipitate formed is removed immediately except in the case of the last fraction (90 − 100 % saturation) which is left overnight in the cold room before centrifugation. In the calculation of the degree of saturation, we have admitted that the total amount of salt added correspond only to ammonium sulphate and we have taken account of the variations of volume occurring: the degree of saturation used corresponds to the percentage of the salt concentration of the saturated solution at 0°C (*cf.* Brenner-Holzach and Staehelin [17]). After dialysis the protein content of the solutions is determined with a dipping refractometer. Samples of the protein solution and of the dialysis fluid are placed in a water bath of constant temperature (25°C) and illuminated with a sodium lamp (589 mμ). The refractive increment corresponding to a concentration of 1 g/100 ml has been taken equal to 182×10^{-5} according to determinations by Hamoir [3] on carp myogen.

Electrophoresis is conducted at 1°C in a Tiselius apparatus slightly modified [18], the recording of the patterns beeing made according to the Longsworth's procedure [19,20]. The mobilities are calculated from the mean conductivity of the buffer and the protein solution and from the maximum ordinate of the gradients. At the low ionic strength used, the ascending mobilities notably exceed the descending ones and depend on the protein concentration. A correction according to Longsworth and Mac-Innes [21] has however not been applied. We will thus consider mainly the descending values. The relative concentrations of the different gradients have been determined according to the perpendicular extrapolation method of Tiselius and Kabat [22]. At the low ionic strength used, an overestimation of the quick gradients at the expense of the slow ones may occur particularly on the ascending side [23]. Our surface determinations did not, however, disclose notable discrepancies between the ascending and the descending values (*cf.* Table 3). This anomaly appears thus usually not very pronounced here. A further check of its importance by use of higher ionic strength has not been made.

The ultracentrifugations are carried out with an ultracentrifuge Spinco Model E in the department of Physical Chemistry (Prof. V. Desreux). The rates of sedimentation are corrected for water and 20°C, use being made of the mean partial specific volume given in Svedberg and Pedersen's [24] Appendix II. The relative concentrations are also calculated according to Tiselius and Kabat [22]. No account is taken of the decrease of concentration of the slower components occurring at the different boundaries observed which lead to an overestimation of the slow components at the expense of the quick ones [25].

Some measurements of absorption in the ultraviolet have been made with a D. U. Beckman spectrophotometer.

RESULTS

A. The protein composition of whole extracts and myogen

a) *Content in albumins and globulins.* The amounts of proteins isolated after one or several extractions with water and subsequent dialysis against a phosphate buffer of μ 0.05 and pH 7.5 or water are given in Table 1. Although each extract was made from about 10 fishes, notable variations occur from one extraction to the other reminiscent of those observed on the carp [3]. The amounts of globulin X and myogen obtained after exhaustive extraction are definitely lower than the values given for the carp [3]. The content of myogen of plaice muscle is lower than the ones of carp white or red muscles; if one assumes a protein content of 16 %, it would represent only 13 % of the muscle proteins. In the case of globulin X, the difference is perhaps not significant owing to the lower ionic strength used in the present experiments.

b) *Electrophoretic analysis.* The electrophoretic pattern of the whole extract at μ 0.05 and pH 7.5 is given in Fig. 1 a. Its peaks are numbered according to Connell's nomenclature. Four gradients are visible on the ascending side. The quickest small ascending peak of Connell [10] is not observable here. It is not always present in the electrophoretic patterns. The quickest fraction of Fig. 1 a is heterogeneous and corresponds to the component 1 a of Connell [10]. Peak 2 is well defined on the ascending and descending sides; it is separated

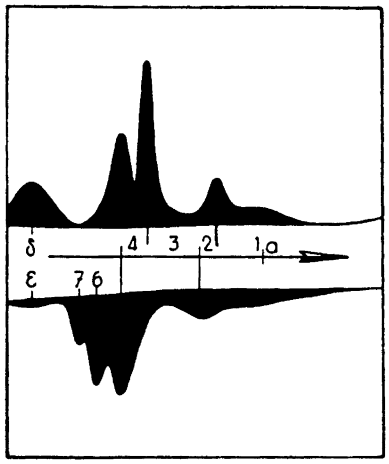
Table 1. Content in albumins and globulin X of plaice muscles (g protein/100 g fresh muscle).

Expt. No.	A			B		
	$Q_{sol.}$ at μ 0.05 pH 7.5	$Q_{sol.}$ at μ 0	% globulin X	$Q_{sol.}$ at μ 0.05 pH 7.5	$Q_{sol.}$ at μ 0	% globulin X
1	2.00	1.44	28			
2	1.70	1.06	38			
3	1.81	1.43	21	2.53	1.98	22
4	1.95	1.58	19	2.50	1.97	21
5	1.87	1.44	23			
6	2.19	1.61	26			
7				3.32	2.26	32
8				3.18	1.92	40
9				3.28	2.08	37
Mean	1.92	1.43	25	3.26 *	2.04	36
Carp white muscles. Mean (from Hamoir [3]).				3.82	2.70	29

A Extraction with one vol. of water and subsequent dialysis against μ 0.05 of pH 7.5 or water.

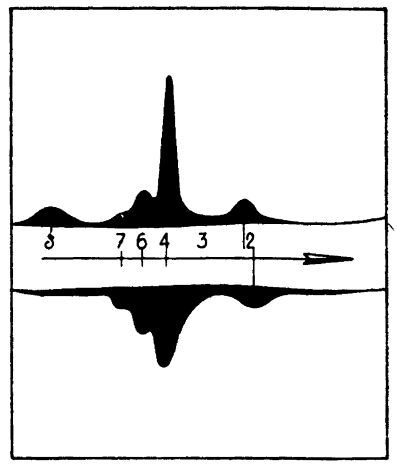
B Successive extractions with one, two and one vol. of water and subsequent dialysis against μ 0.05 of pH 7.5 or water.

* Mean of experiments 7, 8 and 9. The lower values of experiments 3 and 4 are apparently due to a partial extraction of the globulins.



a

Fig. 1 a. Electrophoretic pattern of a water extract of plaice muscle dialysed at μ 0.05 and pH 7.5. Upper diagram: ascending limb. Lower diagram: descending limb. Migration to the right. Conditions of electrophoresis: conc. 1.54 %; μ 0.05; pH 7.5; electrical field 6.6 V/cm; duration: 125 min.



b

Fig. 1 b. Electrophoretic pattern of the myogen fraction of a water extract of plaice muscle. Upper diagram: ascending limb. Lower diagram: descending limb. Migration to the right. Conditions of electrophoresis: conc. 0.96 %; μ 0.05; pH 7.5; electrical field field 6.6 V/cm; duration: 130 min.

from the main peak 4 by a heterogeneous fraction numbered 3. The slowest ascending gradient appears fairly symmetrical; prolonged electrophoresis reveals, however, that it splits into two gradients on the descending side. As some fractionations have shown that a component of intermediate mobility occurs between gradient 4 and this slow peak, it has been represented by the numbers 6 and 7 instead of 5 and 6 previously used [10].

If the extract of Fig. 1 a is dialysed against water, the globulin X fraction precipitates. The supernatant obtained after centrifugation gives a somewhat different electrophoretic pattern (Fig. 1 b). The components 1 and 1 a disappear. The relative proportion of peak 4 increases definitely. The following slow ascending peak is notably reduced and the two gradients corresponding to numbers 6 and 7 separate now clearly on both limbs.

The mobilities of these different gradients are given in Table 2 together with the data obtained by Connell [10] in a single experiment. Connell's values are much lower. In view of the larger number of experiments and of the good agreement of our results, our data are probably more correct. The percentages of the different gradients are given in Table 3. They are similar to the ones given earlier [10]. The measurements made on myogen and the determinations of the proportion of globulin X in these extracts allow to estimate the amount of globulin X corresponding to each peak (*cf.* Table 1). As the total content in globulins varies notably from one extract to the other (*cf.* Table 1), these evaluations are approximate. They show nevertheless that the peaks 3 and 4

Table 2. Electrophoretic mobilities ($\times 10^5$) of plaice muscle extracts of low ionic strength, of the corresponding albumin fraction and of the fractions isolated from plaice myogen with neutral ammonium sulphate at μ 0.05 (phosphate) and pH 7.5.

Protein comp.	Number of electroph.	Prot. conc. in %		Components							
				1	1 a	2	3	4	5	6	7
Whole extract (myogen + globulin x)	3	1.75 ± 0.2	D	8.5	—	6.66	—	3.73	—	2.86	2.26
Connell's data	1	—		9.45	6.55	5.4	4.3	3.05	—	2.35	1.60
Myogen	5	1.11 ± 0.15	D	—	—	6.74	—	3.75	—	2.86	2.13
Myogen fractions precipitated between:											
0—40 % saturation	2	1.5	A	7.74 *	7.17	—	—	4.61	3.76 (1)	3.16 (1)	—
			D	7.55 *	6.95 (1)	—	—	3.87	3.08 (1)	2.46 (1)	—
40—55 » »	3	1.5	A	10.44 ± 0.55 (2)	8.55 ± 0.8 (2)	—	—	4.40	4.07	3.63	—
			D	9.2 ± 0.4 (2)	7.0 ± 0.3 (2)	—	—	3.66	2.86		
40—48 » »	3	1.5	A	—	—	7.13 (2)	—	4.38	—	3.56	—
			D	—	—	6.40 (2)	—	3.57	—	2.77	—
48—55 » »	3	1.5	A	9.3	—	—	—	4.47	3.98	3.67	—
			D	8.37 (1)	—	6.75 (2)	—	3.72	3.0		—
55—65 » »	3	1.5	A	—	8.46 (1)	6.78 (2)	4.93 (1)	4.42	3.74	3.49	2.84 (2)
			D	—	—	6.38 (2)	—	3.62	2.80		2.16 (2)
70—80 » »	3	1.5	A	—	—	7.14	—	4.54	—	3.48	2.77
			D	—	—	6.68	—	3.68	—	2.81	2.10
80—90 » »	3	1.5	A	—	—	7.19	5.0 (1)	4.49	—	3.56	2.70
			D	—	—	6.78	—	3.63	—	2.78	2.07
90—100 » »	3	1.5	A	—	—	7.75	—	4.89	4.12 (1)	3.67	2.73
			D	—	—	6.55	—	3.47	2.71 (1)	2.46	2.0

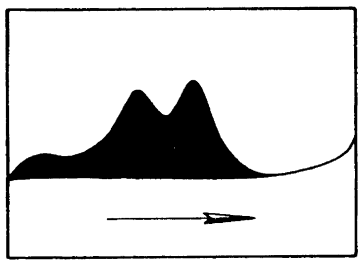
* Mobility intermediate between 1 and 1 a.
When a small gradient is not regularly present, the number of electrophoresis in which it occurs, is given between brackets.
A = Ascending values
D = Descending values

Table 3. Electrophoretic analysis of plaice muscle extracts of low ionic strength, of the corresponding albumin fraction and of the fractions isolated from plaice myogen with neutral ammonium sulphate at μ 0.05 (phosphate) and pH 7.5.

Protein comp.	Number of electroph.	Prot. conc. in %		Components							
				1	1a	2	3	4	5	6	7
Whole extract (myogen + globulin X)	3	1.75 ± 0.2	A	8.7		12.0	4.3	39.7	35.2		
			D	8.6		14.6	6.0	38.7	24.0		8.0
Connell's data		—	A	4.5	6.6	10.8	5.7	32.8	39.6		
			D	7.2	13	12.2	7.8	32.4	16.6		10.8
Myogen	4	1.11 ± 0.15	A	—	—	14.5	6.6	51.9	20.6		6.0
			D	—	—	14.4	7.6	50.5	20.6		6.6
Myogen fractions precipitated between:											
0—40 % saturation	1	1.5	A	—	4.6	5.4		76.7	9.6	3.8 *	—
			D	—	6.3	7.5		69.6	11.1	5.6	—
40—48 » »	2	1.5	A	—	—	—	—	—	—	—	—
			D	—	—	9.5	11.8	68.1	—	10.5	—
48—55 » »	2	1.5	A	4.9 *	8.1 *			44.0	30.3	12.6	—
			D	6.9 *	9.0 *			47.8	36.2		—
55—65 » »	2	1.5	A	—	8.0	15 *	5.9 *	25.0	16.0	30.1	—
			D	—	5.5	14.6 *	2 *	29.4	46.4		2.1
70—80 » »	2	1.5	A	—	—	5.2 *	12.1 *	65.6	—	5.6	11.6
			D	—	—	7	9.9 *	65.7	—	7.5 *	10.9
80—90 » »	2	1.5	A	—	—	14.4	3.3	53.4	—	11.8	17.0
			D	—	—	13.4	5.6 *	51.7	—	12.9 *	16.4
90—100 » »	3	1.5	A	—	—	44.2	2.5 *	23.7	5.3	18.6	9.5
			D	—	—	37.6	0.8	26.9	—	26.5	8.3

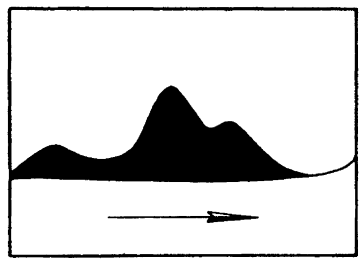
A = Ascending values
D = Descending values

* = heterogeneous



a

Fig. 2 a. Ultracentrifugal pattern of a water extract of plaice muscle dialysed at μ 0.05 and pH 7.5. Direction of migration: from left to right. Protein conc. 1 %; μ 0.05; pH 7.5. Duration of the ultracentrifugation: 56 min.



b

Fig. 2 b. Ultracentrifugal pattern of the myogen fraction of a water extract of plaice muscle. Direction of migration from left to right. Protein conc. 1 %; μ 0.05; pH 7.5. Duration of the ultracentrifugation: 64 min.

of whole extracts are entirely soluble in water while a partial precipitation of peak 2 occurs. Globulin X is formed mainly by the slow moving components 5, 6 and 7 and by the quick ones 1 and 1 a.

c) *Ultracentrifugal analysis.* Two ultracentrifugations of plaice myogen made by Henrotte [12] have shown that the three components sedimenting with rates of about 7, 5 and 1.5 *S* observed in carp myogen [3,4] are also present in this case. We have carried out further experiments on myogen and have examined whole extracts similar to those analysed by electrophoresis too.

The whole extract (Fig. 2 a) does not differ qualitatively from myogen (Fig. 2 b); it includes the three peaks already mentioned. Their corrected rates of sedimentation and relative concentrations are given in Table 4, together with similar data relative to the myogen. Notable variations occur in the rate of sedimentation of each peak. Two of the experiments of myogen carried out on two different concentrations and the surface measurements show that these variations cannot be due to changes in concentration. They also obviously exceed the experimental errors, particularly in the case of the slow and the median peaks. Several components of close rates of sedimentation are present in these two gradients. The comparison of the surface determinations relative to whole extracts and myogen allows also to estimate the amount of globulins occurring in each peak of the whole extract. After dialysis against water the slow peak remains entirely in solution, the medium one precipitates in the proportion of about 25 % (*cf.* Table 1) and the third one about 50 %. Fish muscle extracts of low ionic strength appear thus to behave very similarly in the ultracentrifuge. They seem to contain generally the slow sedimenting components first observed by Deuticke [26] on frog muscle extracts which do not occur in rabbit muscles and therefore not in muscles of warm-blooded animals. They differ only from each other in the proportions of the different gradients observed.

Table 4. Ultracentrifugal analysis of plaice muscle extracts of low ionic strength, of the corresponding albumin fraction and of the fractions isolated from plaice myogen with neutral ammonium sulphate at μ 0.05 (phosphate) and pH 7.5.

Protein comp.	Total conc. in % protein	Number of ultracent.	Corrected rate of sedimentation			Composition in % of the total area		
			Slow peak	Median peak	Quick peak	Slow peak	Median peak	Quick peak
Whole extract	1	2	1.53 ± 0.31	4.97 ± 0.01	7.08 ± 0.03	11.6 ± 0.7	43.2 ± 3.3	45.3 ± 2.2
(myogen + globulin_X)	0.71	1	1.47	5.10	7.24	13.3	47.1	39.6
Myogen	1	3	1.39	5.07	7.23	20.6	50.8	28.3
	0.5	2	1.55	5.28	7.55	22.6	51.1	26.3
Myogen of carp	1.32 [a]	3	1.45 [a]	4.85 [a]	7.10 [a]	48 [a]	39 [a]	13 [a]
Myogen fractions precipitated between								
0— 40 % sat.	1	1	1.58	5.96	8.03	4.1	7.3	88.5
0— 48 » »	1	1	1.97	5.53	7.76	8.6	7.3	84.2
	0.5 [b]	1	2.14	5.72	8.09			
40— 48 » »	1	1	—	—	7.64	5	10.7	84.3
48— 55 » »	1	1	—	5.63	7.52	0	15.9	84.1
55— 65 » »	1	1	—	5.32	7.05—9.29 [c]	0	63.1	23.1—13.8 [c]
70— 80 » »	1	2	—	4.73	—	8.3 [d]	84.8	6.8
	0.5 [b]	1	—	4.91	—	0	92.6	7.4
80—100 » »	1	1	1.15	5.36	6.99	50.2	34.5	15.3
	0.5 [b]	1	1.23	5.38	—			
90—100 » »	1	1	1.29	—	7.07			
	0.5 [b]	1	1.42	4.10	7.18	58.4	17.5	24.1

[a] Mean of three ultracentr. at 1.07, 1.1 and 1.8 %. [b] Preparation obtained by dilution of the preceding one of 1 %. [c] Component moving ahead of the quick component not apparent in whole myogen. [d] Heterogeneous.

B. The fractionation of myogen with neutral ammonium sulphate

The range of salt fractionation up to 65 % has already been briefly examined by Henrotte [12,14]. He has considered the fractions corresponding to 0—40, 40—48, 48—55 and 55—65 % of saturation at pH 6.5 and analysed the fractions 40—48 and 48—55 by ultracentrifugation. The first one sediments as a single peak of about 7 *S*; the second one is a mixture of the previous component and of another one of about 5 *S* which could be crystallized [12,14].

We have fractionated plaice myogen at neutral pH in the whole range of saturation. In order to avoid arbitrariness in the determination of the zones of fractionation, a salting-out curve has first been measured by ultraviolet absorption up to 65 % saturation. The precipitation starts at 35 % saturation and increases progressively without definite break of the curve. There was therefore no reason to change the earlier zones of fractionation. The main fractions examined are 0—40, 40—48, 48—55, 55—65, 70—80, 80—90 and 90—100 %. The amount of protein isolated in each of these fractions is given in Table 5. Two major ranges of precipitation appear clearly separated by the small zone 65—70 % of lower precipitation.

a) *Electrophoretic analysis of the fractions.* All the fractions considered except fraction 65—70 % have been isolated several times in order to check the reproducibility of the electrophoretic results. Many overlapping fractions have also been considered such as 40—55, 48—65, 65—80, 70—100, 80—100 whose results are mainly confirmatory of the previous ones and will therefore usually not be described here. The relative positions of some peaks pertaining to different fractions have been controlled by mixing some fractions and examining the elctrophoretic pattern of the mixture. The results obtained are summarized in Fig. 3.

The fractionation discloses some new peaks which are not observed in plaice myogen. Small quick heterogeneous gradients are visible on the ascending side in the fractions isolated up to 65 % saturation (Fig. 3a, b, c, d); the only ones which appear more or less homogeneous migrate at the level of 1a in fractions 0—40 and 55—65 %. Component 2 remains soluble up to

Table 5. Partition of myogen by ammonium sulphate fractionation at neutral pH.

Zone of saturation		0—40	40—48	48—55	55—65	65—70	70—80	80—90	90—100
% of myogen precipitated	a)	4.5	8.05	11.15	10.7	2.0	28.4	26.5	8.8
	b)	4.2	6.0	10.5	11.8	3.0	32.2	22.4	8.7
	c)	4.1	6.9	14.3	13.8	2.4	25.6	25.1	8.1
Mean		4.3	7.0	12.0	12.1	2.5	28.7	24.7	8.5
Content in g/100 g fresh muscle *		0.09	0.14	0.24	0.25	0.05	0.59	0.50	0.17

* Assuming the myogen content of 2.04 g/100 g fresh muscle of Table 1.

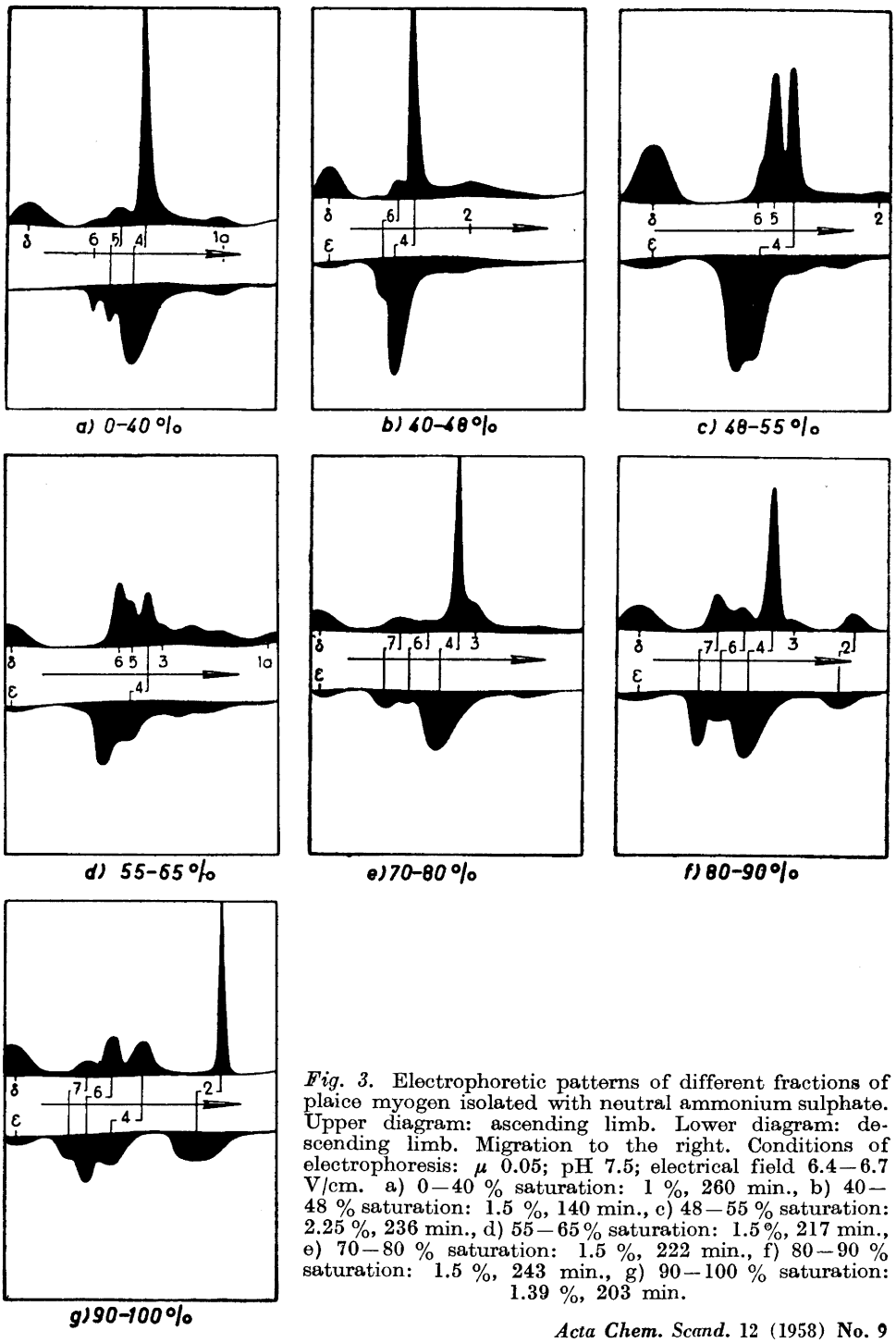


Fig. 3. Electrophoretic patterns of different fractions of plaice myogen isolated with neutral ammonium sulphate. Upper diagram: ascending limb. Lower diagram: descending limb. Migration to the right. Conditions of electrophoresis: μ 0.05; pH 7.5; electrical field 6.4—6.7 V/cm. a) 0—40 % saturation: 1 %, 260 min., b) 40—48 % saturation: 1.5 %, 140 min., c) 48—55 % saturation: 2.25 %, 236 min., d) 55—65 % saturation: 1.5 %, 217 min., e) 70—80 % saturation: 1.5 %, 222 min., f) 80—90 % saturation: 1.5 %, 243 min., g) 90—100 % saturation: 1.39 %, 203 min.

70 % saturation; it precipitates mainly in the fractions 80—90 % and 90—100 %. Component 3 which does not appear clearly in whole myogen is somewhat better defined in Fig. 3d, e and f. The proportion of component 4 in the different fractions varies suprisingly: it decreases up to 55—65 % and increases again in fractions 70—80 and 80—90 %. This behaviour suggests that this peak corresponds to at least two components of different solubilities. Component 5 separates from component 6 after prolonged electrophoresis on the ascending side in fractions 48—55 and 55—65 % (Fig. 3c and d) as a sometimes important peak although it is not visible in myogen. It corresponds probably to a rapid portion of the gradient 6 of myogen which does not clearly resolve from this gradient because of its low concentration in myogen. Component 7 is fairly soluble and precipitates in the range 70—100 % (Fig. 3e, f, g). The analysis of these electrophoretic patterns is given in Tables 2 and 3.

Our results differ somewhat from those obtained by fractionation of pike muscle extracts with ethanol [11]. It was found in this case that the solubility of the various components increases with the electrophoretic mobility. The

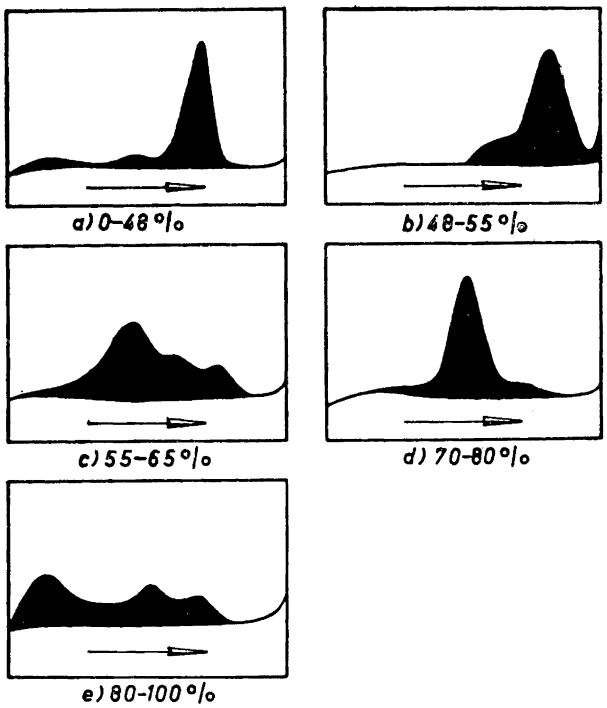


Fig. 4. Ultracentrifugal patterns of some fractions of plaice myogen isolated with neutral ammonium sulphate. Direction of migration: from left to right. Protein conc. 1 %; μ 0.05; pH 7.5. a) 0—48 % saturation, 56 min., b) 48—55 % saturation, 48 min., c) 55—65 % saturation, 56 min., d) 70—80 % saturation, 64 min., e) 80—100 % saturation, 76 min.

behaviour of components 7 and 4 of plaice myogen are in disagreement with these results. The quicker electrophoretic components are apparently generally more soluble but it is clear that this regularity has many exceptions.

b) *Ultracentrifugal analysis of the fractions.* Henrotte [12,14] has already shown that a single component of about 7 *S* is present in fraction 40—48 % while two of about 5 and 7 *S* are observed in the fraction 48—55 %. We have extended this analytical work to the various fractions described in the preceding paragraph. The main results obtained are summarized in Fig. 4.

The three ultracentrifugal gradients of plaice myogen precipitate on ammonium sulphate fractionation in very different ranges. The fractions 0—40 and 40—48 % contain nearly exclusively the quick peak (Fig. 4a). The median ones begin to precipitate in significant amounts in the range 48—55 % (Fig. 4b *) and occur as a single peak in the fraction 70—80 (Fig. 4d) with sometimes a slight admixture of the quick peak. The intermediate fraction 55—65 % is a mixture of the quick and the median components. It reveals also the occurrence of a new gradient sedimenting more quickly than the quick one of whole myogen. The slow gradient is present as an impurity in fraction 0—48 % and is the major component of the fraction 80—100 %. A nearly complete isolation of the quick and the median gradients and a considerable increase in concentration of the slow gradient are thus obtained by ammonium sulphate fractionation at neutral pH. The corrected rates of sedimentation and the relative amounts of the different peaks are given in Table 4. The surface determinations are somewhat inaccurate because of the difficulty in determining the exact position of the base line. In the case of the small gradients, a quick spreading is also usually observed which makes the determinations of the rates of sedimentation less precise. Some conclusions are nevertheless possible. The slow peak of plaice myogen precipitates in a small proportion in the range 35—48 % and mainly in the range 80—100 %. The two other peaks are more heterogeneous; they precipitate in the whole range. The rates of sedimentation are of the same order of magnitude for the fractions as for the total myogen. The higher variations observed are apparently due to the quick spreading of some gradients and to changes in concentration except perhaps for the quick peak which shows a progressive decrease of the rate when higher salting-out ranges are considered.

The comparison of the ultracentrifugal results with the electrophoretic patterns obtained with the same preparations is very instructive. It shows that the main parts of the fractions 0—48 % and 70—80 % which sediment so differently, migrate with the mobility of peak 4. The wide range of precipitation of this peak is due to the successive salting-out of two different types of molecules. The electrophoresis of a fraction 70—80 % which appeared pure by ultracentrifugation has shown that few percent of the median ultracentrifugal gradient migrate in the electrical field more slowly than peak 4.

The major part of the slow sedimenting component of plaice myogen precipitates in the range 80—100 % as the electrophoretic gradient 2. This gradient isolated by preparative electrophoresis sediments effectively at the rate of

* The apparently higher rates of sedimentation observed in this picture are due to the incomplete filling of the cell.

about 1.5 *S* (*cf.* following paragraph). The higher proportion of the slow ultracentrifugal peak (*cf.* Tables 3 and 4) in fraction 80—100 % suggests however the occurrence in this peak of proteins migrating more slowly in the electrical field.

C. Isolation of some components

The electrophoretic and ultracentrifugal analyses just described have shown the occurrence of some fractions which are fairly homogeneous under the conditions of our experiments. The precipitates which separate between 0—48 % and between 70—80 % contain relatively low percentages of impurities. Further experiments would, however, be necessary to determine their degree of homogeneity. A first step has been made in this direction by isolating crystalline components sedimenting with the rate of these two fractions. Henrotte and Dresse [14] have crystallized from plaice myogen a component of a rate of sedimentation of about 5 *S*. We have reproduced these results and made a preliminary examination of two other components.

1. *Crystalline component of 5.5 S.* Our method of preparation differs somewhat from the one described earlier [12,14]. Myogen is brought to 48 % saturation and about pH 6.5 by addition of solid ammonium sulphate. A saturated solution of ammonium sulphate at neutral pH is then added up to 55 % saturation. This second precipitate is also removed. The fraction precipitating between 55 % and 62 % saturation is redissolved in a minimum amount of water. Rhombohedra similar to those obtained by Henrotte [12,14] separate usually within a day (Fig. 5). They have also been prepared from fractions precipitating with neutral saturated ammonium sulphate in the range of 59—65 or 65—70 % saturation. The slight acidity of 6.5 recommended by Henrotte probably favours the crystallization but seems not very critical.

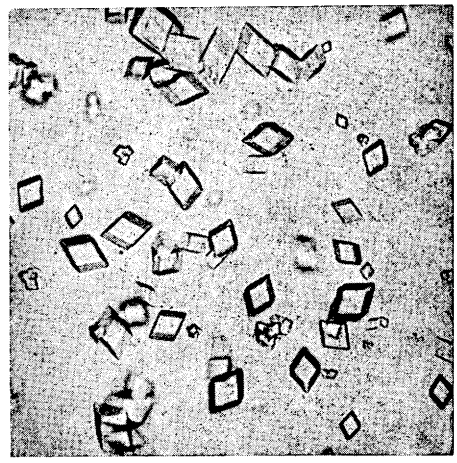


Fig. 5. Crystalline component of 5.5 *S*. Magnification: × 190.

Fig. 6. Crystalline component of 7.1 *S*. Magnification: × 1 800.

Three preparations have been examined by ultracentrifugation. An impurity sedimenting more quickly than the main peak and amounting to less than 5 % of the total protein content is observed. The corrected rates of sedimentation of the main peak at a concentration of 1 % are of 5.48 *S*, 5.48 *S* and 5.46 *S* and at 0.5 % of 5.54 *S* (one determination). The corresponding values of the impurity have been measured on a single preparation at a total concentration of 1 and 0.5 %; they are, respectively, of 8.11 and 8.31 *S*. The values for the main peak are in agreement with the determinations made on the corresponding fractions (Table 4). They are somewhat higher than the rates of the median peak of myogen. This crystalline component seems to correspond to a quick fraction of this median peak. In the electrical field, this crystalline material migrates at μ 0.05 and pH 7.5 as a single peak, moving at the rate of peak 4. A good degree of purity has therefore been achieved in this preparation.

2. *Crystalline component of 7.1 S.* In the course of the previous preparation, another crystalline material could be isolated. This result was first observed by Henrotte [12]. The method of preparation of this new type of crystals differs only slightly from the preceding one. The same fraction 55—62 % redissolved in a minimum amount of water sometimes gives rise to bunches of needles instead of rhombohedra. An important amorphous precipitate appears also at about the same speed. The aspect of the preparations is shown in the Fig. 6. Needles could also been obtained from precipitates isolated in the ranges 48—53 and 53—59 %. We have also been able recently to prepare these crystals in an easier way: myogen has been precipitated at neutral pH with our usual mixture of solid ammonium sulphate and disodium phosphate up to 55 % saturation. The precipitate is partially redissolved with water and the supernatent is separated by centrifugation. Bunches of needles soon appear in the solution together with an amorphous precipitate. The formation

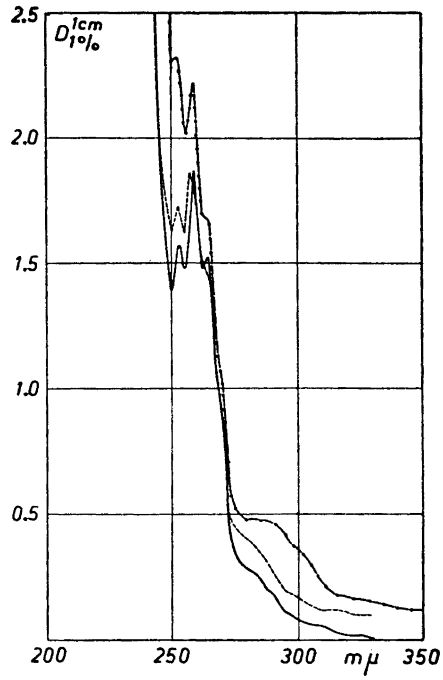


Fig. 7. Ultraviolet absorption spectra of the component of 1.5 *S* at neutral pH (——) and in presence of 0.1 N NaOH (—·—·—) and 0.1 N HCl (— — —).

of these crystals occurs therefore at neutral pH. This protein has a lower range of precipitation than the preceding one. The mixture of crystals and amorphous material shown in Fig. 6 has been separated by centrifugation, redissolved and examined by ultracentrifugation. A single peak has been observed on four different preparations sedimenting at a concentration of 1 % with corrected rates of 6.92 *S*, 7.24 *S*, 7.1 *S* and 7.17 *S*. The last preparation has also been examined at a concentration of 0.5 %; a somewhat higher corrected rate of 7.37 *S* was observed. These values are in agreement with those obtained for the quick component of myogen (Table 4) but somewhat smaller than those of the corresponding fractions.

A preliminary electrophoresis on the other hand suggests that this mixture of crystals and amorphous material is still fairly heterogeneous. A further fractionation should be necessary to isolate this crystalline material in a satisfactory degree of purity.

3. *Component of 1.5 S.* The myogens of cold-vertebrates appear to contain generally a fraction sedimenting very slowly in the ultracentrifuge. A crystalline component of this fraction has been isolated from carp muscles [7]. It precipitates with ammonium sulphate at very high salt concentration, contains about 15 % phenylalanine and no tyrosine and tryptophane [7,13]. This abnormal amino acid composition allowed to determine if a similar protein exists also in plaice myogen. In the fraction precipitating with neutral ammonium sulphate between 90 and 100 % saturation, a sharp ascending peak

(*cf.* Fig. 3g) migrates much quicker than the other gradients. We have been unable to isolate this component by zone electrophoresis on starch columns but small amounts could be obtained by preparative electrophoresis. They have been examined by ultracentrifugation and spectrophotometry. A preliminary report on this part of our work has already been published [15].

a. *Ultracentrifugal behaviour:* A single peak is observed which sediments with a corrected rate of 1.4—1.5 *S*. The values obtained with three different preparations are 1.45 *S* at 0.88 %, 1.52 *S* at 0.5 % and 1.43 *S* at 0.4 %. They do not differ essentially from those obtained previously for the slow peak (Table 4).

b. *Ultraviolet spectrum:* This component presents the abnormal amino acid composition of the corresponding component of carp muscle. Several preparations have been examined at pH 7.5. The three maxima of phenylalanine at 252, 257 and 263 mμ are observed at 253, 259 and 265 mμ and a small absorption which varies from one preparation to the other occurs at higher wave length suggesting the presence of an impurity containing tyrosine, tryptophane or cystine (Fig. 7). In presence of 0.1 N NaOH, the absorption due to this impurity is notably increased. We are therefore not dealing with tryptophane but possibly with tyrosine or cystine. The absence of a definite maximum at about 295 mμ suggests the presence of cystine. In 0.1 N HCl, the spectrum does not differ notably from the one observed at neutral pH.

The content in phenylalanine has been evaluated spectrophotometrically: if the absorption at 259 mμ is attributed exclusively to phenylalanine and if use is made of Fromageot and Schneck's data [27] for the absorption of this amino acid, it amounts to 16 % and is therefore approximatively equal to the one of the carp component. The comparison of the electrophoretic data on the other hand suggests a notable difference in charge: its descending mobility at μ 0.05 and pH 7.5 is -6.5×10^{-5} cm^2 volt^{-1} sec^{-1} while that of the carp protein at μ 0.075 (0.05 phosphate and 0.025 NaCl) and pH 7.3, is -3.2×10^{-5} cm^2 volt^{-1} sec^{-1}. The content in basic and acid amino acids appears thus variable according to the origin.

DISCUSSION

The present investigation has attempted a general survey of the protein composition of plaice myogen by electrophoresis and ultracentrifugation. In order to increase the resolution, the electrophoresis has been carried out at low ionic strength. The resulting improvement of the qualitative analysis, however, increases the boundary anomalies and decreases the precision of the quantitative data. Although myogen contains about 50 different enzymes according to several authors [28,29], the resolution obtained by electrophoresis at pH 7.5 is particularly poor in this case. Five components are observed in the whole extracts instead of 7 in the cod [8] and 6 in the carp [12] and the major one represents a particularly high percentage of the total amount: 50 to 55 % instead of about 40 % in the two other fishes. Electrophoresis carried out at different pH values would perhaps give a better separation but this point has not been investigated. On the other hand, the resolution obtained by

ultracentrifugation is small and similar to the one previously obtained with the carp [3,4].

The fractionation of plaice myogen with neutral ammonium sulphate has made possible an improvement of this analysis: peaks could be observed which were initially too small to be visible and some components could be separated. The examination of the different fractions by electrophoresis und ultracentrifugation shows the occurrence of a general trend: when the degree of saturation of the range considered increases, the electrophoretic mobilities of the components isolated increase and the ultracentrifugal ones decrease. A similar electrophoretic result has already been observed after ethanol fractionation of a pike muscle extract [11]. The range of fractionation suitable to the isolation of a component identified by ultracentrifugation or electrophoresis can thus bee foreseen. This paper shows, however, that discrepancies are not uncommon neither from the electrophoretic nor from the ultracentrifugal point of view.

The comparative electrophoretic studies of Connell [10] and Nikkilä and Linko [11] have shown the great heterogeneity of fish muscle extracts of low ionic strength. This heterogeneity has not been observed so far by ultracentrifugation. Furthermore, when a protein such as the component of S 1.5 can be characterized by some peculiar properties, it seems to occur generally but with different electrophoretic mobilities according to its origin. The charge of these proteins seems to play a secondary role and not to be related to their function in the cell. The further comparative study of the muscle albumins will certainly show more clearly the properties of these proteins which are directly related to their enzymatic functions and must not differ according to their origin.

We are much indebted to *Fiskeribedriftens Forskningsfond*, Norway (J. W. Jebsen) and to the *Fonds National de la Recherche Scientifique* (G. Hamoir) for financial support of this investigation, and wish to thank Prof. Dubuisson and Desreux for facilities put at our disposal. One of us (J. W. J.) expresses his sincere gratitude to Prof. Dubuisson for his kind hospitality, and to Director Eirik Heen, *Norwegian Fisheries Research Institute*, for making this study possible.

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Received February 28, 1958.